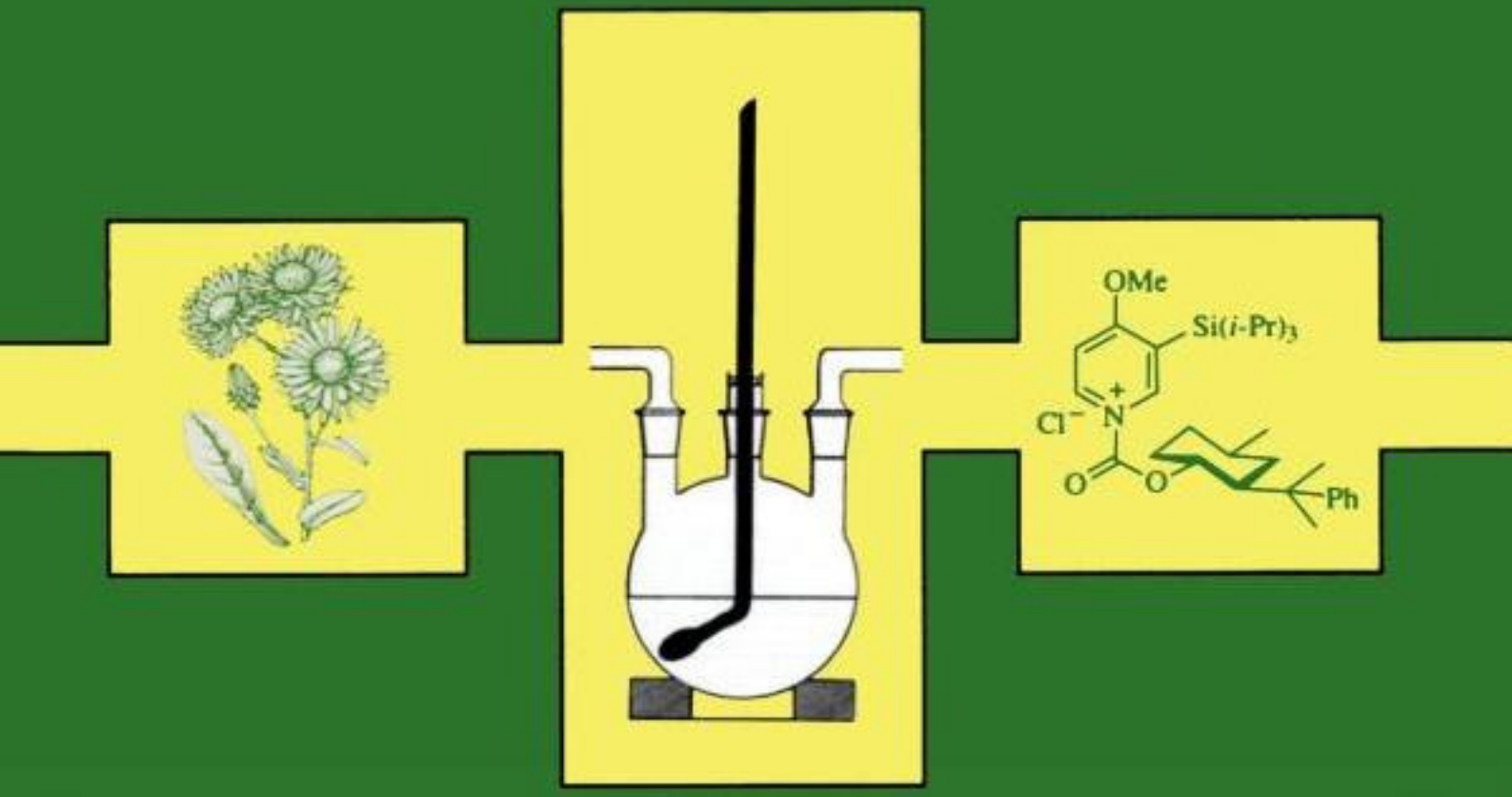
Studies in Natural Products Chemistry

Atta-ur-Rahman/Editor



Volume 19

Structure and Chemistry (Part E)

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FOREWORD

The present volume of Studies in Natural Products Chemistry which is the 19th in the series presents important research work carried out over a wide front of natural product chemistry. It includes the synthesis of dendrobatid alkaloids, aspidosperma and related alkaloids and some important natural products via aliphatic nitro derivatives, cannabinoids, brassinosteroids, quinocarcin and its related compounds, mannostatins and cyclophellitols and derivatives of grandiflorenic acid and some related diterpenes. The volume also presents marine cyclic halo ether compounds, oxidative ring transformation of 2-furylcarbinols and stereoselective synthesis of *C*-branched nucleoside analogues. The present work in the area of marine chemistry also includes reviews on bioactive marine macrolides, hormones in the red swamp crayfish. Work on novel polysaccharides from microorganisms and plants, the dereplication of plant-derived natural products, and siderophores from fluorescent pseudomonas is also presented.

Like the previous volumes of this series, it is hoped that the present volume will provide readers with exciting new chemistry in whole areas on natural product chemistry and help them to keep abreast with important recent developments.

I wish to express my thanks to Dr Ather Ata for his assistance in the preparation of the index and Mr Mahmood Alam for secretarial assistance.

June 1997 Atta-ur-Rahman

PREFACE

The international scientific community interested in natural products has already expressed its gratitude toward one of its members, Professor Atta-Ur-Rahman. Through his ongoing efforts, our colleague has been able to constitute a unique collection, "Studies in Natural Product Chemistry" resulting from the active participation of all those who count in this area. The series is now up to volumes 19 and 20, an impressive achievement.

Natural products constitute today, and no doubt for a long time to come,a domain of intense scientific activity. In fact, the term "natural products" must be taken in a broad sense, that is, molecules which originate in the living world, in Nature. Even though, for a long time, it has been especially secondary metabolites which have been the object of the majority of studies, the field of research has been observed, in recent years, to be moving towards more and more complex molecules : large polypeptides, proteins, polyosides, etc. The frontier between chemistry and biology is getting smaller to the point of disappearing altogether. The different chapters of this new work are of a very varied nature and breadth. Topics include the study of Dendrobatid alkaloids and brassisterols, of general synthesis and of the inhibitory mechanisms of glycoprotein and glycolipid formation.

Each reader will find something of value. The present volume will be welcomed with the same enthusiasm as the preceding ones, reminding us that natural products still have a bright future.

July, 1997 Pierre Potier

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Recent Advances in the Synthesis of Dendrobatid Alkaloids

Chihiro Kibayashi and Sakae Aoyagi

1. INTRODUCTION

Over two hundred alkaloids that possess interesting pharmacological activity on nerve and muscle have been characterized from the skin extracts of frogs of the neotropical family Dendroibatidae and, hence, have been referred to as dendrobatid alkaloids. The scarcity of natural material and biological significances of these alkaloids have prompted considerable synthetic efforts toward their total synthesis. An excellent review article by Daly and Spande covers all that was known about the chemistry, pharmacology, and biology of dendrobatid alkaloids by the early 1980s (ref. 1). Around the late 1980s, an international treaty to protect endangered species was enacted (ref. 2) that has prevented collecting the neotropical poison-dart frogs whose habitat is Central and nearby South America. In this respect, the lack of availability of natural products as well as the remarkable pharmacological activity (ref. 3) make the synthesis of the alkaloids from the poison-dart frogs an extremely important and urgent goal, and during 1990s there has been an impressive growth in the number of publications dealing with the synthesis of the dendrobatid alkaloids in particular the indolizidine alkaloids.

The object of the present review is to document synthetic development in the field of dendrobatid alkaloids accumulated during 1990s under the following six sub-divisions.

(1) 2,5-Disubstituted decahydroquinoline alkaloids
(2) Histrionicotoxins
(3) Indolizidine alkaloids
(4) Pumiliotoxin A class alkaloids
(5) Epibatidine
(6) Miscellaneous

2. SYNTHESES OF 2,5-DISUBSTITUTED DECAHYDROQUINOLINE ALKALOIDS

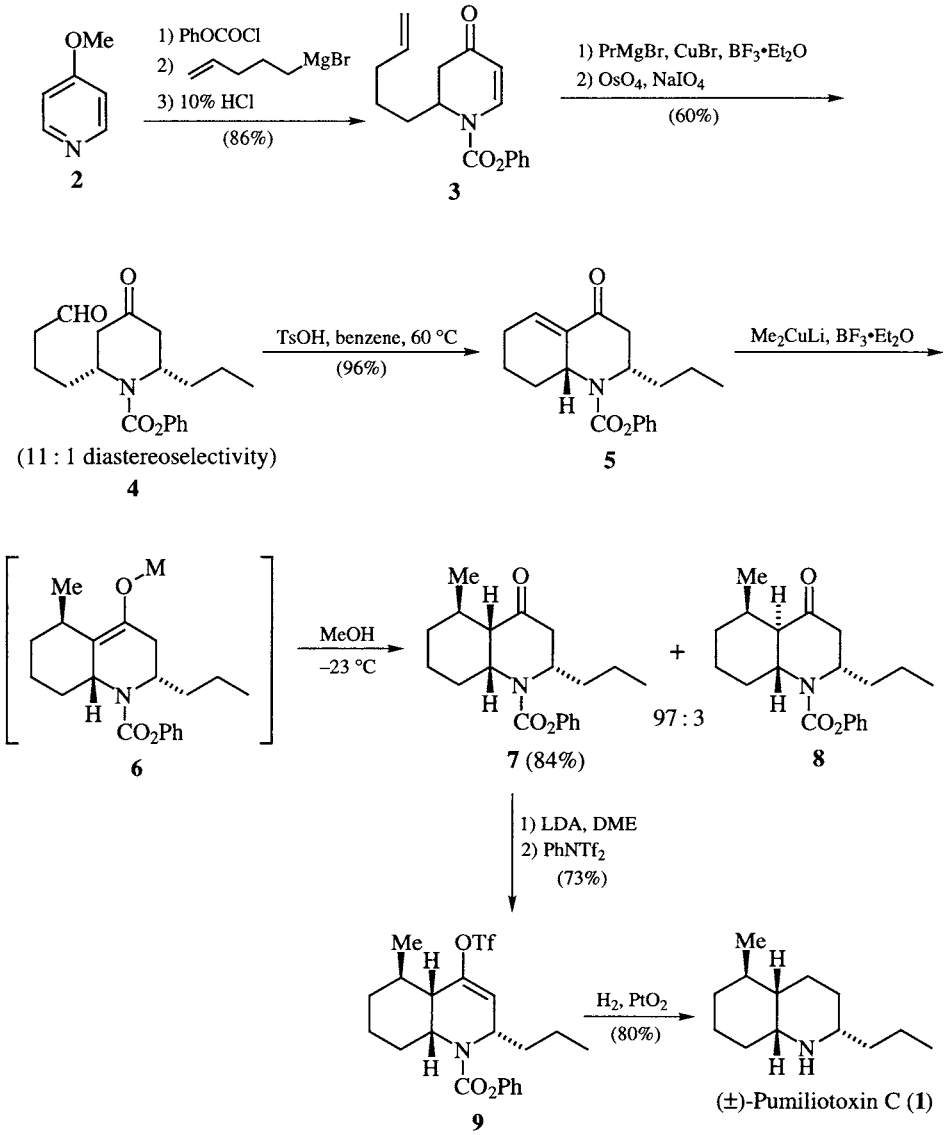
One major class of dendrobatid alkaloids is the 2,5-disubstituted decahydroquinolines. The first member of this group named pumiliotoxin C (**1**) was isolated from the skin secretions of

Panamanian population of *Dendrobates pumilio* (ref. 4) and *D. auratus* (ref. 5) and more recently found in skin extracts of Madagascan genus of ranid frogs *Mentella* (ref. 6). Although there had been some confusions in the literature, the absolute configuration of natural (–)-pumiliotoxin C was unambiguously established as shown in **1** by X-ray crystallographic analysis of the hydrochloride salt (refs. 4b and 7) and its total synthesis (ref. 8). The unusual chemical and neuromuscular characteristics (refs. 1 and 9) have prompted chemists to develop numerous approaches to nonchiral and chiral syntheses of the 2,5-disubstituted decahydroquinoline alkaloids mainly pumiliotoxin C in recent years.

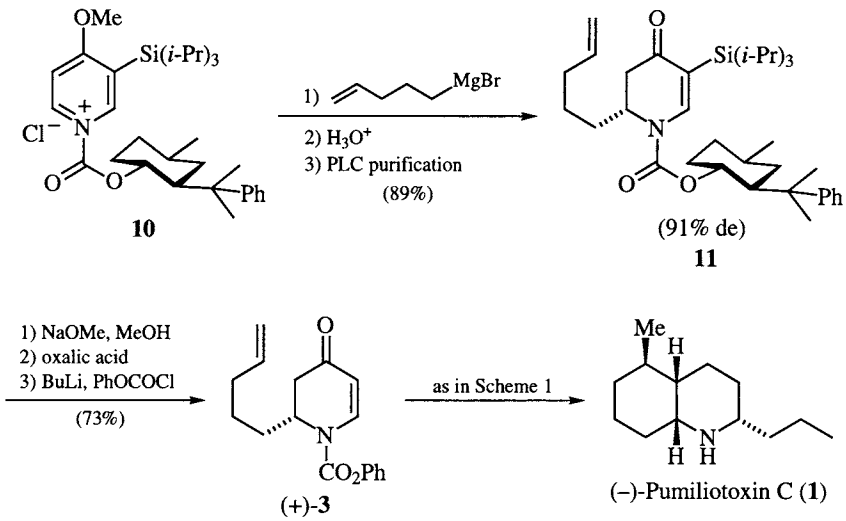
The racemic synthesis of pumiliotoxin C (**1**) using an intermediate *N*-acyldihydropyridone **3** as a useful building block has been developed by Comins and Dehghan (ref. 10) and this is shown in Scheme 1. The dihydropyridone **3** was prepared from 4-methoxy pyridine **2** by sequential treatment with phenyl chloroformate, [5-(1-pentenyl)]magnesium bromide, and hydrochloric acid. Copper-mediated conjugate addition of propyl magnesium bromide to **3** proceeded with the diastereoselectivity of 11:1 in favor of the *cis*-product. Oxidative cleavage of the terminal olefin with $OsO_4/NaIO_4$ followed by acid treatment of **4** gave the bicyclic enone **5**. Conjugate addition to the enone **5** with Me_2CuLi and $BF_3{\bullet}Et_2O$ followed by protonation of the enolate **6** led to the desired diastereomer **7**. Removal of the oxygen of the keto carbonyl group at C-4 provided (±)-pumiliotoxin C (**1**).

This strategy was used for the enantioselective synthesis of (–)-pumiliotoxin C by the same authors (ref. 11) (Scheme 2). Reaction of the *N*-acylpyridinium salt **10** bearing the (–)-8-phenylmenthyl group as a chiral auxiliary with the Grignard reagent afforded the *N*-acyldihydropyridone **11** in 91% de. Subsequent radial PLC purification followed by removal of the chiral auxiliary and the silyl group provided the enantiopure building block (+)-**3**, which was converted to (–)-pumiliotoxin C (**1**) based on the above sequence.

These authors have further exploited this enantioselective approach in the first asymmetric synthesis of (+)-*trans*-decahydroquinoline 219A (**12**) (ref. 12) as shown in Scheme 3. Reaction of the *N*-acylpyridinium salt **13** installed by (+)-*trans*-2-(α-cumyl)cyclohexyl group with the Grignard reagent gave the *N*-acyldihydropyridone **14** in 93% de. After radial PLC purification, the pure diastereoisomer **14** was converted to the key intermediate (–)-**3**. In the presence of $BF_3{\bullet}Et_2O$, copper-mediated conjugate addition of [3-(benzyloxy)propyl]magnesium bromide to (–)-**3** provided the *cis*-piperidone **15**. Oxidative cleavage of the terminal olefin followed by intramolecular aldol type condensation yielded the bicyclic enone **16**. The stereocenter at C-5 was introduced stereoselectively by conjugate addition of the higher order cuprate to **16** followed by trapping as the vinyl triflate **17**, which was converted to the amino diol **18** as a diastereomeric mixture in three steps. The diol mixture was treated with tributylphosphine and *o*-nitrophenyl selenocyanate to give the bisselenides **19** in a 87:13 ratio in favor of the *trans*-isomer. Finally, the pure *trans*-somer underwent oxidative elimination followed by deprotection, affording alkaloid (+)-*trans*-219A (**12**).


OMe
N
2
1) PhOCOCl
2)
MgBr
3) 10% HCl
(86%)
O
N
CO2Ph
3
1) PrMgBr, CuBr, BF3•Et2O
2) OsO4, NaIO4
(60%)
CHO
O
N
CO2Ph
(11 : 1 diastereoselectivity)
4
TsOH, benzene, 60 °C
(96%)
H
5
Me2CuLi, BF3•Et2O
Me
O
M
6
MeOH
–23 °C
7 (84%)
+
97 : 3
8
1) LDA, DME
2) PhNTf2
(73%)
OTf
9
H2, PtO2
(80%)
(±)-Pumiliotoxin C (1)

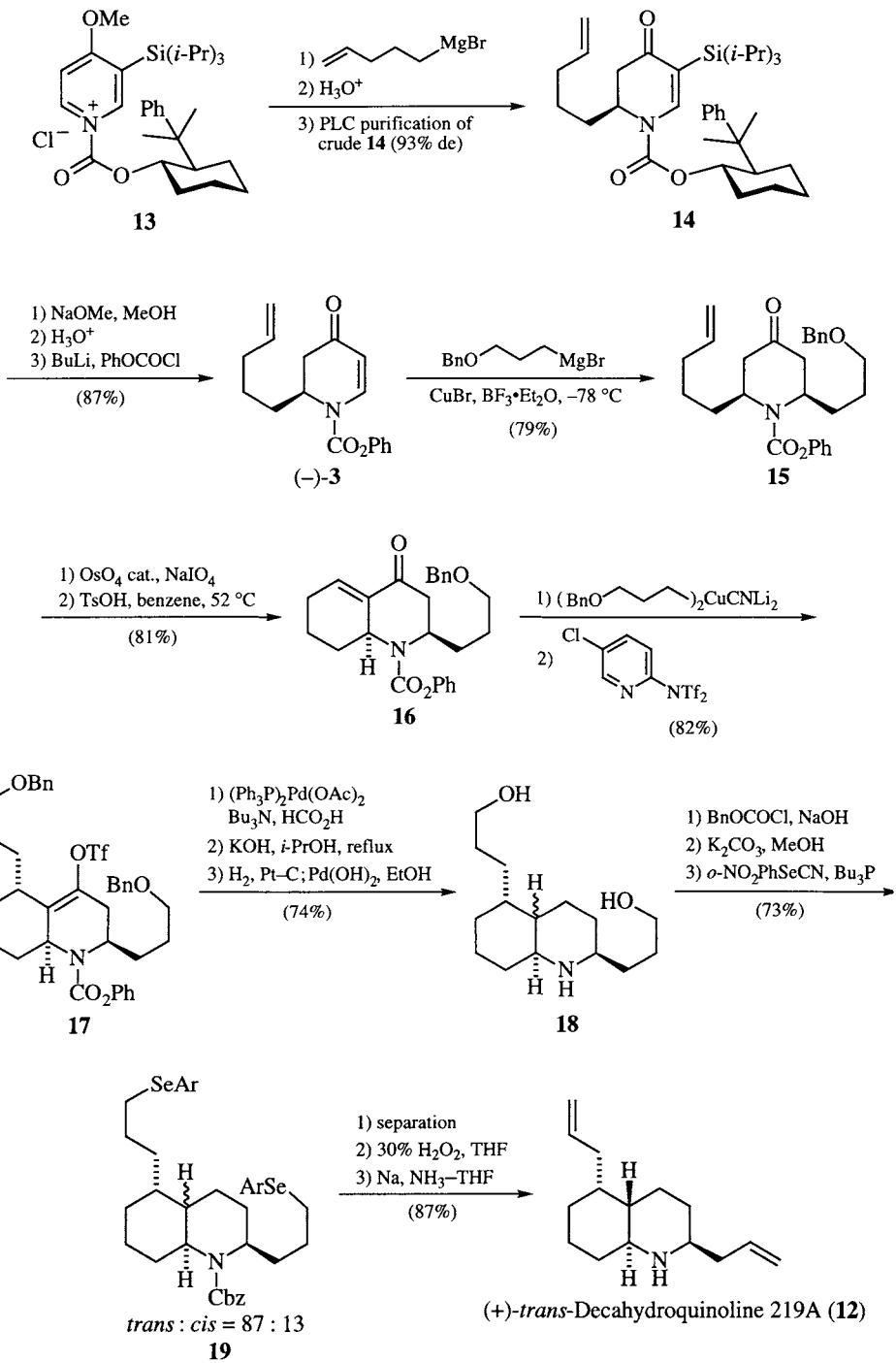
Scheme 1



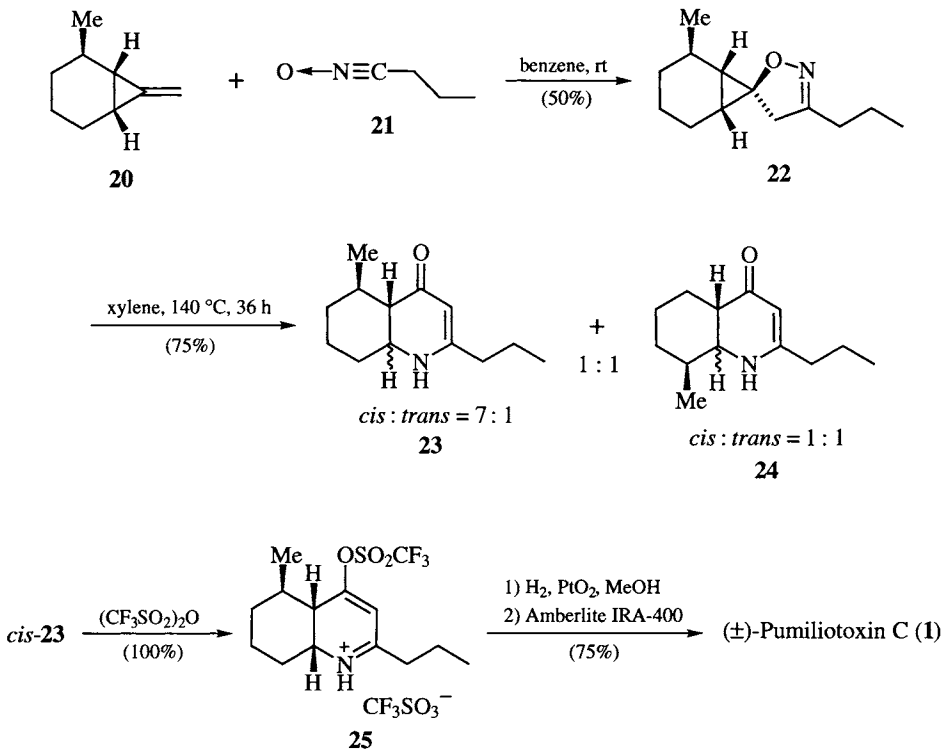
Scheme 2

A new route to (±)-pumiliotoxin C (**1**) by Brandi and co-workers (ref. 13) uses a key thermal rearrangement of an appropriate 5-spirocyclopropane (Scheme 4). The key spiroisoxazoline was obtained by the cycloaddition of butyronitrile **21** to *exo*-2-methyl-7-methylenebicyclo[4.1.0]heptane (**20**). Subsequent thermal rearrangement was performed by refluxing **22** in xylene at 140 °C to provide a 1:1 mixture of octahydroquinolines **23** and **24**, which were separated by flash chromatography. The quinolinone **23** was obtained as a 7:1 mixture of two diastereoisomers. Treatment of the major isomer *cis*-**23** with trifluoromethanesulfonic anhydride gave the iminium salt **25**, which was catalytically hydrogenated to yield (±)-pumiliotoxin C (**1**).

Polniaszek and Dillard (ref. 14) utilized [3,3] sigmatropic rearrangement of isoquinuclidines to construct the *cis*-fused hydroquinoline ring in a stereospecific total synthesis of (±)-pumiliotoxin C (Scheme 5). The *N*-acyl-1,2-dihydropyridine **26**, available from 4-methoxypyridine (**2**), was subjected to a stereospecific Diels-Alder reaction with (*E*)-bis(phenylsulfonyl)ethylene to give the adduct **27**. Sequential ketalization, reductive elimination of the sulfone moieties, and amide reduction converted **27** to the amino acetal **28**. After hydrolysis of **28**, the corresponding ketone underwent a stereospecific carbonyl addition reaction with (*E*)-2-(phenyldimethylsilyl)vinyllithium to give the divinyl carbinol **29**, which, upon treatment with potassium hydride, subjected to anionic oxy-Cope rearrangement to generate the octahydroquinolone **30**. The phenyldimethylsilyl moiety of **30** was then oxidized via the fluorodesilylation–oxidation procedure (ref. 15) to afford the β-hydroxy ketone **31**. The enone **32** obtained by dehydration was subjected to the conjugate addition with lithium dimethyl cuprate and the resultant copper enolate trapped with *N*-phenyltriflamide. Catalytic hydrogenation of the diene triflate **33** resulted in hydrogenolysis of both the *N*-benzyl and enol triflate moieties, with concomitant reduction of both double bonds and produced (±)-pumiliotoxin C (**1**) which was characterized as its hydrochloride salt.


OMe
Si(i-Pr)3
Ph
Cl−
13
1) MgBr
2) H3O+
3) PLC purification of crude 14 (93% de)
14
1) NaOMe, MeOH
2) H3O+
3) BuLi, PhOCOCl
(87%)
CO2Ph
(–)-3
BnO MgBr
CuBr, BF3•Et2O, –78 °C
(79%)
15
1) OsO4 cat., NaIO4
2) TsOH, benzene, 52 °C
(81%)
16
1) (BnO)2CuCNLi2
2)
NTf2
(82%)
OBn
OTf
17
1) (Ph3P)2Pd(OAc)2
Bu3N, HCO2H
2) KOH, i-PrOH, reflux
3) H2, Pt–C; Pd(OH)2, EtOH
(74%)
OH
HO
18
1) BnOCOCl, NaOH
2) K2CO3, MeOH
3) o-NO2PhSeCN, Bu3P
(73%)
SeAr
ArSe
Cbz
trans : cis = 87 : 13
19
1) separation
2) 30% H2O2, THF
3) Na, NH3–THF
(87%)
(+)-trans-Decahydroquinoline 219A (12)

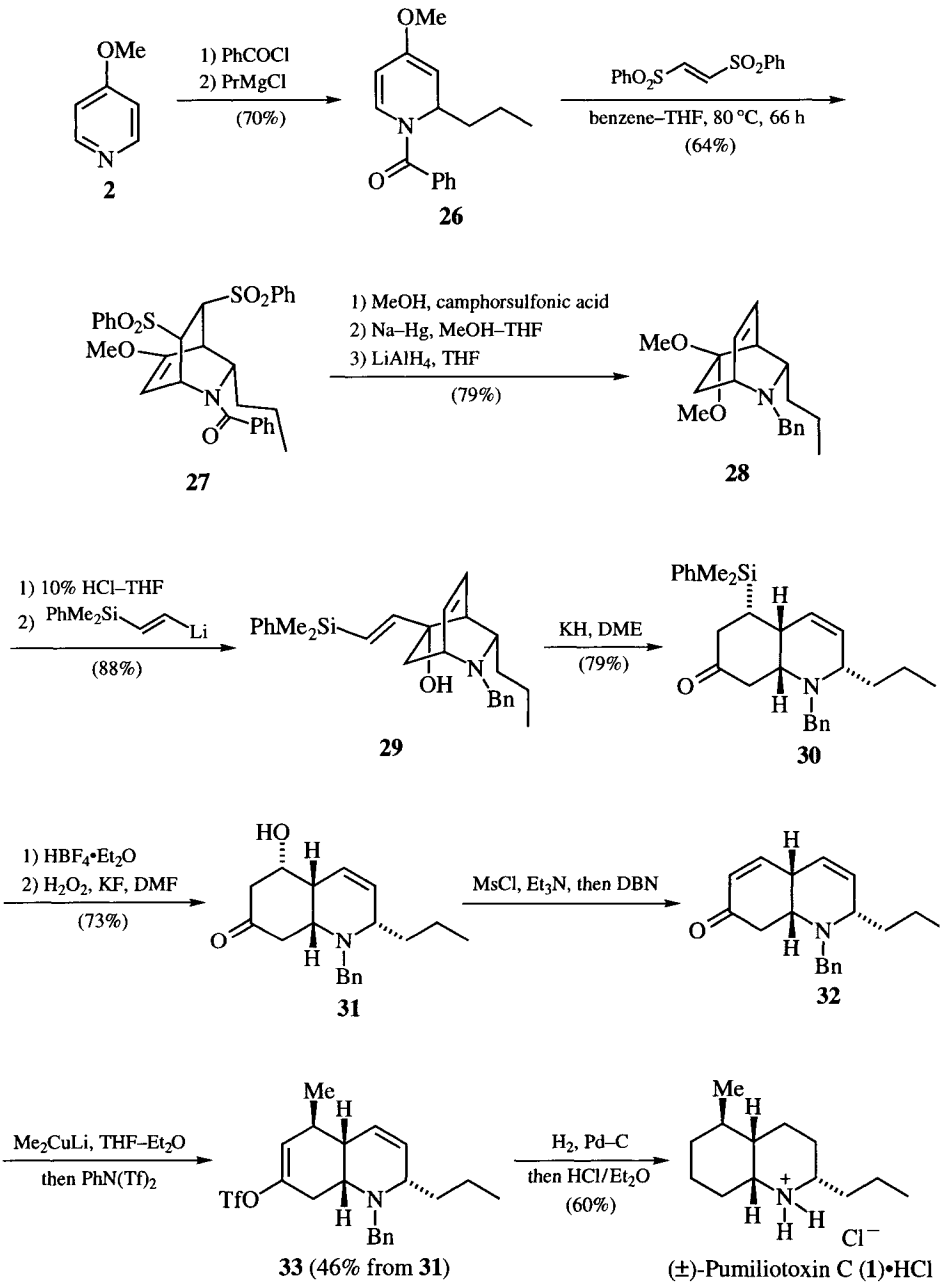
Scheme 3



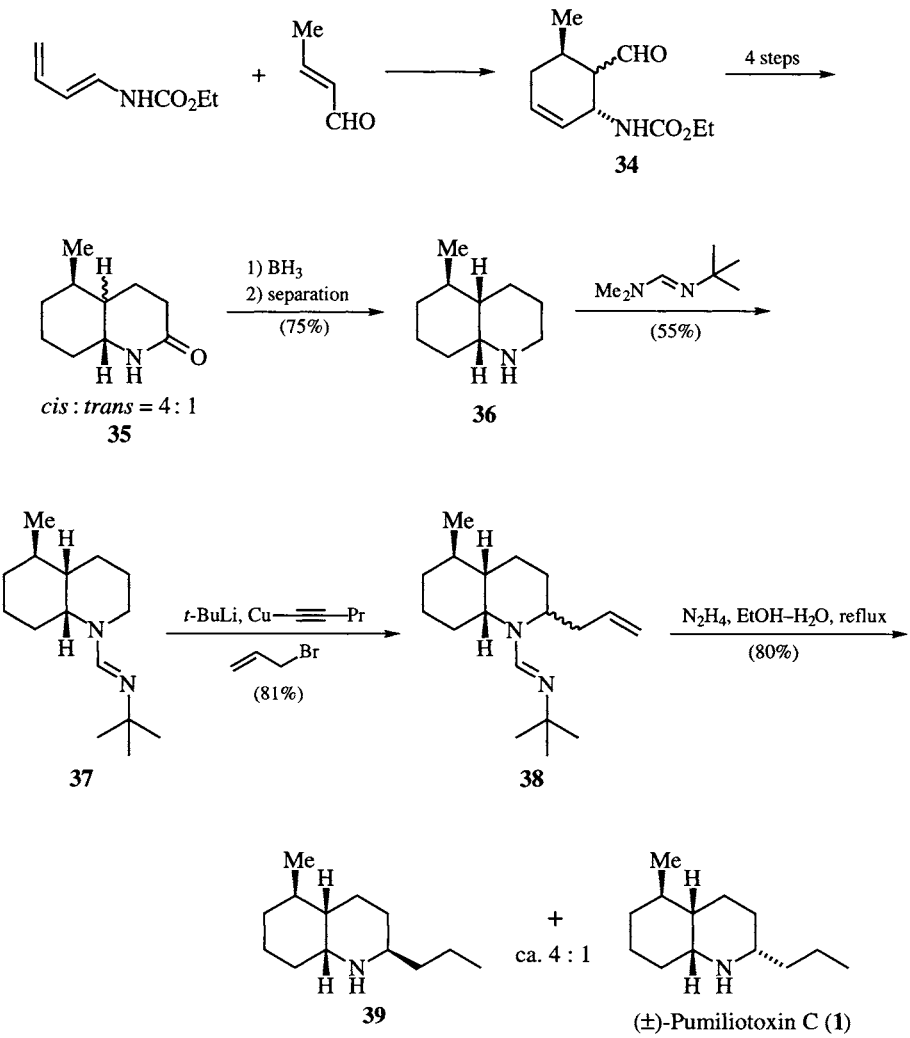
Scheme 4

An alternative synthesis of racemic **1** has been described by Meyers and Milot (ref. 16). This approach shown in Scheme 6 was based on α-alkylation of nitrogen heterocycles containing a formamidine moiety reported earlier (ref. 17). Thus, the bicyclic lactam **35**, prepared from **34** as a 4:1 mixture of *cis*- and *trans*-isomers according to the Diels–Alder strategy described by Overman (ref. 18), underwent borane reduction and chromatographic separation to give the *cis*-decahydroquinoline **36**. Thermal exchange with the (dimethylamino)formamidine converted **35** to the formamidine **37**. Metalation with *tert*-butyllithium followed by introduction of pentynyl copper was then followed by the addition of allyl bromide to afford the diastereomeric mixture **37**. When the mixture **38** was treated with hydrazine–ethanol–water, both the formamidine was removed and the propene side chain was reduced cleanly to furnish (±)-pumiliotoxin C (**1**) and its epimer **39** in a 1:4 ratio.

Mehta and Praveen (ref. 19) have demonstrated that a key precursor of (±)-pumiliotoxin C such as *cis*-hydroindanone **41** could be synthesized from a tricyclic dione such as **40** (Scheme 7). Regioselective Haller–Bauer cleavage (ref. 20) of **40** with 1% aqueous NaOH followed by esterification produced a 7:3 mixture of the bicyclic esters **41** and **42**, which were conveniently separated as the ketals. The ester functionality in the major ketal **43** was transformed to a methyl


OMe
1) PhCOCl
2) PrMgCl
(70%)
OMe
N
O
Ph
PhO2S
SO2Ph
benzene–THF, 80 °C, 66 h
(64%)
2
26
PhO2S
SO2Ph
MeO
N
O
Ph
1) MeOH, camphorsulfonic acid
2) Na–Hg, MeOH–THF
3) LiAlH4, THF
(79%)
MeO
MeO
N
Bn
27
28
1) 10% HCl–THF
2) PhMe2Si
Li
(88%)
PhMe2Si
OH
N
Bn
KH, DME
(79%)
PhMe2Si
H
O
H
N
Bn
29
30
1) HBF4•Et2O
2) H2O2, KF, DMF
(73%)
HO
H
O
H
N
Bn
MsCl, Et3N, then DBN
H
O
H
N
Bn
31
32
Me2CuLi, THF–Et2O
then PhN(Tf)2
Me
H
TfO
H
N
Bn
H2, Pd–C
then HCl/Et2O
(60%)
Me
H
H
N
H
H
Cl−
33 (46% from 31)
(±)-Pumiliotoxin C (1)•HCl

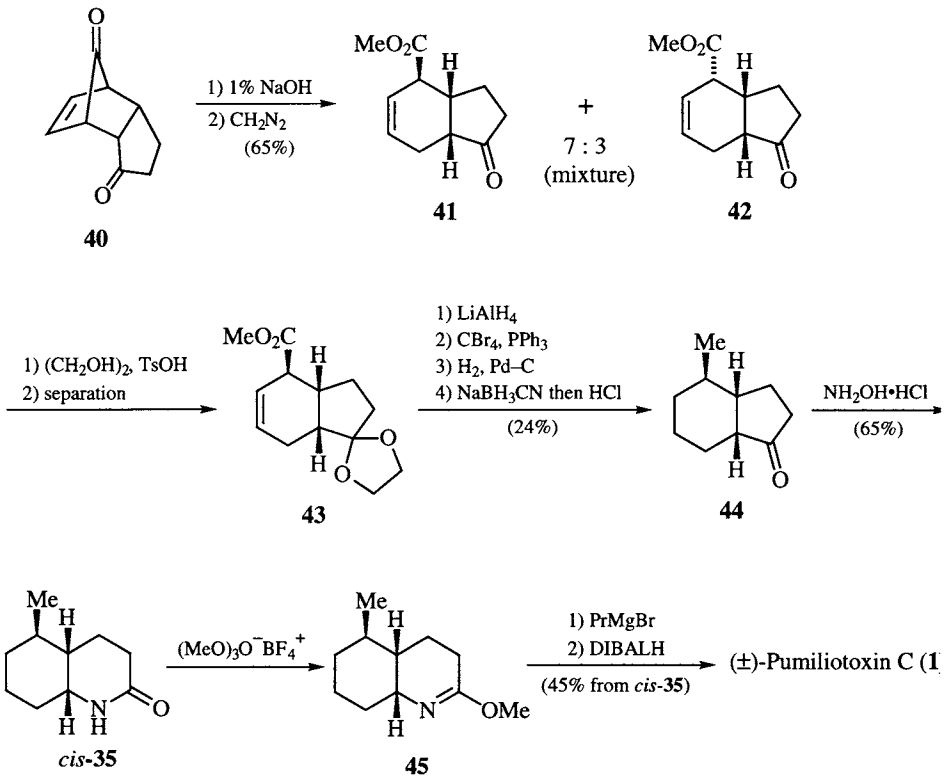
Scheme 5



Scheme 6

group and aqueous acid workup furnished the *cis*-hydroindanone **44** which was converted to (±)-pumiliotoxin C (**1**) via Beckmann rearrangement following a protocol similar to that previously described by Oppolzer's group (ref. 21).

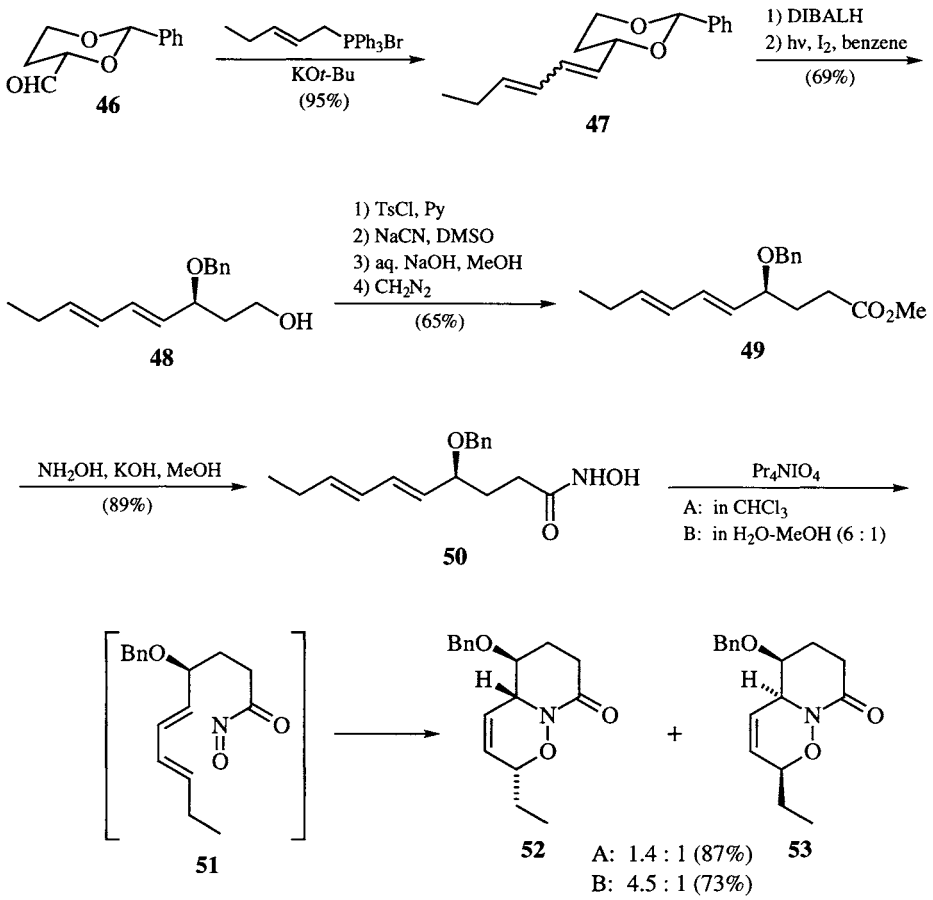
Recently, we have developed an enantioselective synthesis of (–)-pumiliotoxin C (ref. 22) in which the intramolecular hetero Diels–Alder reaction is performed on the acylnitroso compound as shown in Schemes 8 and 9. The Wittig reaction was carried out with (2*S*,4*S*)-4-formyl-2-phenyl-1,3-dioxane (**46**), available from L-malic acid (ref. 23), in which both the 2-phenyl and 4-formyl groups are preserved in equatorial conformation during the reaction without epimerizing to the axially



Scheme 7

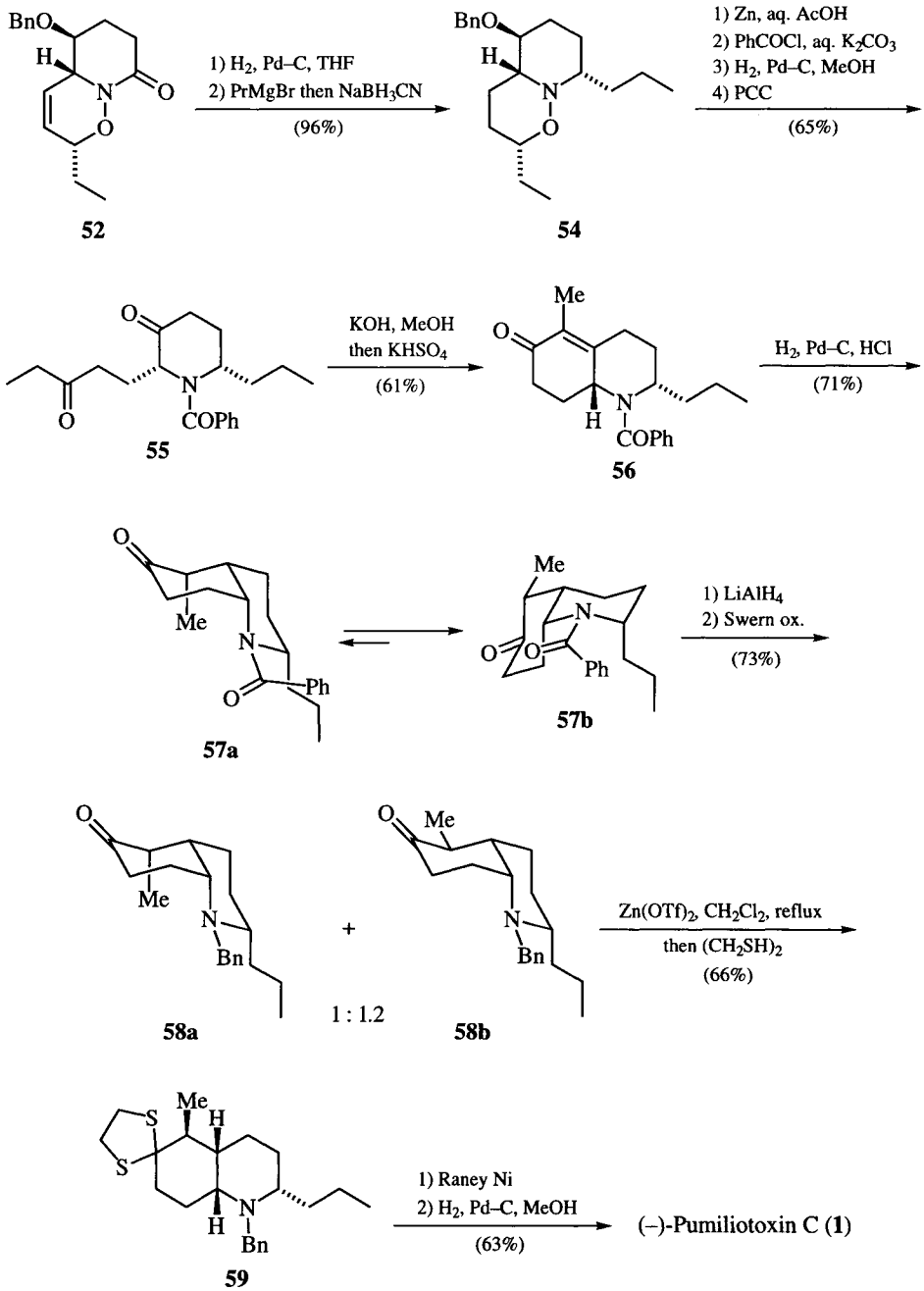
oriented 4-formyl-1,3-dioxane to give a 3:1 mixture of (*Z*)- and (*E*)-dienes **47**. Reductive ring-opening of the benzylidene acetal with DIBALH followed by photoisomerization by UV irradiation generated the geometrically pure (4*E*)-diene **48**, which was converted to the hydroxamic acid **50** by standard procedures. Our previous studies revealed (ref. 24) that use of aqueous media for intramolecular Diels–Alder reaction of chiral 1,3-diene acylnitroso compounds produces significant enhancement on the trans selectivity compared with non aqueous conditions. Consistent with these observations, on the treatment of with Pr_4NIO_4 at 0 °C in water–MeOH (6:1) (Scheme 8, method B) the (*S*)-hydroxamic acid **50** underwent cycloaddition via the in situ generated acylnitroso compound **51**, yielding the *trans* (with respect to C-4a and C-5) cycloadduct **52** as a major isomer with significantly increased diastereoselectivity of 4.5:1 compared with the reaction conducted in a chloroform solution which affords a 1.4:1 *trans/cis* ratio.

After catalytic hydrogenation of the olefin moiety of the *trans*-adduct **52**, introduction of the propyl side chain to the C-8 position to afford **54** was achieved in a completely stereoselective manner according to the tandem Grignard reaction–reduction procedure developed earlier in this laboratory (ref. 25). Reductive N–O bond cleavage with zinc and aqueous acetic acid yielded the amino alcohol which was transformed into the diketone **55** after protection, deprotection, and



Scheme 8

oxidation. Aldol cyclization of **55** followed by catalytic hydrogenation of **56** established the *all-cis*-decahydroquinolone ring system with the incorrect α-methyl substitution at C-5, which should exist preferentially in the ring conformation **57b** in order to minimize $A^{1,3}$ strain (ref. 26) between the *N*-benzoyl group and the C-2 propyl group. Therefore, the C-5 methyl group is presumably placed in the energetically favored equatorial conformation. In an anticipation of epimeric transformation of the α-methyl to β-methyl group, such interaction due to $A^{1,3}$ type strain was released by replacing the *N*-benzoyl group by the *N*-benzyl group to give **58**, in which the stable ring conformation with the C-5 methyl group in axial (as **58a**) would be preponderant. Thus, under these reaction conditions, epimerization of the axial isomer **58a** to the more stable equatorial isomer **58b** occurred to afford a mixture of the 5α- and 5β-methyl isomers **58a** and **58b** in a ratio of 1:1.2. After prolonged heating of this epimeric mixture with zinc triflate, treatment with ethanedithiol resulted in the dithioketal **59** with exclusive epimerization to the 5β-methyl isomer. Finally, **59** was converted to (–)-pumiliotoxin C (**1**) by desulfurization and deprotection.


BnO
H
N
O
O
1) H2, Pd–C, THF
2) PrMgBr then NaBH3CN
(96%)
1) Zn, aq. AcOH
2) PhCOCl, aq. K2CO3
3) H2, Pd–C, MeOH
4) PCC
(65%)
52
54
KOH, MeOH
then KHSO4
(61%)
Me
COPh
H2, Pd–C, HCl
(71%)
55
56
Ph
57a
57b
1) LiAlH4
2) Swern ox.
(73%)
Bn
58a
1 : 1.2
58b
Zn(OTf)2, CH2Cl2, reflux
then (CH2SH)2
(66%)
S
59
1) Raney Ni
2) H2, Pd–C, MeOH
(63%)
(–)-Pumiliotoxin C (1)

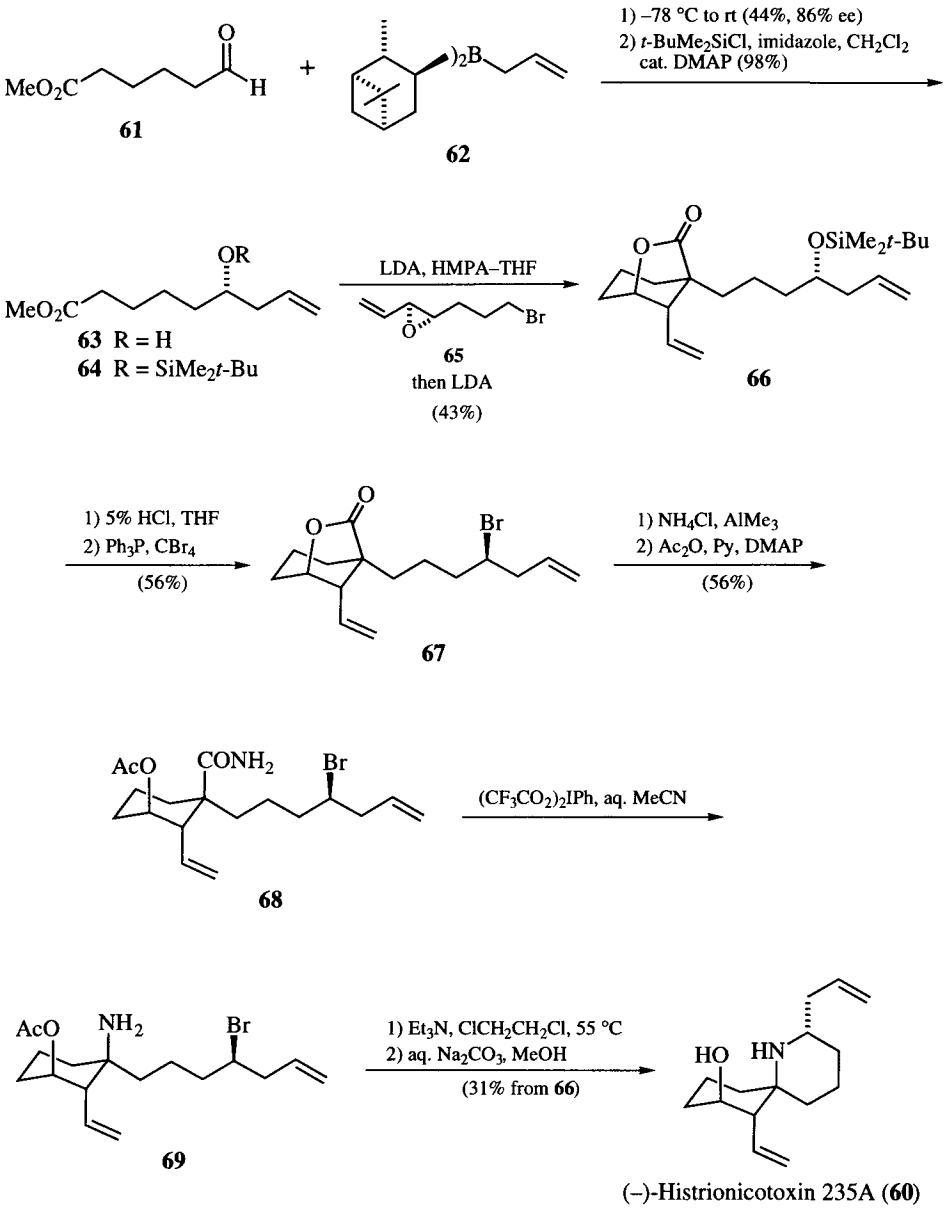
Scheme 9

3. SYNTHESES OF HISTRIONICOTOXINS

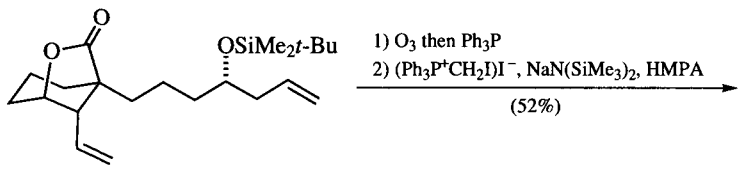
An enantioselective route to (–)-histrionicotoxin 235A (**60**) has been first published by the group of Stork (ref. 27) in which the "allylic epoxide cyclization" method previously disclosed by the same group (ref. 28) was exploited (Scheme 10). The synthesis began with the optically active methyl (*S*)-6-hydroxy-8-nonenoate (**63**), readily accessible by the reaction of methyl 6-oxohexanoate (**61**) and *B*-allyldiisopinocamphenylborane (**62**) in 86% ee. The silyl ether **64** of the alcohol **63** nderwent the allylic epoxide cyclization in a single operation to give the lactone **66**, in which three chiral centers are correctly set for eventual transformation to (–)-histrionicotoxins. Removal of the silyl protecting group followed by inversive bromination provided **67**, which was then converted to the amide **68** by treatment with trimethylaluminum–ammonium chloride followed by acetic anhydride. Hofmann-like rearrangement with phenyliodonium bistrifluoroacetate and subsequent intramolecular cyclization of **69** at 55 °C with triethylamine followed by deprotection resulted in (–)-histrionicotoxin 235A (**60**).

Stork's group (ref. 28) has also described the first enantioselective synthesis of (–)-histrionicotoxin (**70**) through the use of the above strategy starting from the bicyclic lactone **66**. This approach outlined in Scheme 11 began with elaboration of the preparation of the bis((*Z*)-vinyl iodide) **71** via ozonolysis followed by iodomethylenation. In a similar manner as described above for the synthesis of histrionicotoxin 235A, **71** was converted to the spiropiperidine **72**, which was coupled with (trimethylsilyl)acetylene and further converted to the natural (–)-enantiomer of histrionicotoxin (**70**) upon deprotection of the resulting dienyne.

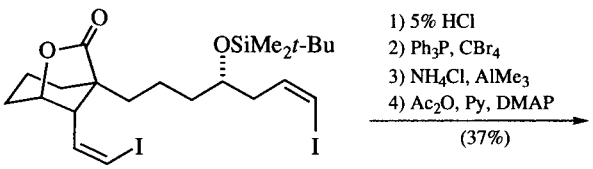
Iwata et al. (ref. 29) have reported a formal synthesis of (+)-perhydrohistrionicotoxin (**74**). The key step is the formation of a spiro skeleton via a carbonyl ene reaction and a palladium-catalyzed carbonyl allylation (Scheme 12). The bromide **75**, derived from cyclopentanone, was lithiated and treated with (–)-menthyl (*S*)-*p*-toluenesulfinate to give the chiral vinylic sulfoxide **76**, which on treatment with allylmagnesium bromide underwent the Pummerer-type reaction to give the vinylic sulfide **77** in 90% ee. Sequential hydroboration–oxidation, mesylation, and cyanation provided the nitrile **78**, which was converted to the aldehyde **79** via deacetalization followed by Horner–Emmons reaction. The palladium catalyzed intramolecular carbonyl allylation of **79** proceeded diastereoselectively via the transition state **80**, affording **81** along with two isomers in 83:17. Conversion to the ketol **82** was performed in 5 steps involving elaboration of the butyl side chain. Subsequent Beckman rearrangement of the oxime of **82** led to the known spiro lactam **83**, providing a formal synthesis of (+)-perhydrohistrionicotoxin (**74**), since this lactam has been converted to **74** by the other group (ref. 30).


MeO2C
O
H
61
+
)2B
62
1) –78 °C to rt (44%, 86% ee)
2) t-BuMe2SiCl, imidazole, CH2Cl2
cat. DMAP (98%)
OR
MeO2C
63 R = H
64 R = SiMe2t-Bu
LDA, HMPA–THF
Br
65
then LDA
(43%)
O
O
OSiMe2t-Bu
66
1) 5% HCl, THF
2) Ph3P, CBr4
(56%)
Br
67
1) NH4Cl, AlMe3
2) Ac2O, Py, DMAP
(56%)
AcO
CONH2
Br
68
(CF3CO2)2IPh, aq. MeCN
AcO
NH2
Br
69
1) Et3N, ClCH2CH2Cl, 55 °C
2) aq. Na2CO3, MeOH
(31% from 66)
HO
HN
(–)-Histrionicotoxin 235A (60)

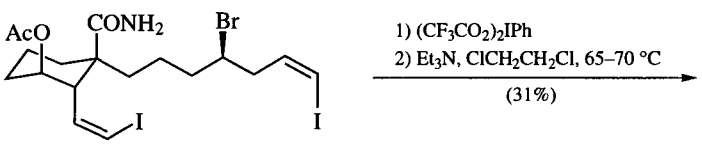
Scheme 10



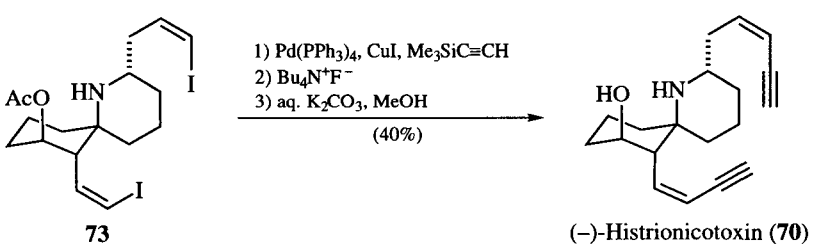
66



71



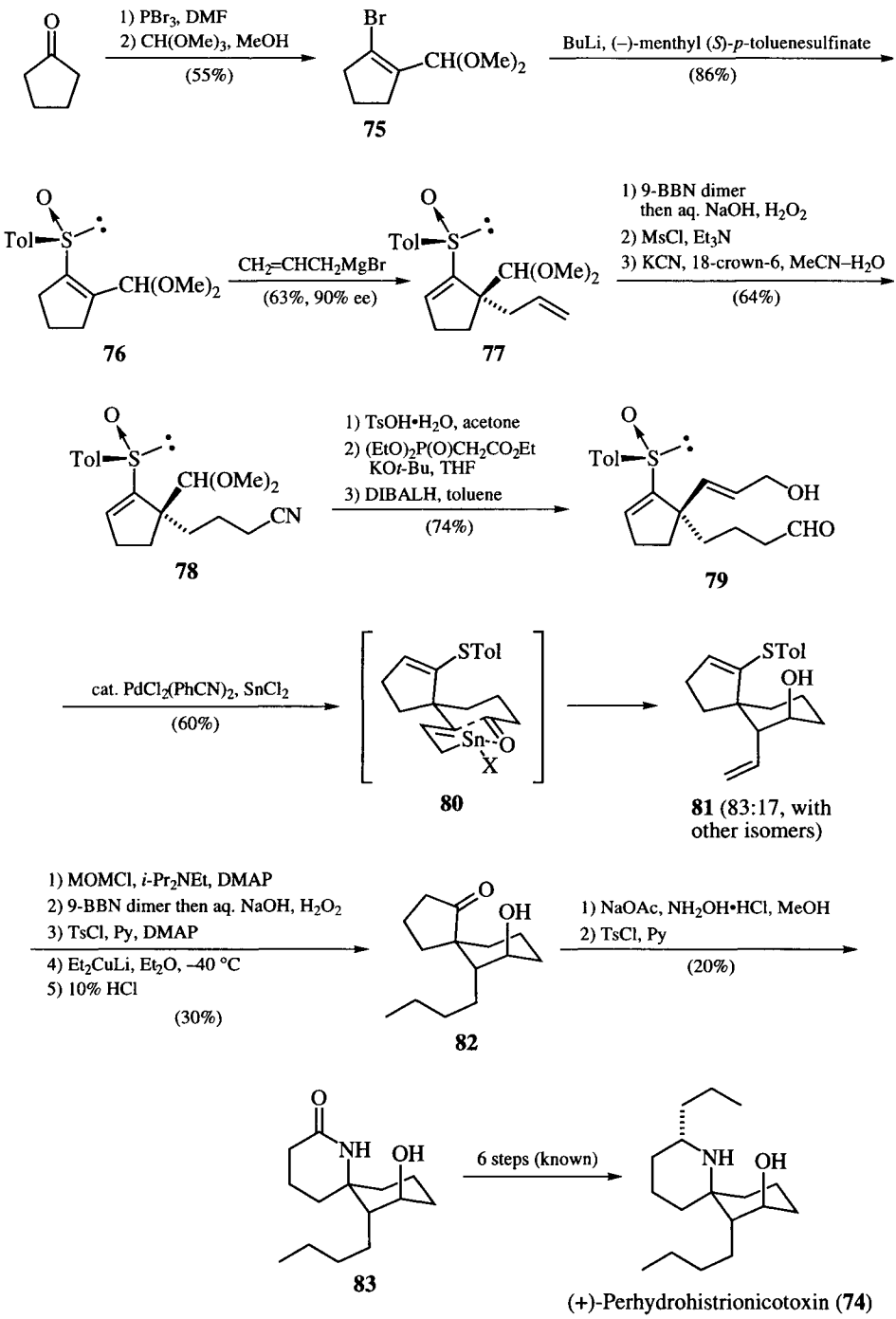
72



73

(–)-Histrionicotoxin (**70**)

Scheme 11


1) PBr3, DMF
2) CH(OMe)3, MeOH
(55%)
Br
CH(OMe)2
75
BuLi, (–)-menthyl (S)-p-toluenesulfinate
(86%)
Tol
S
CH(OMe)2
76
CH2=CHCH2MgBr
(63%, 90% ee)
77
1) 9-BBN dimer
then aq. NaOH, H2O2
2) MsCl, Et3N
3) KCN, 18-crown-6, MeCN–H2O
(64%)
CN
78
1) TsOH•H2O, acetone
2) (EtO)2P(O)CH2CO2Et
KOt-Bu, THF
3) DIBALH, toluene
(74%)
OH
CHO
79
cat. PdCl2(PhCN)2, SnCl2
(60%)
STol
Sn
O
X
80
OH
81 (83:17, with
other isomers)
1) MOMCl, i-Pr2NEt, DMAP
2) 9-BBN dimer then aq. NaOH, H2O2
3) TsCl, Py, DMAP
4) Et2CuLi, Et2O, –40 °C
5) 10% HCl
(30%)
82
1) NaOAc, NH2OH•HCl, MeOH
2) TsCl, Py
(20%)
NH
83
6 steps (known)
(+)-Perhydrohistrionicotoxin (74)

Scheme 12

4. SYNTHESES OF INDOLIZIDINE ALKALOIDS

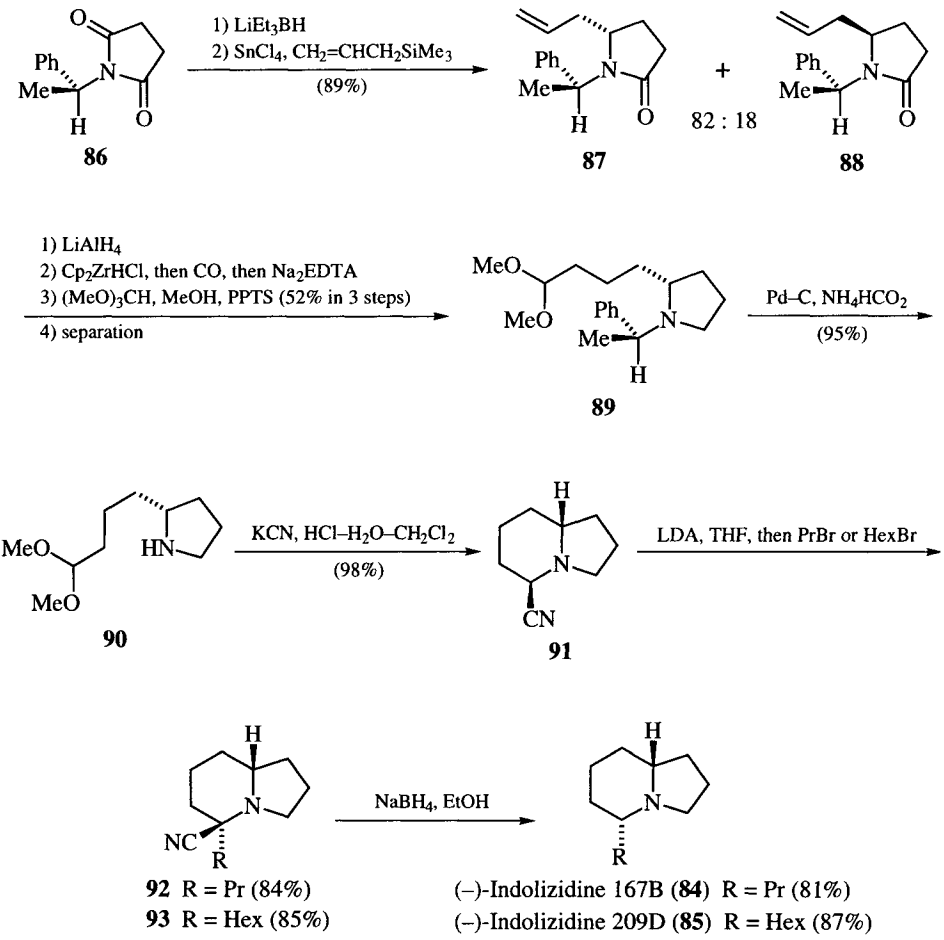
The indolizidine group of the dendrobatid alkaloids is classified into 3 small subgroups based on the substitution pattern of the alkyl group(s) on the indolizidine skeleton as follows: (a) 5-substituted indolizidines; (b) 3,5-disubstituted indolizidines; (c) 5,8-disubstituted indolizidines.

4.1 5-Substituted Indolizidine Alkaloids

The structurally simplest indolizidine alkaloids 167B and 209D bearing a single substituent at the C-5 were detected once as trace components in unidentified dendrobatid frogs found in a single population (ref. 31). Their structures have been tentatively assigned as **84** and **85** based on mass spectral evidence whereas their absolute configurations were simply inferred as 5*R*,9*R* by analogy to the structurally related indolizidine 223AB whose absolute stereochemistry is known.

The first enantioselective syntheses of these alkaloids 167A (**84**) and 209D (**85**) have been reported by Polniaszek and Belmont (ref. 32) by using diastereoselective *N*-acyliminium ion reaction (Scheme 13). Thus, the succinimide **86** bearing (*S*)-α-phenethylamine as a chiral auxiliary was reduced with lithium trietylborohydride to give the hydroxy lactam diastereomers, which was further treated with allyltrimethylsilane in the presence of $SnCl_4$ to generate an 82:18 mixture of allyllactam diastereomers **87** and **88** via *N*-acyliminium ion formation. This mixture was sequentially subjected to reduction with $LiAlH_4$, hydrozirconation, carbonylation, and acetalization to provide a diastereomeric mixture of the dimethyl acetals, which were separated by medium-pressure liquid chromatography to afford **89** as a major isomer. The chiral auxiliary was hydrogenolyzed and the resultant secondary amine **90** was cyclized to **91** on hydrolysis in the presence of hydrogen cyanide according to the Husson protocol (ref. 33). After deprotonation with LDA, alkylation with either propyl bromide or hexyl bromide afforded the alkylated amino nitriles **92** and **93**, which were converted to (–)-indolizidines 167B (**84**) and 209D (**85**), respectively, by reduction with sodium borohydride.

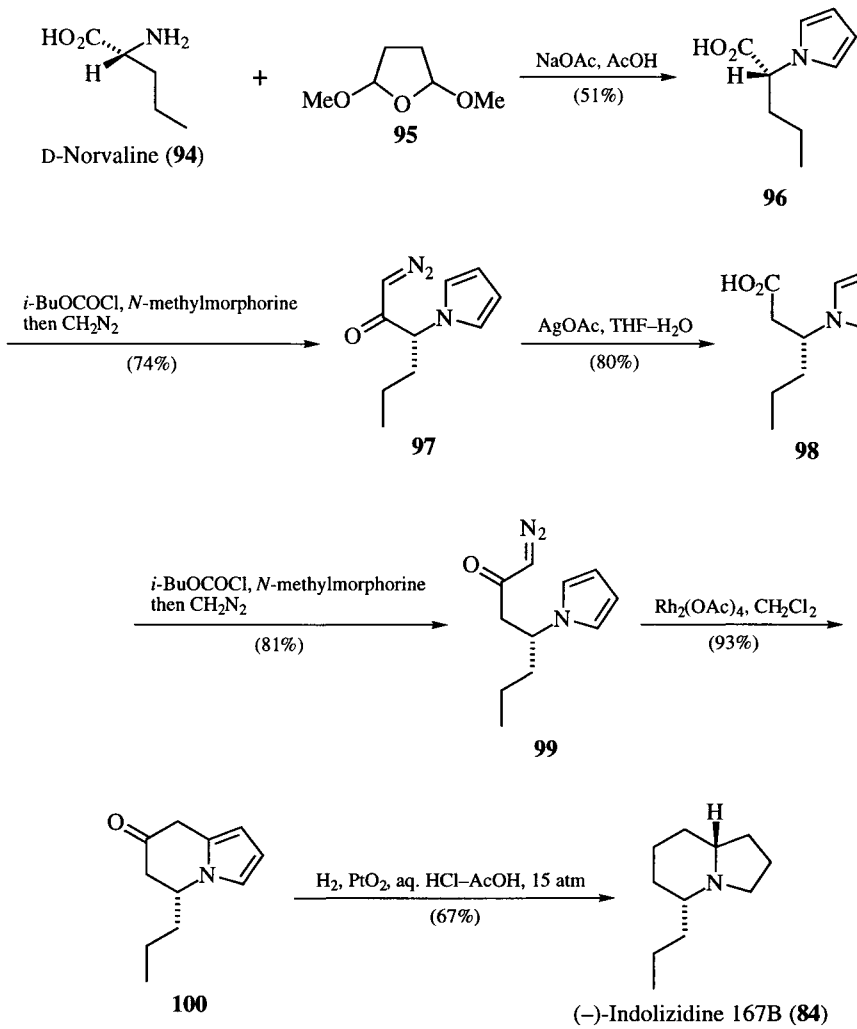
Jefford et al. (ref. 34) have developed an alternative enantiogenic synthesis of (–)-indolizidine 167B utilizing D-norvaline (**94**) as a chiral starting material as shown in Scheme 14. Thus, the desired enantiogenic chirality was installed by condensation of 2,5-dimethoxytetrahydrofuran (**95**) with D-norvaline. The resulting 1-pyrrolylacetic acid **96** was converted to the α-diazo ketone **97** by reaction of its mixed anhydride with diazomethane. Wolff rearrangement and repetition of the mixed anhydride–diazomethane procedure on **97** afforded the corresponding α-diazo ketone **99**, which was catalytically decomposed with rhodium(II) acetate to provide the dihydroindolidine **100**. Desired (–)-indolizidine 167B (**84**) was obtained by hydrogenation with Adams catalyst at 15 atm under acid conditions. The complete reduction of the non-conjugated carbonyl group in **100** is quite unusual and suggested to occur by a Clemmensen-type deoxygenation (ref. 35).



Scheme 13

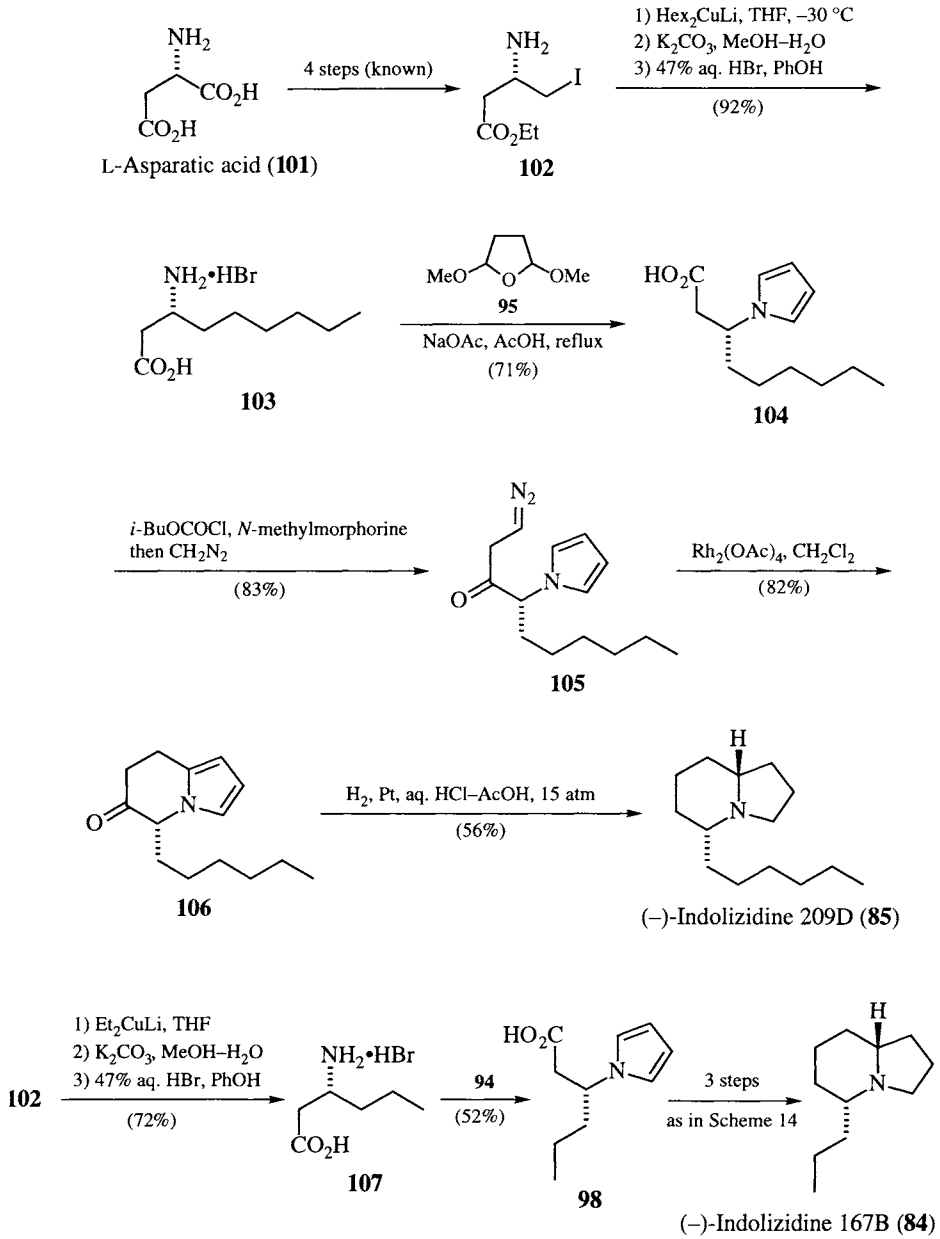
Jefford and his colleague (ref. 36) have also accomplished the enantiogenic syntheses of (–)-indolizidines 167B and 209D from L-aspartic acid (**101**) as a chiral precursor (Scheme 15) by using a very similar reaction sequence as discussed above (see Scheme 14). The chirally modified pyrrol analogue **104** was synthesized by condensation of 2,5-dimethoxytetrahydrofuran (**95**) with (*R*)-β-amino acid hydrobromide **103**, derived from L-aspartic acid (**101**) via the iodo ester **102** in 7 steps. The mixed anhydride–diazomethane procedure was applied to **104** to produce the α-diazoketone **105**, which was subjected to cyclization with catalytic rhodium(II) acetate followed by hydrogenation of the resulting bicyclic keto pyrrole **106** to afford (–)-indolizidine 209D.

Furthermore, (–)-indolizidine 167B (**84**) was formally prepared by following the same synthetic sequence employing the iodo ester **102** as a precursor. The chirally modified pyrrol analogue **98**, derived via the (*R*)-β-amino acid hydrobromide **107**, had been converted to (–)-indolizidine 167B in 3 steps as discussed above (see Scheme 14).

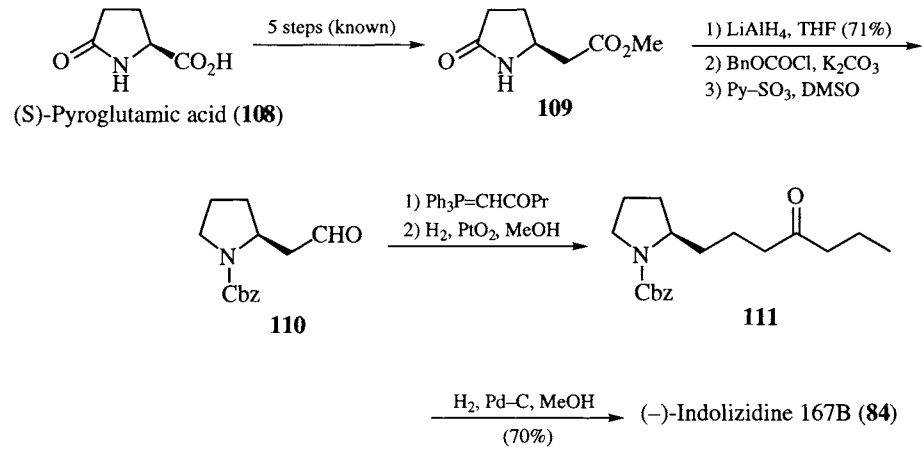


Scheme 14

A versatile and practical approach to (–)-indolizidine 167B has been published by Lhommet et al. (ref. 37). This approach (Scheme 16) employed (*S*)-pyroglutamic acid (**108**) as a chiral precursor which was converted to the ester **109** in five steps in a straightforward manner (ref. 38): esterification followed by reduction, tosylation, cyanation, then acidic methanolysis. Reduction of **109** with $LiAlH_4$ followed by protection and oxidation with sulfur trioxide–pyridine complex provided the aldehyde **110**, which was subsequently transformed into the ketone **111** via Wittig reaction with 1-triphenylphosphoranylidenepentan-2-one followed by hydrogenation. Upon hydrogenation of **111** over palladium on carbon in methanol, cyclization proceeded by way of an iminium intermediate to lead to (–)-indolizidine 167B.


NH2
CO2H
CO2H
L-Asparatic acid (101)
4 steps (known)
NH2
I
CO2Et
102
1) Hex2CuLi, THF, –30 °C
2) K2CO3, MeOH–H2O
3) 47% aq. HBr, PhOH
(92%)
NH2•HBr
CO2H
103
MeO
OMe
95
NaOAc, AcOH, reflux
(71%)
HO2C
104
i-BuOCOCl, N-methylmorphorine
then CH2N2
(83%)
N2
105
Rh2(OAc)4, CH2Cl2
(82%)
106
H2, Pt, aq. HCl–AcOH, 15 atm
(56%)
(–)-Indolizidine 209D (85)
102
1) Et2CuLi, THF
2) K2CO3, MeOH–H2O
3) 47% aq. HBr, PhOH
(72%)
NH2•HBr
CO2H
107
94
(52%)
HO2C
98
3 steps
as in Scheme 14
(–)-Indolizidine 167B (84)

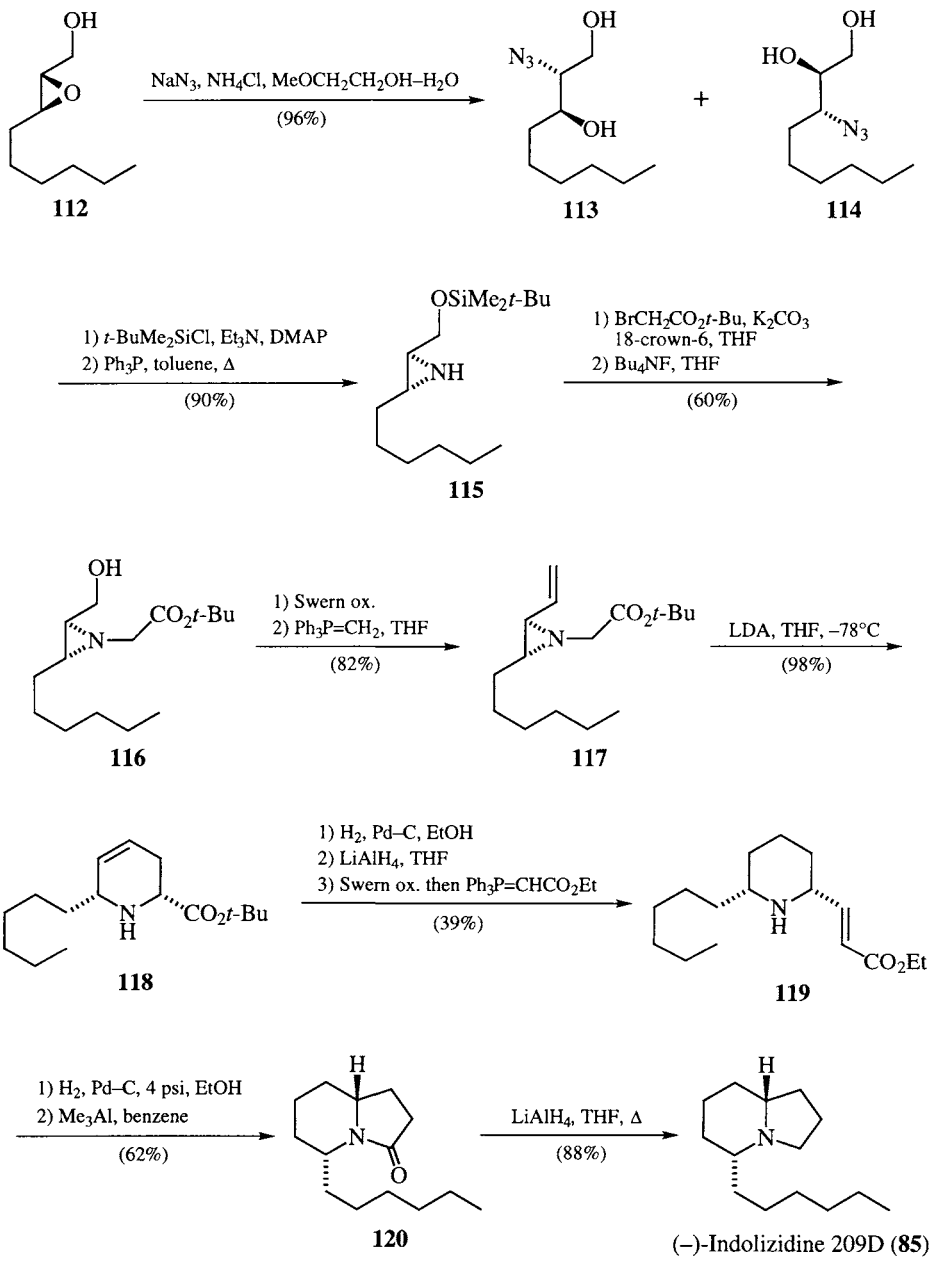
Scheme 15



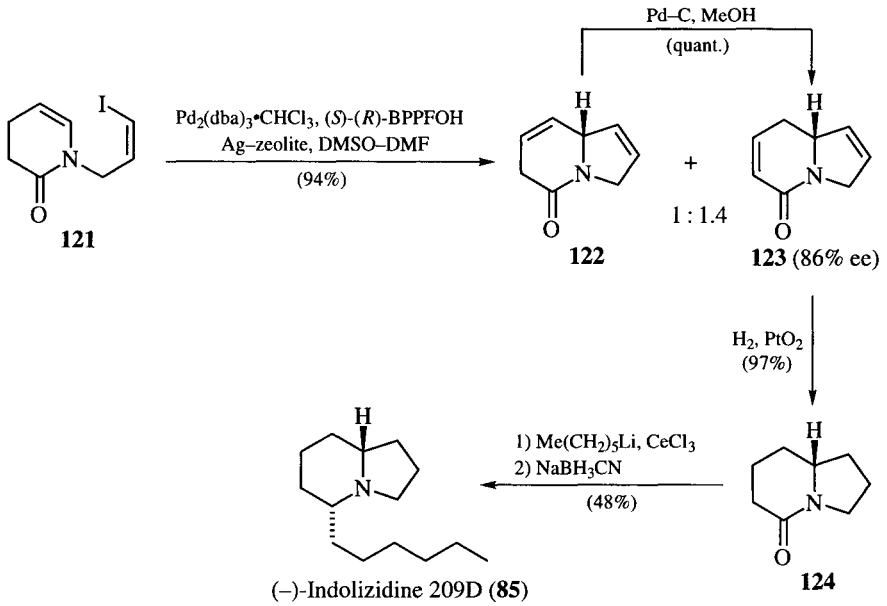
Scheme 16

Recently, Somfai and Åhman (ref. 39) have developed a novel enantioselective entry to (–)-indolizidine 209D employing an aza-[2,3]-Wittig rearrangement as a key step (ref. 40) and based on a different disconnection involving the elaboration of the five-membered ring on to a substituted piperidine system (Scheme 17). The key intermediate, rearrangement precursor **117**, was prepared in a straightforward manner from the known epoxy alcohol **112** (ref. 41). Exposure of **112** to sodium azide gave the corresponding azidodiols **113** and **114** as a mixture of regioisomers. Selective protection of the primary hydroxy group as a silyl ether followed by reductive cyclization with triphenylphosphine (ref. 42) gave the aziridine **115**. The required anion-stabilizing group for the rearrangement was introduced by reaction of **115** with *tert*-butyl bromoacetate which was followed by removal of the silyl group to yield the alcohol **116**, which was then converted to the vinylaziridine **117** by Swern oxidation followed by Wittig olefination. Treatment of **117** with LDA at –78 °C led to aza-[2,3]-Wittig rearrangement to provide the *cis*-2,6-disubstituted tetrahydropyridine **118** as a single diastereomer. Hydrogenation followed by reduction of the ester moiety afforded the amino alcohol, which was subjected to the one-pot Swern–Wittig protocol developed by Ireland (ref. 43) to yield the α,β-unsaturated ester **119**. Hydrogenation of **119** followed by Me_3Al induced lactam formation (ref. 44) and $LiAlH_4$ reduction resulted in (–)-indolizidine 209D (**85**).

Another enantioselective entry to (–)-indolizidine 209D has been published by Shibasaki et al. (ref. 45) (Scheme 18). This approach is based on the catalytic, asymmetric Heck reaction previously developed by Shibasaki's group (ref. 46). The reaction has been applied to the iodide **121** with catalytic amounts of $Pd_2(dba)_3$•$CHCl_3$ and (*S*)-(*R*)-BPPFOH to generate a 1:1.4 mixture of the (*R*)-tetrahydroindolizinones **122** and **123** in 86% ee, the former product of which could completely be isomerized to **123** by treatment with a catalytic amount of palladium on carbon in methanol. Preparation of (–)-indolizidine 209D (**85**) was completed by hydrogenation of **123** followed by stereoselective introduction of the hexyl side chain to the C-5 of the indolizidinone **124**.


OH
O
NaN3, NH4Cl, MeOCH2CH2OH–H2O
(96%)
112
OH
N3
OH
113
+
OH
HO
N3
114
1) t-BuMe2SiCl, Et3N, DMAP
2) Ph3P, toluene, Δ
(90%)
OSiMe2t-Bu
NH
115
1) BrCH2CO2t-Bu, K2CO3
18-crown-6, THF
2) Bu4NF, THF
(60%)
OH
CO2t-Bu
N
116
1) Swern ox.
2) Ph3P=CH2, THF
(82%)
CO2t-Bu
N
117
LDA, THF, –78°C
(98%)
N
H
CO2t-Bu
118
1) H2, Pd–C, EtOH
2) LiAlH4, THF
3) Swern ox. then Ph3P=CHCO2Et
(39%)
N
H
CO2Et
119
1) H2, Pd–C, 4 psi, EtOH
2) Me3Al, benzene
(62%)
H
N
O
120
LiAlH4, THF, Δ
(88%)
H
N
(–)-Indolizidine 209D (85)

Scheme 17



Scheme 18

4.2 3,5-Disubstituted Indolizidine Alkaloids

The 3,5-disubstituted indolizidine alkaloids so far fully characterized are indolizidines 195B (**125**), 223AB (**126**), 239AB (**127**), and 239CD (**128**) (Figure 1). The latter three natural alkaloids 223AB and its ω-hydroxy side chain congeners 239AB and 239CD are all levorotatory. The synthesis of 3*R*,5*R*,8a*R* enantiomer of indolizidine 223AB achieved by Husson et al. (ref. 33) established the absolute stereochemistry of natural indolizidine 223AB. The absolute configuration of both the natural ω-hydroxy congeners 239AB and 239CD have been inferred to be 3*R*,5*S*,8a*R* and 3*R*,5*R*,8a*R*, respectively, in analogy with that of 223AB.

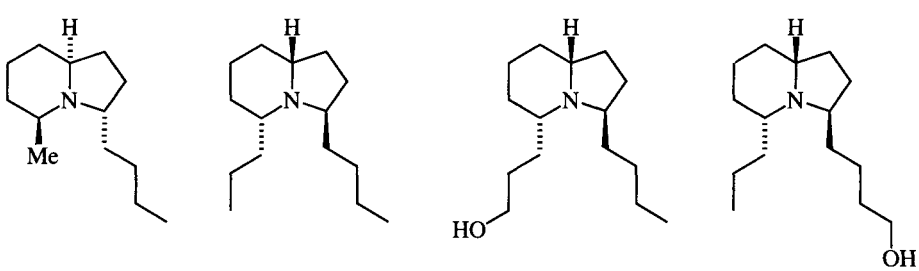
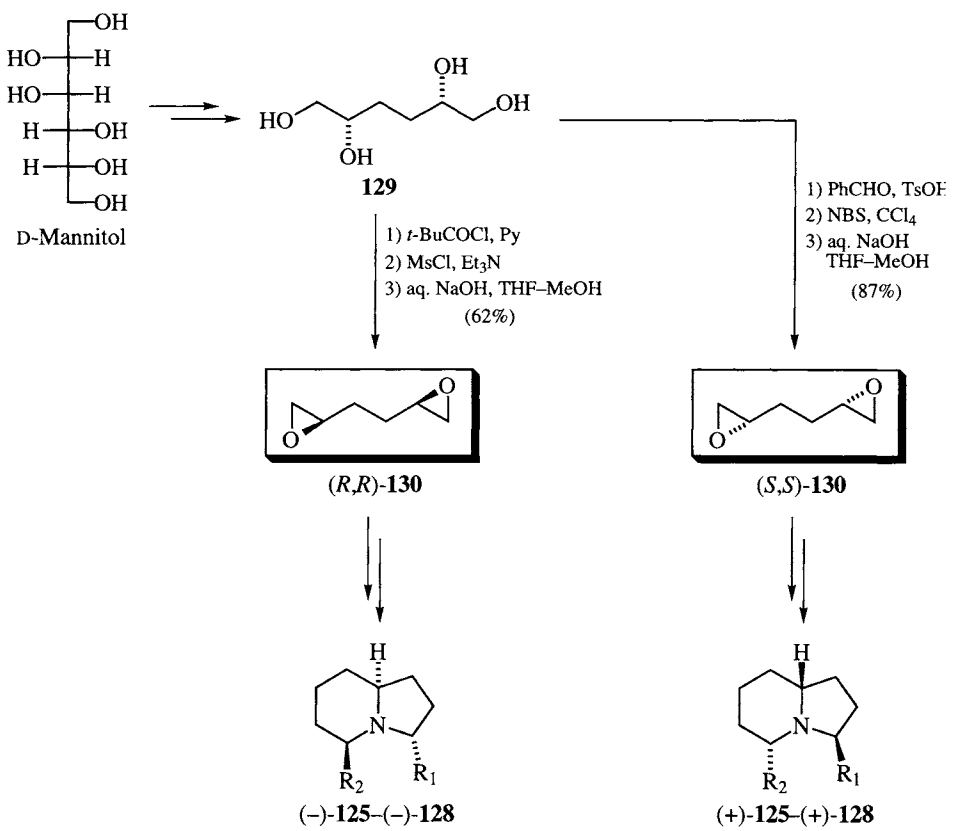


Figure 1. Naturally occurring 3,5-disubstituted indolizidine alkaloids.

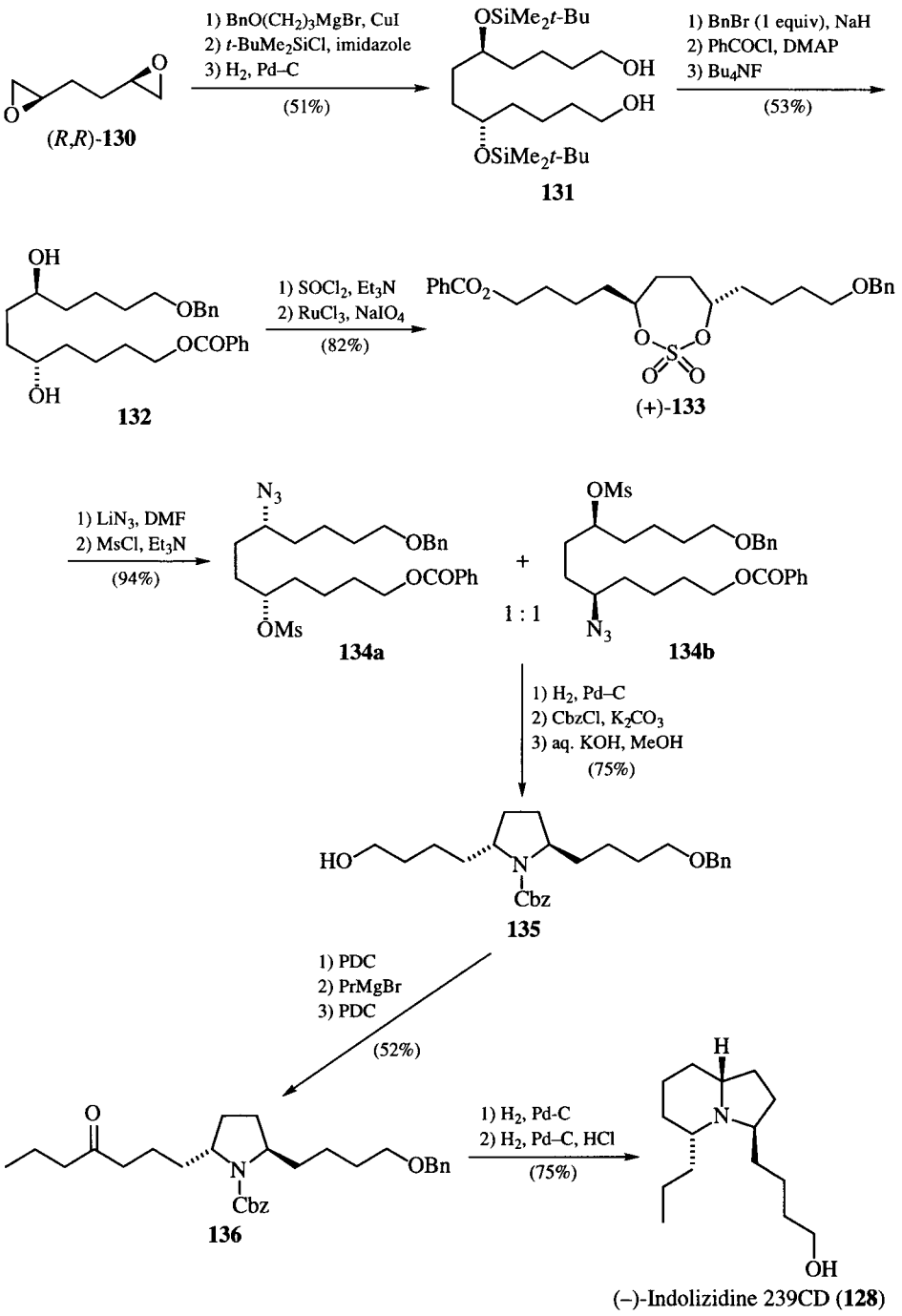
Another alkaloid 195B, unlike 223AB, 239AB, and 239CD, is dextrorotatory, which suggests that this alkaloid has the opposite, namely 3*S*,5*S*,8a*S*, configuration compared to the other 3,5-disubstituted indolizidines. The proposed absolute stereochemistry of 195B has previously been confirmed by the syntheses of both enantiomers of 195B achieved in our laboratory (ref. 47).

Our approach to the syntheses of the both enantiomers of all four indolizidine alkaloids has utilized 3,4-dideoxy-D-*threo*-hexitol (**129**) as a single common chiral synthon, derived from D-mannitol (Scheme 19) (ref. 48). In this enantiodivergent synthesis, **129** is stereoselectively converted to the versatile chiral building blocks of C_2 symmetry (*R*,*R*)- and (*S*,*S*)-bisepoxides (**130**) (ref. 49) each in three steps (ref. 49), which respectively serve as the precursors for the (–) and (+)-series of indolizidines 195B (**125**), 223AB (**126**), 239AB (**127**), and 239CD (**128**) (Figure 1).

With the (*R*,*R*)-bisepoxide (*R*,*R*)-**130** in hand, (–)-indolizidine 239CD [(–)-**128**] was initially prepared as outlined in Scheme 20. The (*R*,*R*)-**130** was converted to the diol (**131**) via epoxide ring-opening with the Grignard reagent and a catalytic amount of copper(I) iodide followed by protection and hydrogenolysis. After selective protection of the primary hydroxyl function by the benzyl group and the benzoyl group followed by desilylation, the resulting diol **132** was converted to



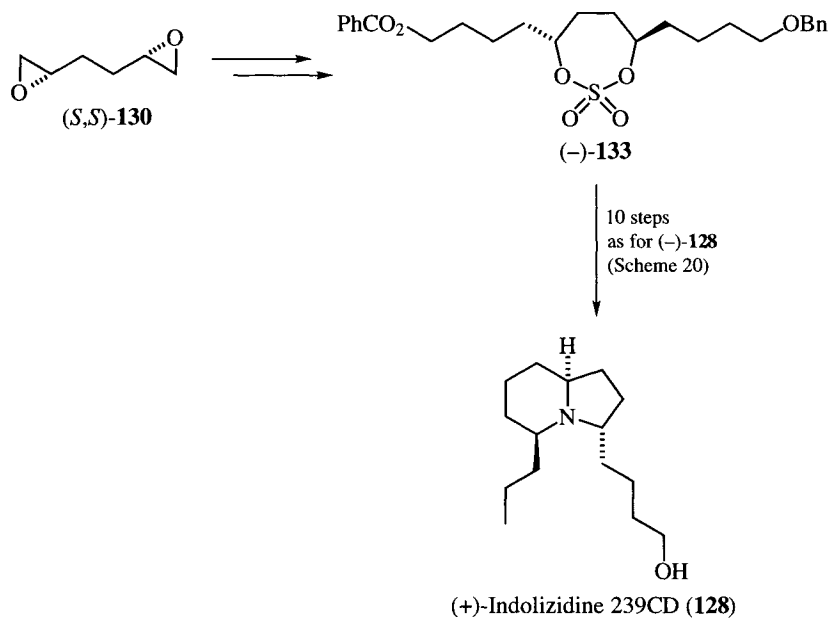
Scheme 19


1) BnO(CH2)3MgBr, CuI
2) t-BuMe2SiCl, imidazole
3) H2, Pd–C
(51%)
OSiMe2t-Bu
OH
OH
OSiMe2t-Bu
1) BnBr (1 equiv), NaH
2) PhCOCl, DMAP
3) Bu4NF
(53%)
(R,R)-130
131
OH
OBn
OCOPh
OH
132
1) SOCl2, Et3N
2) RuCl3, NaIO4
(82%)
PhCO2
OBn
(+)-133
1) LiN3, DMF
2) MsCl, Et3N
(94%)
N3
OBn
OCOPh
OMs
134a
1 : 1
OMs
OBn
OCOPh
N3
134b
1) H2, Pd–C
2) CbzCl, K2CO3
3) aq. KOH, MeOH
(75%)
HO
N
Cbz
OBn
135
1) PDC
2) PrMgBr
3) PDC
(52%)
O
N
Cbz
OBn
136
1) H2, Pd-C
2) H2, Pd–C, HCl
(75%)
H
N
OH
(–)-Indolizidine 239CD (128)

Scheme 20

the cyclic sulfate (+)-**133** by treatment with thionyl chloride and subsequently RuO_4. Nucleophilic ring opening of (+)-**133** with LiN_3 in DMF followed by mesylation of an inseparable 1:1 mixture of the resulting alcohols afforded the corresponding mesylates **134a** and **133b**, which were, without separation, subjected to hydrogenation of the azide function leading to in situ cyclization to give the *trans*-pyrrolidine as a single product and subsequent protection and ester hydrolysis converted this product to the alcohol **135**. Hydrogenation of the ketone **135**, obtained from **135** by oxidation and subsequent three-carbon homologation on the side chain, caused intramolecular cyclization via iminium ion formation and was followed by hydrogenolytic debenzylation to provide (–)-indolizidine 239CD [(–)-**128**] as a single isomer.

The same pathway was applied starting with the (*S*,*S*)-bisepoxide (*S*,*S*)-**130** for the synthesis of (+)-indolizidine 239CD [(+)-**128**] (Scheme 21). These syntheses using the two enantiomeric building blocks (*R*,*R*)-**130** and (*S*,*S*)-**130** serve to confirm the absolute configuration of natural alkaloid (–)-239CD as 3*R*,5*R*,8a*R*.



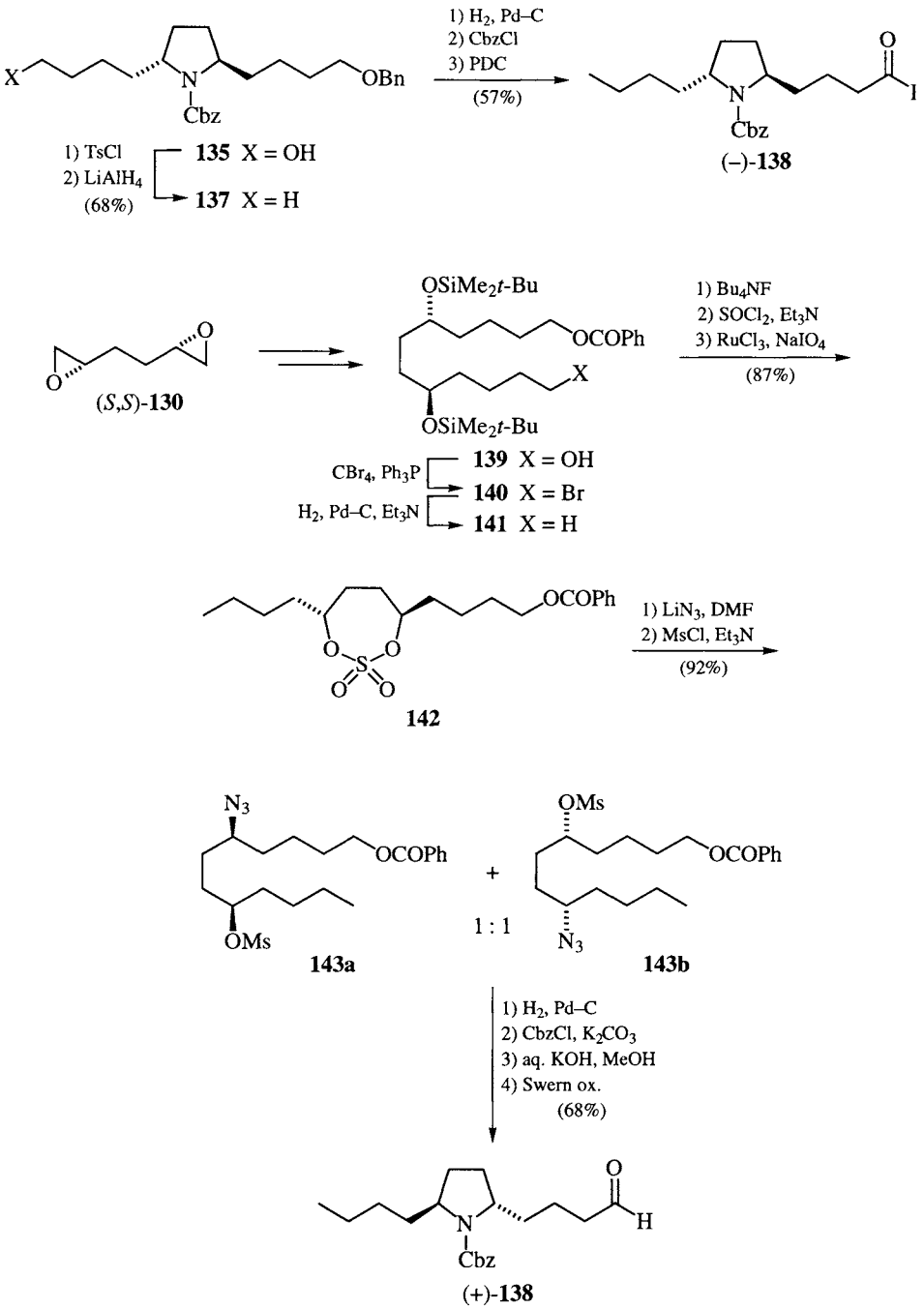
(*S*,*S*)-**130**

(–)-**133**

(+)-Indolizidine 239CD (**128**)

Scheme 21

Conceptually analogous strategy was applied for the enantiodivergent syntheses of the rest of the 3,5-disubstituted indolizidine alkaloids 195B, 223AB, and 239AB, where the common key intermediates for the both (–) and (+) series of all three alkaloids are the enantiomeric aldehydes (–)-**138** and (+)-**138**, respectively. Thus, the alcohol **135**, described above in the synthesis of (–)-239CD from the (*R*,*R*)-bisepoxide (*R*,*R*)-**130** (see Scheme 20), was deoxygenated to **137** in a straightforward manner via tosylation followed by reduction with $LiAlH_4$ (Scheme 22).


1) H2, Pd–C
2) CbzCl
3) PDC
(57%)
X
OBn
N
Cbz
O
I
(–)-138
1) TsCl
2) LiAlH4
(68%)
135 X = OH
137 X = H
(S,S)-130
OSiMe2t-Bu
OCOPh
X
1) Bu4NF
2) SOCl2, Et3N
3) RuCl3, NaIO4
(87%)
CBr4, Ph3P
139 X = OH
140 X = Br
H2, Pd–C, Et3N
141 X = H
OCOPh
1) LiN3, DMF
2) MsCl, Et3N
(92%)
142
N3
OMs
143a
1 : 1
143b
1) H2, Pd–C
2) CbzCl, K2CO3
3) aq. KOH, MeOH
4) Swern ox.
(68%)
(+)-138

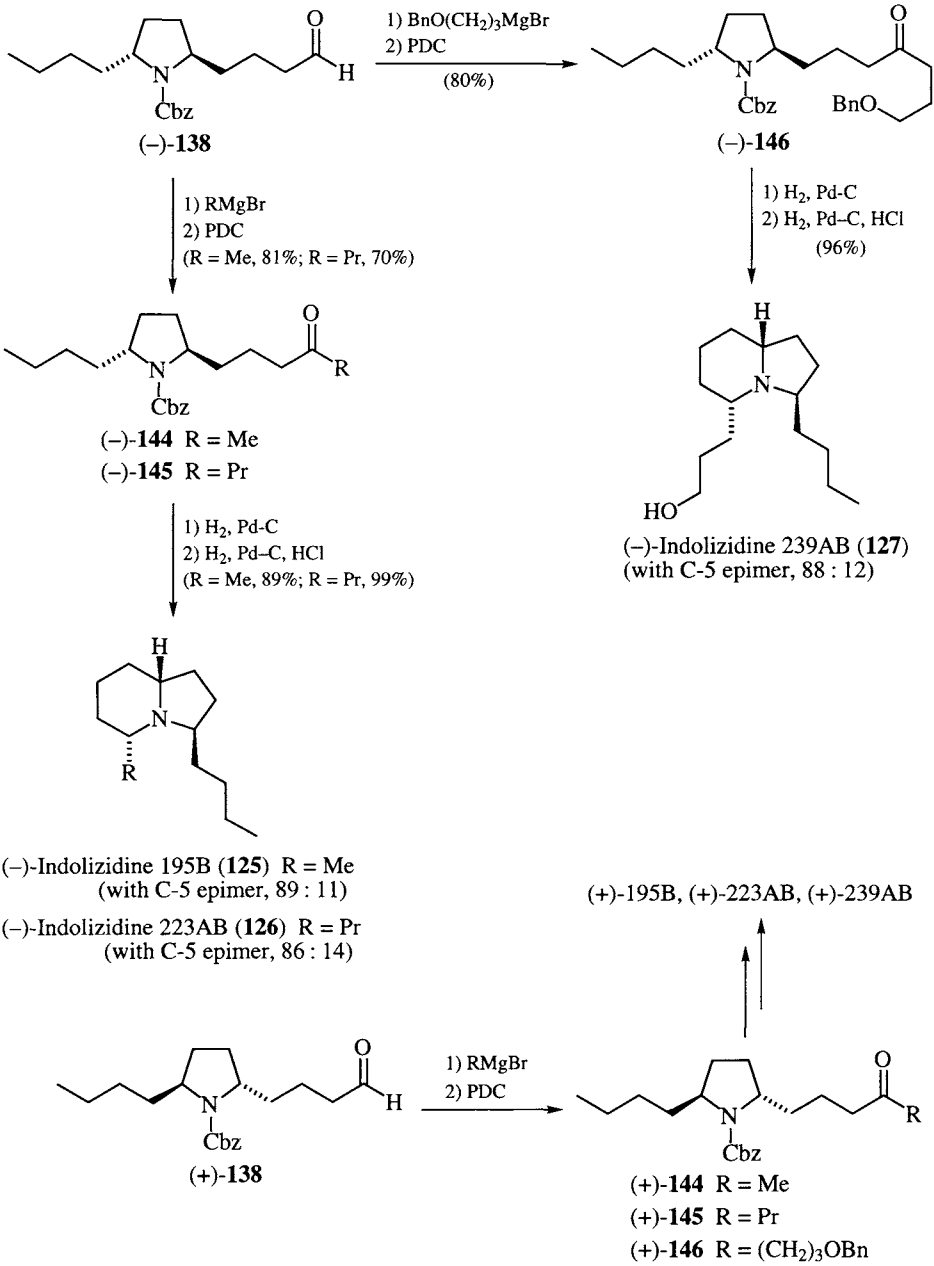
Scheme 22

The antipodal aldehyde (+)-**138** was prepared from (*S*,*S*)-bisepoxide (*S*,*S*)-**130** by a modified sequence (Scheme 22), compared to the sequence employed for the preparation of the (–)-enantiomer (–)-**138**. In this case, deoxygenation of the primary alcohol on the side chain was carried out via the bromide **140** prior to the process of the ring closing to the *trans*-pyrrolidine.

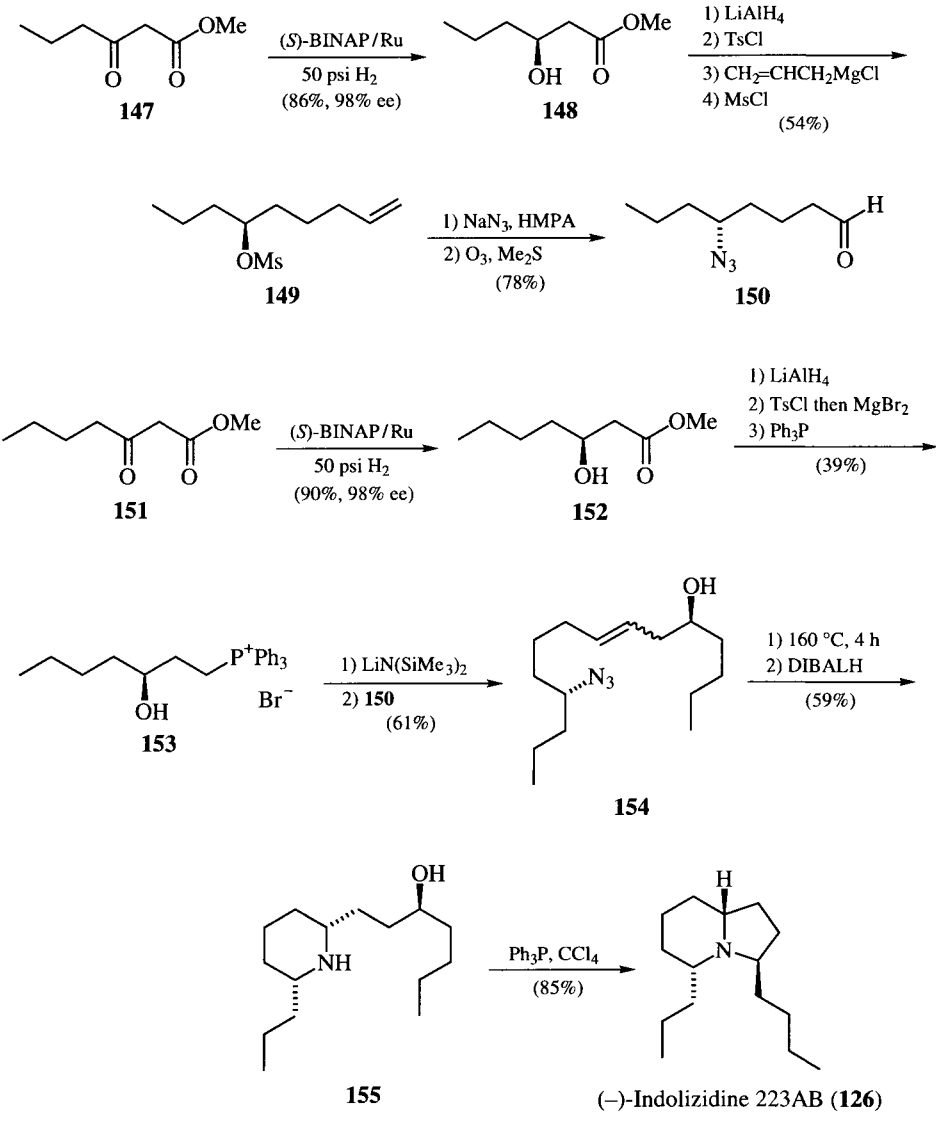
Either enantiomeric aldehyde (–)-**138** or (+)-**138** was transformed, upon suitable homologation of the side chain by the Grignard reaction–oxidation protocol and subsequent ring closure of the amino ketones **144**, **145**, and **146** based on reductive alkylation of the secondary amines, to the corresponding (–) or (+)-series of indolizidine alkaloids 195B (**125**), 223AB (**126**), and 239AB (**128**) (Scheme 23). The absolute configuration of the naturally occurring (–)-enantiomer of alkaloid 239AB was thus confirmed as 3*R*, 5*S*, 8a*R*.

Shortly after our synthesis described above, the enantioselective synthesis of (–)-indolizidine 223AB [(–)-**126**] has been reported by Taber et al. (ref. 51). The basic approach used in this synthesis involves enantioselective BINAP/Ru-mediated hydrogenation of β-keto esters originally developed by the Noyori group (ref. 52). As outlined in Scheme 24, the approach was designed on the basis of the Wittig coupling of the chiral phosphonium salt with the chiral aldehyde. Thus, the preparation of the aldehyde unit **150** began with (*S*)-BINAP/Ru-mediated hydrogenation of β-keto ester **147** to form the (*S*)-β-hydroxy ester **148** with 98% ee. Compound **148** was converted to the mesylate **149** by $LiAlH_4$ reduction, tosylation of the primary alcohol function, coupling with allylmagnesium chloride, and mesylation. Subsequent azidation followed by ozonolysis provided the aldehyde **150**. On the other hand, the (*S*)-β-hydroxy ester **152**, obtained by (*S*)-BINAP/Ru-mediated hydrogenation of β-keto ester **151** in 98% ee, was converted to the phosphonium salt **153** via $LiAlH_4$ reduction and bromination, which was coupled with the aldehyde **150** under Wittig conditions to give a geometrically isomeric mixture of the azide alcohols **154**. Upon heating at 160 °C, **154** underwent intramolecular dipolar cycloaddition/thermal fragmentation (ref. 53) and subsequent reduction of the resultant cyclic imine stereoselectively afforded the *cis*-dialkylpiperidine **155**. The cyclization of **155** with Ph_3P–CCl_4 proceeded with single inversion to afford (–)-indolizidine 223AB [(–)-**126**].

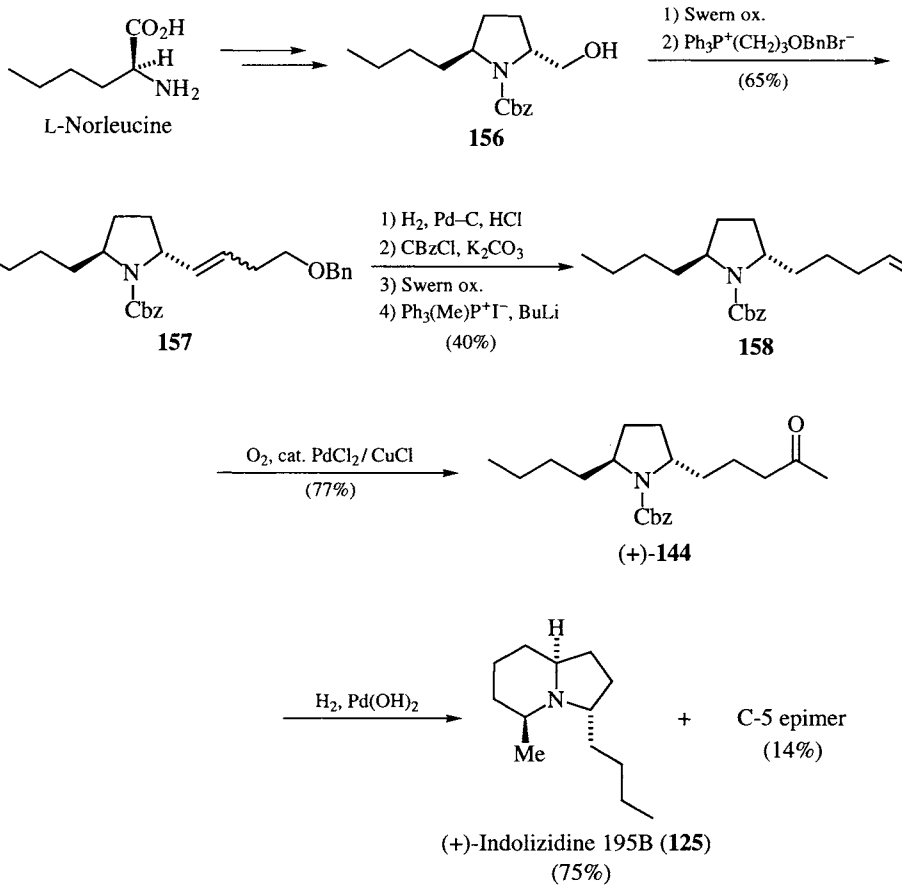
A simplified approach to the chiral ketone (+)-**144**, which was used as a pivotal precursor for the synthesis of (+)-indolizidine 195B [(+)-**125**] in our approach describe above (Scheme 23), has been presented by Takahata, Momose et al. (ref. 54) as outlined in Scheme 25. Their approach uses *trans*-2-alkyl-5-hydroxymethylpyrrolidine (**156**) as a homochiral building block which was previously prepared by the same group (ref. 55) by a stereoselective intramolecular amidomercuration of *N*-alkenylurethanes available from L-norleucine. Four-carbon extension of **156** was carried out by repetition of the Swern oxidation–Wittig reaction protocol to give the olefin **158**. Wacker oxidation of **158** afforded the methyl ketone (+)-**144**, which was upon catalytic hydrogenation cyclized to (+)-indolizidine 195B [(+)-**125**] as a 5:1 mixture of epimers.


1) BnO(CH2)3MgBr
2) PDC
(80%)
(–)-138
(–)-146
1) RMgBr
2) PDC
(R = Me, 81%; R = Pr, 70%)
1) H2, Pd-C
2) H2, Pd–C, HCl
(96%)
(–)-144 R = Me
(–)-145 R = Pr
1) H2, Pd-C
2) H2, Pd–C, HCl
(R = Me, 89%; R = Pr, 99%)
(–)-Indolizidine 239AB (127)
(with C-5 epimer, 88 : 12)
(–)-Indolizidine 195B (125) R = Me
(with C-5 epimer, 89 : 11)
(–)-Indolizidine 223AB (126) R = Pr
(with C-5 epimer, 86 : 14)
(+)-195B, (+)-223AB, (+)-239AB
1) RMgBr
2) PDC
(+)-138
(+)-144 R = Me
(+)-145 R = Pr
(+)-146 R = (CH2)3OBn

Scheme 23


OMe
O
O
147
(S)-BINAP/Ru
50 psi H2
(86%, 98% ee)
OMe
OH
O
148
1) LiAlH4
2) TsCl
3) CH2=CHCH2MgCl
4) MsCl
(54%)
OMs
149
1) NaN3, HMPA
2) O3, Me2S
(78%)
H
N3
O
150
OMe
O
O
151
(S)-BINAP/Ru
50 psi H2
(90%, 98% ee)
OMe
OH
O
152
1) LiAlH4
2) TsCl then MgBr2
3) Ph3P
(39%)
P+Ph3
Br−
OH
153
1) LiN(SiMe3)2
2) 150
(61%)
OH
N3
154
1) 160 °C, 4 h
2) DIBALH
(59%)
OH
NH
155
Ph3P, CCl4
(85%)
H
N
(–)-Indolizidine 223AB (126)

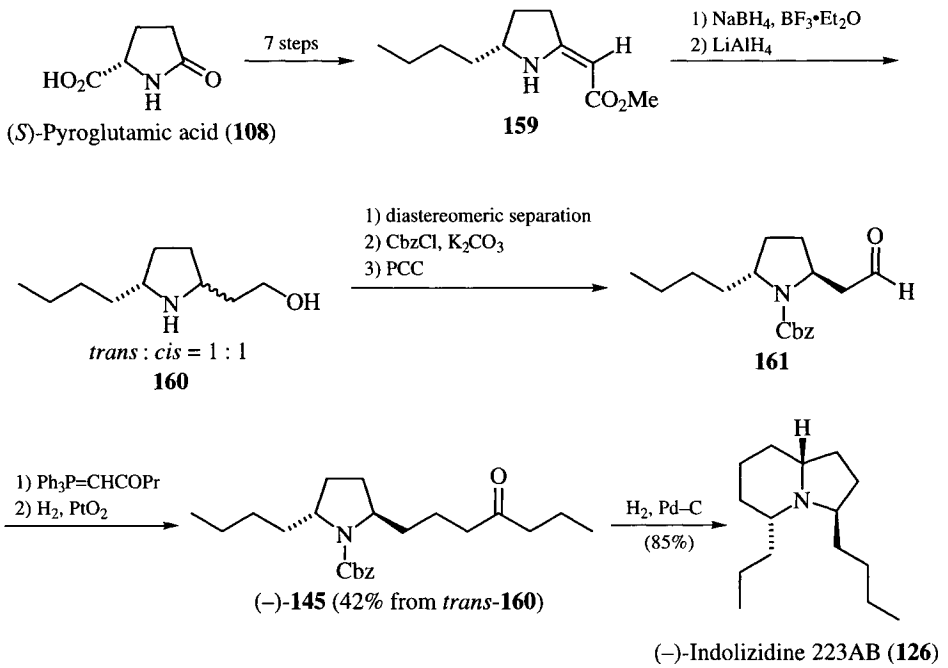
Scheme 24



Scheme 25

The synthetic utility of the ketone (–)-**145** as a direct precursor of (–)-indolizidine 223AB [(–)-**126**] has been again recognized by the Lhommet group (ref. 56). This approach outlined in Scheme 26 utilized the chiral pool (*S*)-pyroglutamic acid (**108**), which was converted to the β-enamino ester **159** in seven steps according to the procedure previously developed by this group (ref. 57). Reduction of **159**, producing a 1:1 diastereomeric mixture of the *trans*- and *cis*cyclic amino alcohols **160**, and subsequent separation provided the *trans*-**160**, which was transformed into the amino aldehyde **161** via protection followed by oxidation. Homologation of the side chain was performed by Wittig reaction followed by catalytic hydrogenation to afford the ketone (–)-**145** which could be converted to (–)-indolizidine 223AB [(–)-**126**] by catalytic hydrogenation.

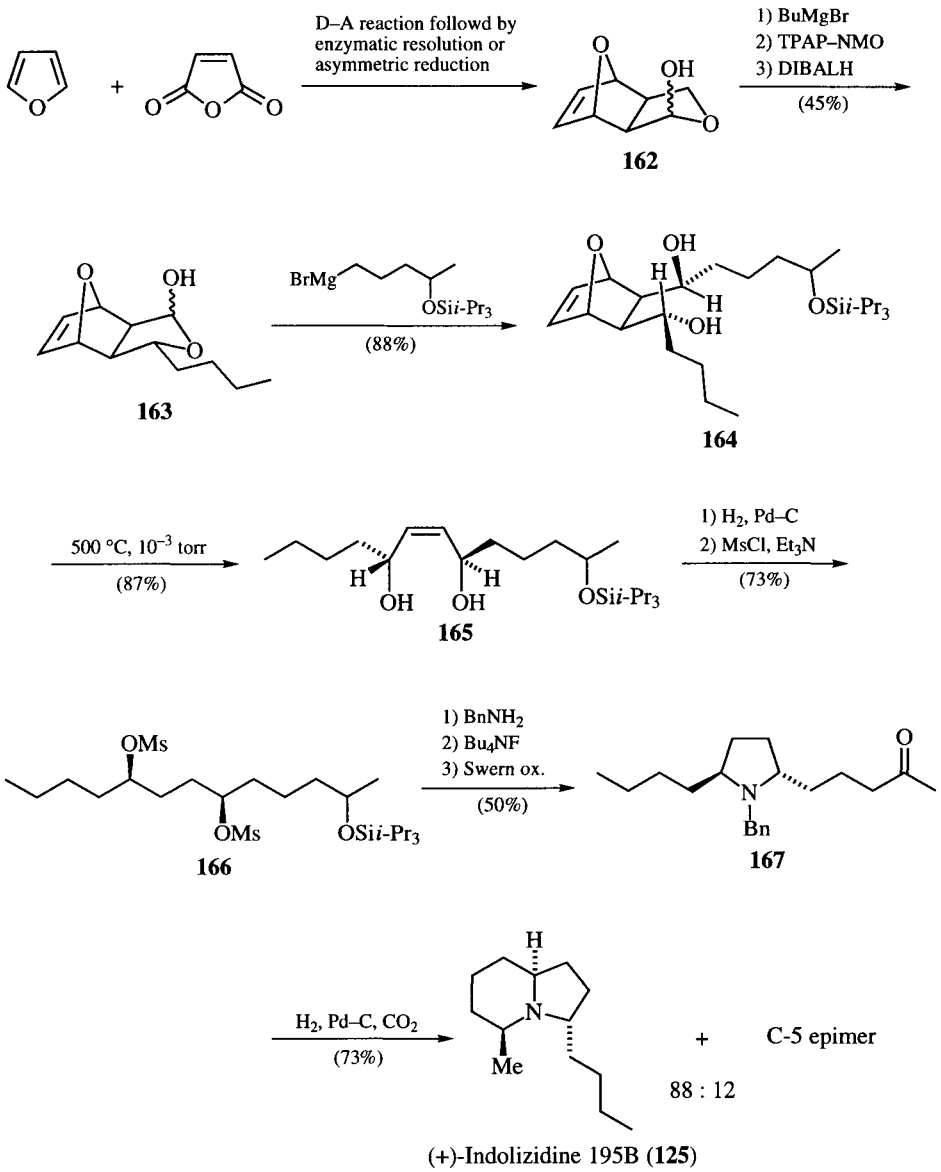
The useful precursor keto pyrrolidine **167** for the synthesis of (+)-indolizidine 195B has been also elaborated by Bloch et al. (ref. 58) based on a different approach with respect to pyrrolidine ring formation (Scheme 27). The synthesis started from the chiral lactol **162**, readily available from the Diels–Alder adduct of furan and maleic anhydride, either via an enzymatic resolution (ref. 59) or an



Scheme 26

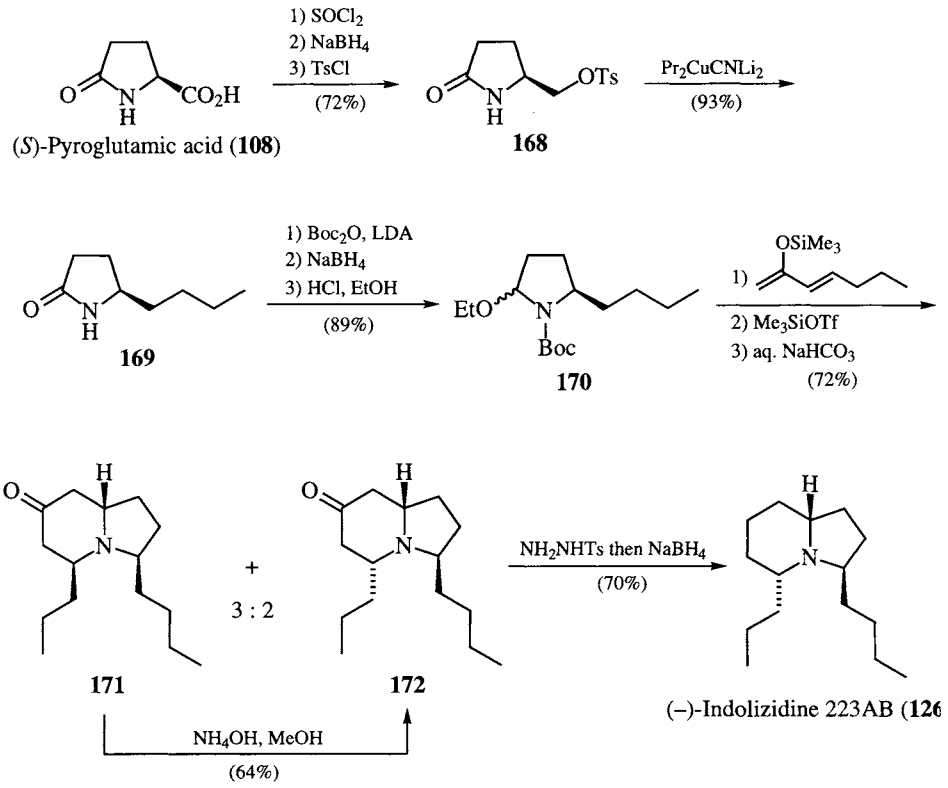
asymmetric reduction (ref. 60). Stereoselective addition of butylmagnesium bromide to **162** followed by oxidation to the lactone and DIBALH reduction gave rise to the lactol **163**. Chelation controlled addition of the Grignard reagent to **163** led to the diol **164**, which upon flash thermolysis was subjected to retro Diels–Alder reaction, yielding the unsaturated diol **165**. After catalytic hydrogenation followed by mesylation, treatment of the resulting bismesylate **166** with benzylamine resulted in the ring formation to give the *trans*-2,5-disubstituted pyrrolidine, which was converted to the ketone **167** by deprotection followed by Swern oxidation. Catalytic hydrogenation of **167** led to (+)-indolizidine 195B [(+)-**125**] along with the C-5 epimer in an 88:12 ratio.

Pilli et al. (ref. 61) have reported an alternate route to the synthesis of (–)-indolizidine 223AB starting from the chiral pool (*S*)-pyroglutamic acid (**108**) via a cyclic *N*-acyliminium ion based on a one-pot preparation of the indolizidin-7-one ring system (Scheme 28). Thus, **108** was converted to the tosylate **168** in three steps according to a previously described procedure (ref. 62) and three-carbon homologation of **168** provided the lactam **169**, which was further converted to the ethyl carbamate **170**. Addition of the silyloxy diene to the *N*-acyliminium ion, in situ generated from **170** by treatment with trimethylsilyl triflate, afforded a 3:2 mixture of indolizidinones **171** and **172**. Treatment of the major isomer **171** or the 3:2 mixture of **171** and **172** with aqueous NH_4OH afforded **172** as the major isomer, which was transformed into (–)-indolizidine 223AB by reduction of the corresponding tosylhydrazone with $NaBH_4$.



Scheme 27

Muraoka, Momose, and colleagues (ref. 63) have reported an alternative preparation of the optically active *cis*-dialkylpiperidine **179** (Scheme 29) previously used as a key intermediate in the synthesis of (–)-indolizidine 223AB (ref. 64). Their synthesis of **179** is based upon dissymetrization of the bicyclic ketone **173** according to the enantioselective deprotonation of cyclic carbonyl compounds with the homochiral lithium amide **174** developed by Koga's group (ref. 65) to form the

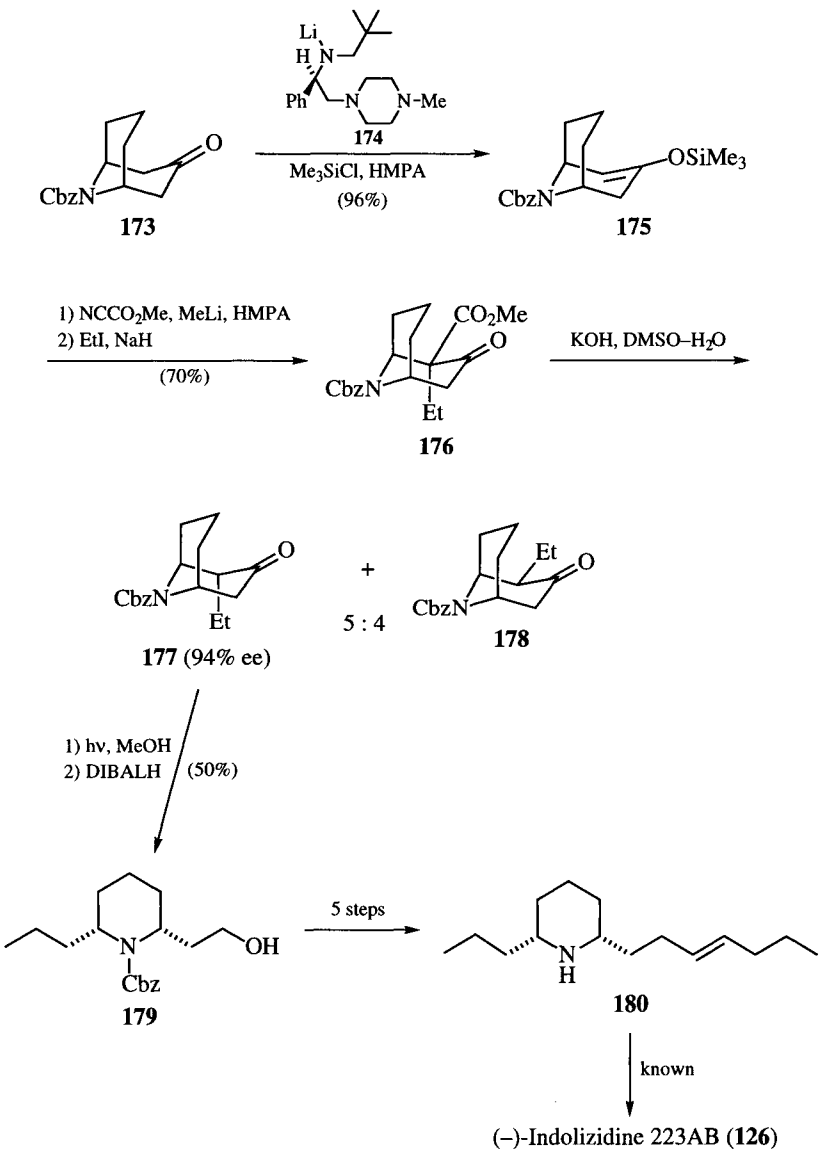


Scheme 28

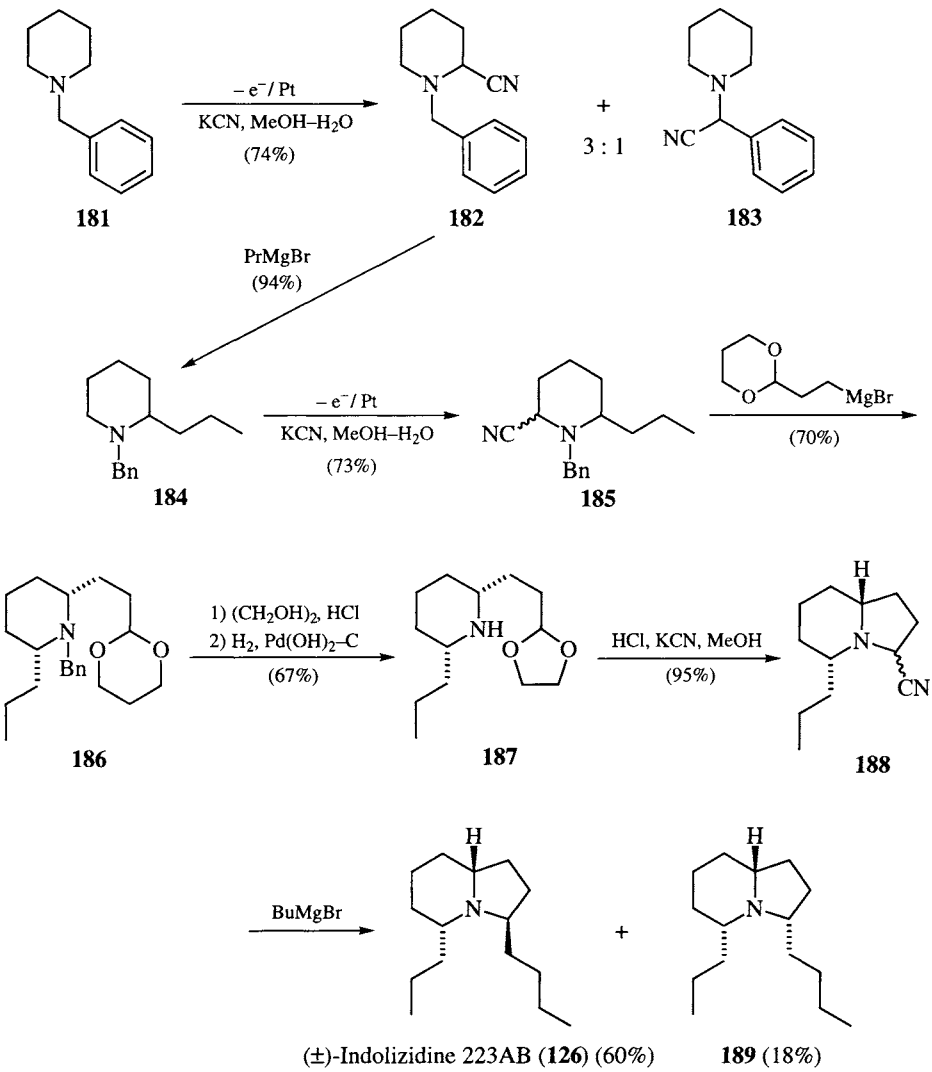
trimethylsilyl enolate **175**, which was then subjected to Claisen condensation followed by alkylation to give the bicyclic ketone **176**. The ketonic cleavage of **176** under alkaline conditions afforded the two epimeric 2-ethyl ketones **177** and **178** in a ratio of 5:4, the former of which, the β-isomer with 94% ee, was subjected to the Norrish type 1 photo-cleavage upon UV irradiation and the resulting ester was converted to the alcohol **179** by selective reduction. After homologation of the hydroxy ethyl side chain according to the reported procedure (ref. 64), the resulting piperidine **180** could be converted to (–)-indolizidine 223AB by applying the known method involving homolytic cyclization of an alkenyl substituted *N*-chloropiperidine previously developed by Broka (ref. 66).

The important precursor the *cis*-2,5-dialkylpiperidine **186** used in the former synthesis of (–)-indolizidine 223AB by Husson's group (ref. 33) has been synthesized by Yang and co-workers (ref. 67) in a racemic form (Scheme 30) via application of electro-oxidative α-cyanation of piperidines developed by Shono (ref. 68). Thus, electrolysis of *N*-benzylpiperidine (**181**) was performed in the presence of KCN to give the α-cyanoamines **182** and **183** in a ratio of 3:1, the former of which was allowed to react with the Grignard reagent to give **184**. Application of the similar electrolytic conditions to **184** generated regioselectively the α-cyanopiperidine **185** as a *cis*-and *trans*-

stereoisomeric mixture, and further substitution of **185** with the Grignard reagent produced the *cis*-2,5-disubstituted piperidine **186**. Replacement to the 1,3-dioxolane followed by catalytic hydrogenolysis led to the secondary amine **187**, which was converted to (±)-indolizidine 223AB according to the literature procedure (ref. 33) involving an intramolecular Strecker reaction and another substitution of the cyanoamine **188** with the Grignard reagent.



Scheme 29


– e⁻ / Pt
KCN, MeOH–H2O
(74%)
3 : 1
181
182
183
PrMgBr
(94%)
– e⁻ / Pt
KCN, MeOH–H2O
(73%)
MgBr
(70%)
184
185
1) (CH2OH)2, HCl
2) H2, Pd(OH)2–C
(67%)
HCl, KCN, MeOH
(95%)
186
187
188
BuMgBr
(±)-Indolizidine 223AB (126) (60%)
189 (18%)

Scheme 30

4.3 5,8-Disubstituted Indolizidine Alkaloids

5-Substituted 8-methylindolizidines occur in a wide range of dendrobatid species and represent one of the many class of dendrobatid alkaloids. Recently, synthetic routes to this group of alkaloids, e.g. indolizidines 205A (**190**), 207A (**191**), 209B (**192**), 235B (**193**), and 235B' (**194**), have been developed.

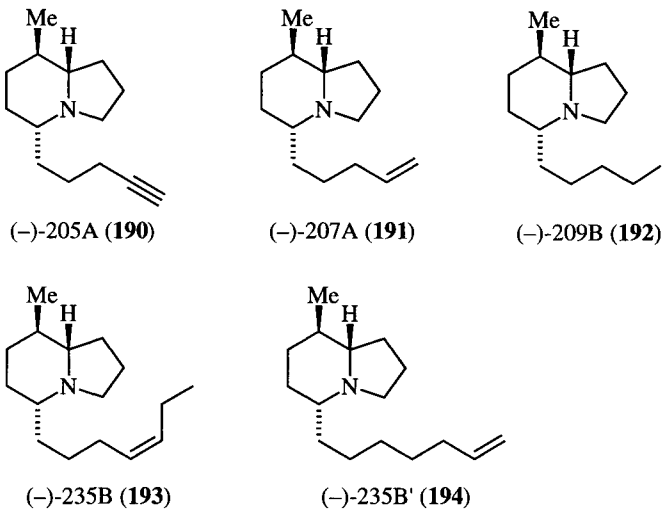
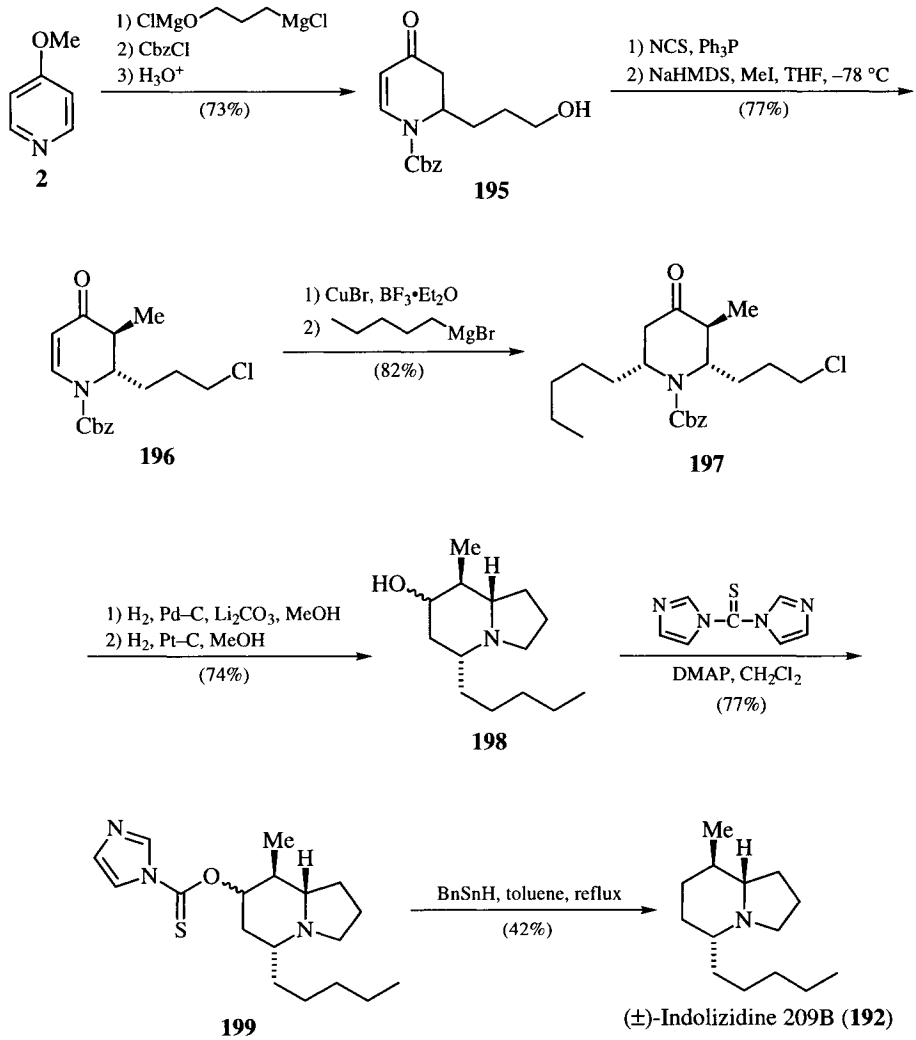


Figure 2. Naturally occurring 5,8-disubstituted indolizidine alkaloids.

Comins group (ref. 69) has applied the *N*-acyldihydropyridone based strategy, broadly developed by this group (see Section 2), to the synthesis of (±)-indolizidine 209B (**192**) (Scheme 31). The desired *N*-acyldihydropyridone **195** was prepared from 4-methoxypyridine (**2**), Grignard reagent, and benzylchloroformate. Chlorination of the alcohol function followed by C-3 methylation converted **195** to **196**, which was subjected to copper-mediated conjugate addition of pentylmagnesium bromide to afford the *cis*-2,5-disubstituted piperidone **197**. After cyclization by one-pot procedure involving catalytic hydrogenolysis and subsequent catalytic hydrogenation of the ketone function, deoxygenation of the resulting indolizidine **198** was achieved by treatment of the thiocarbonyl derivative **199** with Bu_3SnH/AIBN to provide (±)-indolizidine 209B (**192**).

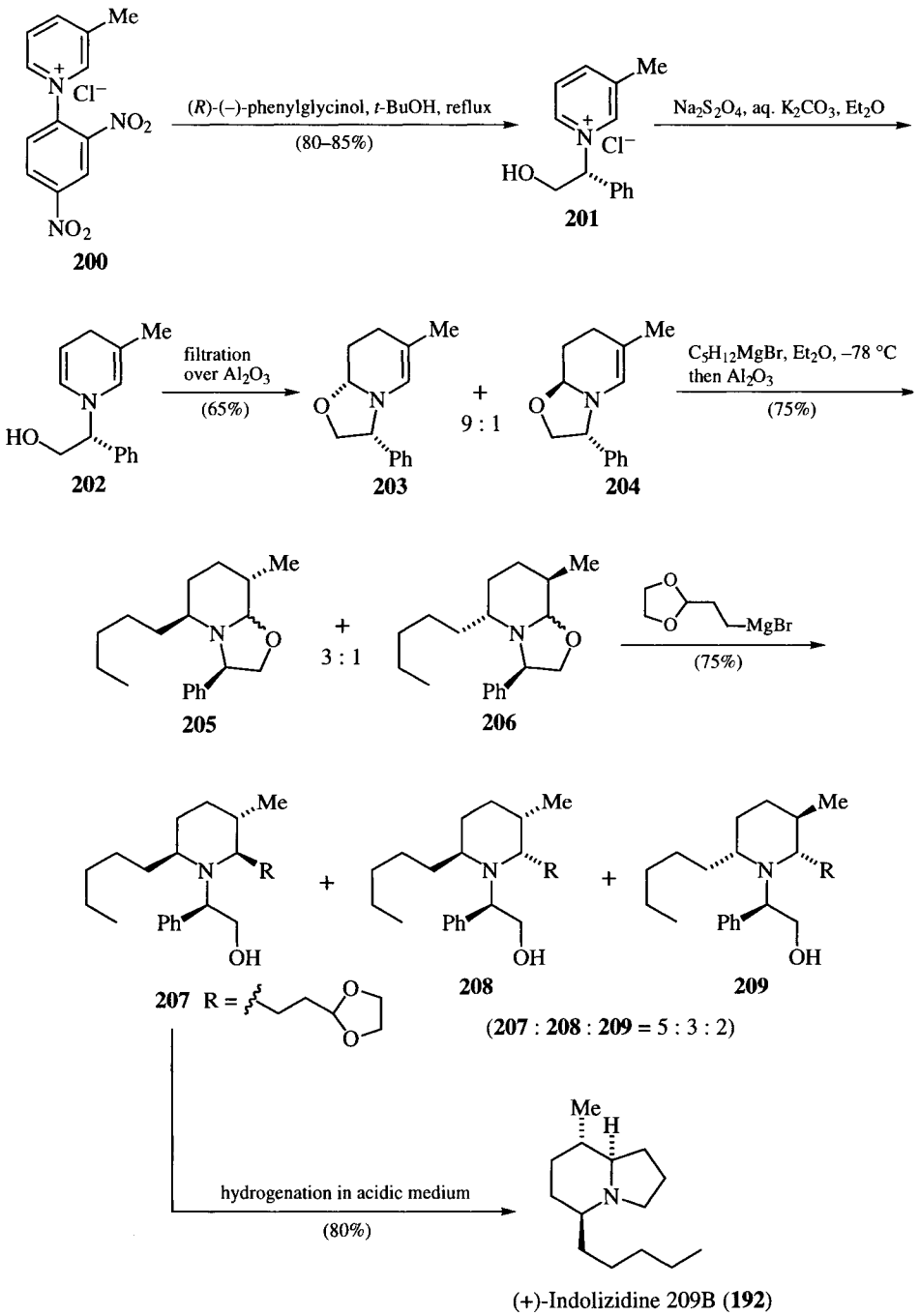
The enantioselective synthesis of (+)-indolizidine 209B has been reported by Marazano and colleagues (ref. 70) (Scheme 32). The chiral 1,4-dihydropyridine intermediate **202**, prepared by treatment of the crystalline Zincke's salt **200** with (*R*)-phenylglycinol followed by partial reduction of the resulting chiral pyridinium salt **201**, plays the central role in the synthesis. Filtration of **203** over alumina generated a mixture of the oxazolidines **203** and **204** in a ratio of 9:1 with the thermodynamically more stable **203** predominating. This oxazolidine mixture was treated with the Grignard reagent and filtered over alumina affording a 3:1 mixture of the oxazolidines **205** and **206**,



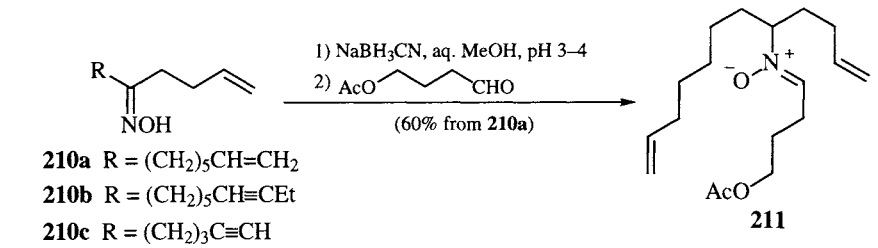
Scheme 31

which was further subjected the Grignard reaction to give a mixture of **207**, **208**, and **209** in a ratio of 5:3:2. Hydrogenation of the major isomer **207** in an acidic medium directly furnished (+)-indolizidine 209B (**192**).

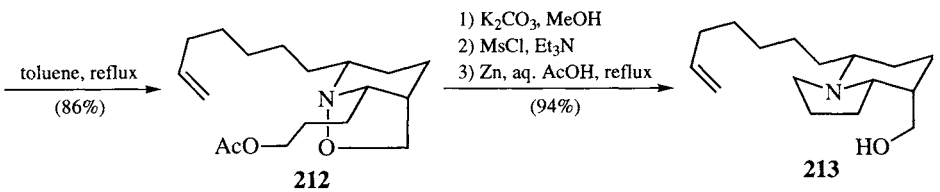
Holmes group (refs. 62b and 71) has developed a general approach to the racemic 5-substituted 8-methylindolizidine alkaloids, which employs as a key step the stereoselective intramolecular nitrone cycloaddition (Scheme 33). Typically for (±)-indolizidine 235B' (**194**), the synthesis began with the preparation of the required nitrone **211** from the corresponding oxime **210a** via $NaBH_3CN$ reduction followed by condensation of the resulting hydroxylamine with the aldehyde.

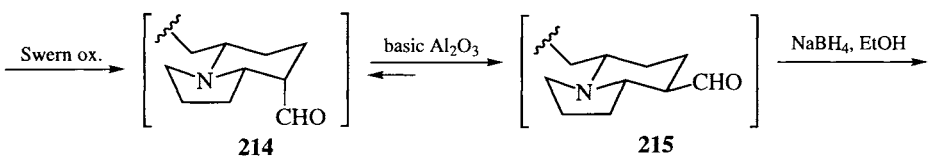

Me
Cl⁻
NO2
NO2
200
(R)-(−)-phenylglycinol, t-BuOH, reflux
(80–85%)
Me
Cl⁻
HO
Ph
201
Na2S2O4, aq. K2CO3, Et2O
Me
HO
Ph
202
filtration
over Al2O3
(65%)
Me
O
N
Ph
203
+
9 : 1
Me
O
N
Ph
204
C5H12MgBr, Et2O, –78 °C
then Al2O3
(75%)
Me
N
O
Ph
205
+
3 : 1
Me
N
O
Ph
206
O
O
MgBr
(75%)
Me
N
R
Ph
OH
207 R =
O
O
+
Me
N
R
Ph
OH
208
+
Me
N
R
Ph
OH
209
(207 : 208 : 209 = 5 : 3 : 2)
hydrogenation in acidic medium
(80%)
Me
H
N
(+)-Indolizidine 209B (192)

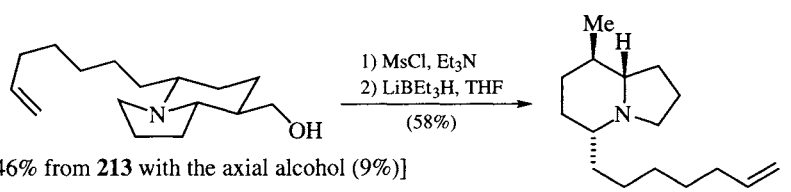
Scheme 32



210a R = $(CH_2)_5CH{=}CH_2$
210b R = $(CH_2)_5CH{\equiv}CEt$
210c R = $(CH_2)_3C{\equiv}CH$

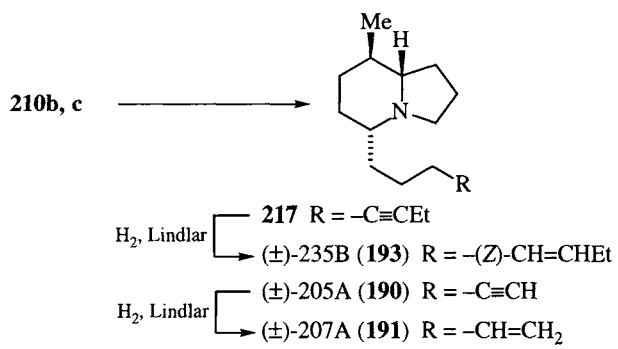






216 [46% from **213** with the axial alcohol (9%)]

(±)-Indolizidine 235B' (**194**)



Scheme 33

Intramolecular dipolar cycloaddition of the (*Z*)-*N*-alkenylnitrone **211**, thus obtained, gave the isoxazolidine **212**. Methanolysis followed by mesylation led to spontaneous cyclization to the quaternary ammonium salt, which was converted to the axial indolizidine alcohol **213** by reductive N–O bond cleavage. Swern oxidation followed by epimerization on basic alumina afforded predominantly (between 16:1 and 10:1) the equatorial aldehyde **215**, which was reduced and the separated equatorial alcohol **216** was deoxygenated to provide (±)-indolizidine 235B' (**194**).

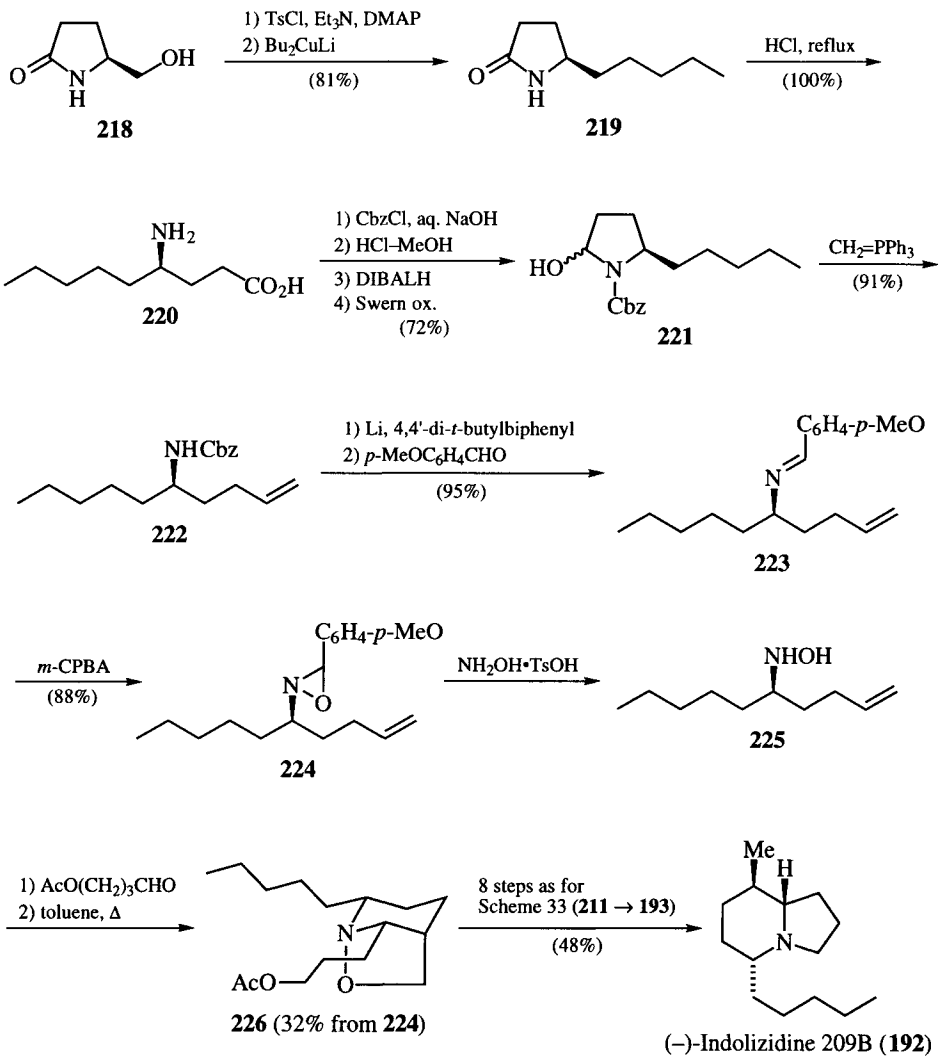
Following the same line of the nitrone strategy, the oximes **210b** and **210c** were converted to the corresponding racemic indolizidines, **217** and (±)-indolizidine alkaloid 205A (**190**), partial hydrogenation of which with Lindler catalyst yielded (±)-indolizidines 235B (**193**) and 207A (**191**), respectively (Scheme 33).

Holmes group (ref. 62b) further applied the nitrone strategy to the first enantioselective synthesis of (–)-indolizidine 209B (**192**) (Scheme 34). This approach requires methodology for the asymmetric synthesis of the enantiomerically pure hydroxylamine **225**. The synthesis therefore started with chain-extension of the hydroxymethyl group of (*S*)-5-(hydroxymethyl)-2-pyrrolidinone (**218**) to give the 5-pentylpyrrolidinone **219**, which was hydrolyzed to form the γ-amino acid **220** and converted to the hemiaminal **221** sequentially by N-protection, methanolysis, reduction, and then Swern oxidation of the primary alcohol function. After Wittig methylenation, removal of the Cbz protecting group from **222** using the lithium radical anion of 4,4'-di-tetrabutylbiphenyl (ref. 72), followed by Schiff formation afforded **223**, which was chemoselectively oxidized with *m*-chloroperbenzoic acid to give the oxaziridine **224**. Treatment of **224** with hydroxylamine produced the *N*-alkenylhdyroxylamine **225**, which underwent condensation with 4-acetoxybutanal to generate the *N*-alkenylnitrone and then thermal cycloaddition to give the single isoxazolidine **226**. Subsequent elaboration using the strategy developed for the synthesis of the racemic indolizidine 235B' (**194**) (see Scheme 33) resulted in the synthesis of (–)-indolizidine 209B (**192**).

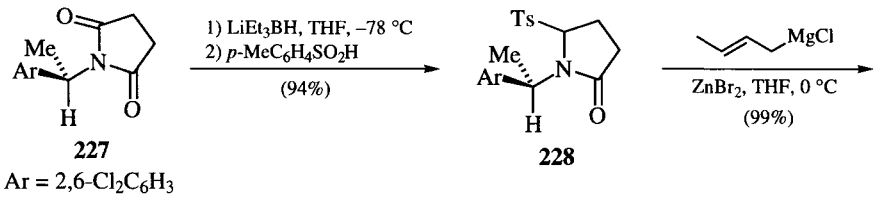
The first enantioselective synthesis of (–)-indolizidines 205A (**190**) and 235B (**193**) (Scheme 35) has been accomplished by Polniaszek and Belmont (ref. 73). This synthesis parallels their synthesis of (–)-indolizidines 167B (**84**) and 209D (**85**) discussed in Section 4.1 (see Scheme 13) and in fact employs similar diastereoselective *N*-acyliminium reaction. The succimide **227** bearing the (*R*)-1-(2,6-dichlorophenyl)ethyl auxiliary was reduced with lithium tirethylborohydride and the resulting hydroxy lactam was converted to the *p*-toluenesulfonyl lactam **228** with *p*-toluenesulfinic acid (ref. 74). Reaction of **228** with crotylmagnesium chloride proceeded by way of an *N*-acyliminium ion intermediate to produce a mixture of the two crotyl lactams **229** and **230**, which was without separation reduced to a readily separable mixture of the primary alcohols. The major isomer **231** underwent Swern oxidation followed by Wittig olefination to give the enol ether **232**, which was further converted to the dimethyl acetal **233** by acetalization followed by reduction of the lactam. Hydrogenolysis of the chiral directing group and subsequent hydrolysis in the presence of HCN according to Husson (ref. 33) led to cyclization via Strecker type reaction to the key intermediate, the α-amino nitrile **234**. Alkylation of **234** produced the alkynyl and alkenyl indolizidines **235** and **237**, the former of which was desilylated to give **236**. Reduction of **236** and

237 with sodium borohydride resulted in (–)-indolizidines 205A (**190**) and 235B (**193**), respectively.

Our group (ref. 25d) has developed a general strategy for the enantioselective synthesis of a series of the 5-substituted 8-methylindolizidine alkaloids, i.e., (–)-indolizidines 205A (**190**), 207A (**191**), 209B (**192**), and 235B (**193**), in which intramolecular hetero Diels–Alder reaction of a chiral *N*-acylnitroso compound played a central role (Scheme 36). The synthesis started with (*R*)-citronellol (**238**) as a chiral building block, which was via the known unsaturated carboxylic acid **239** converted to the diene **240** by ozonolysis followed by Wittig reaction. The hydroxamic acid **241**, derived from the ester **240**, was subjected to periodate oxidation to generate the *N*-acylnitroso

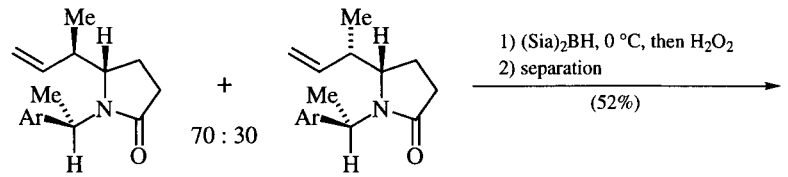


Scheme 34

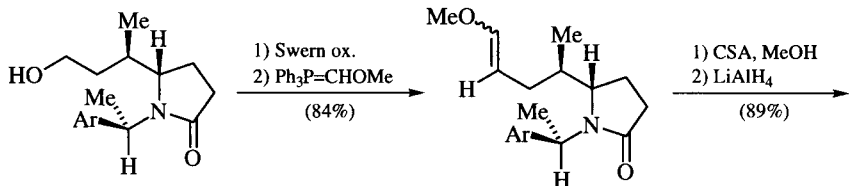


227
Ar = 2,6-$Cl_2C_6H_3$

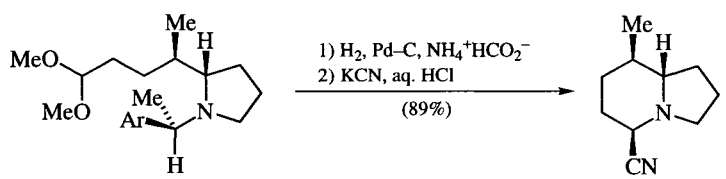
228



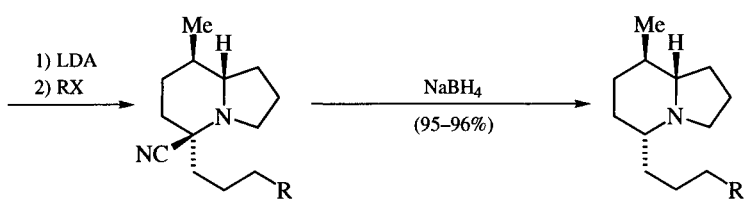
229 **230**



231 **232**



233 **234**



KF (89%) → **235** R = –C≡CSiMe_3 (64%)
236 R = –C≡CH
237 R = –(Z)-CH=CHEt (84%)

(–)-Indolizidine 205A (**190**) R = –C≡CH
235B (**193**) R = –(Z)-CH=CHEt

Scheme 35

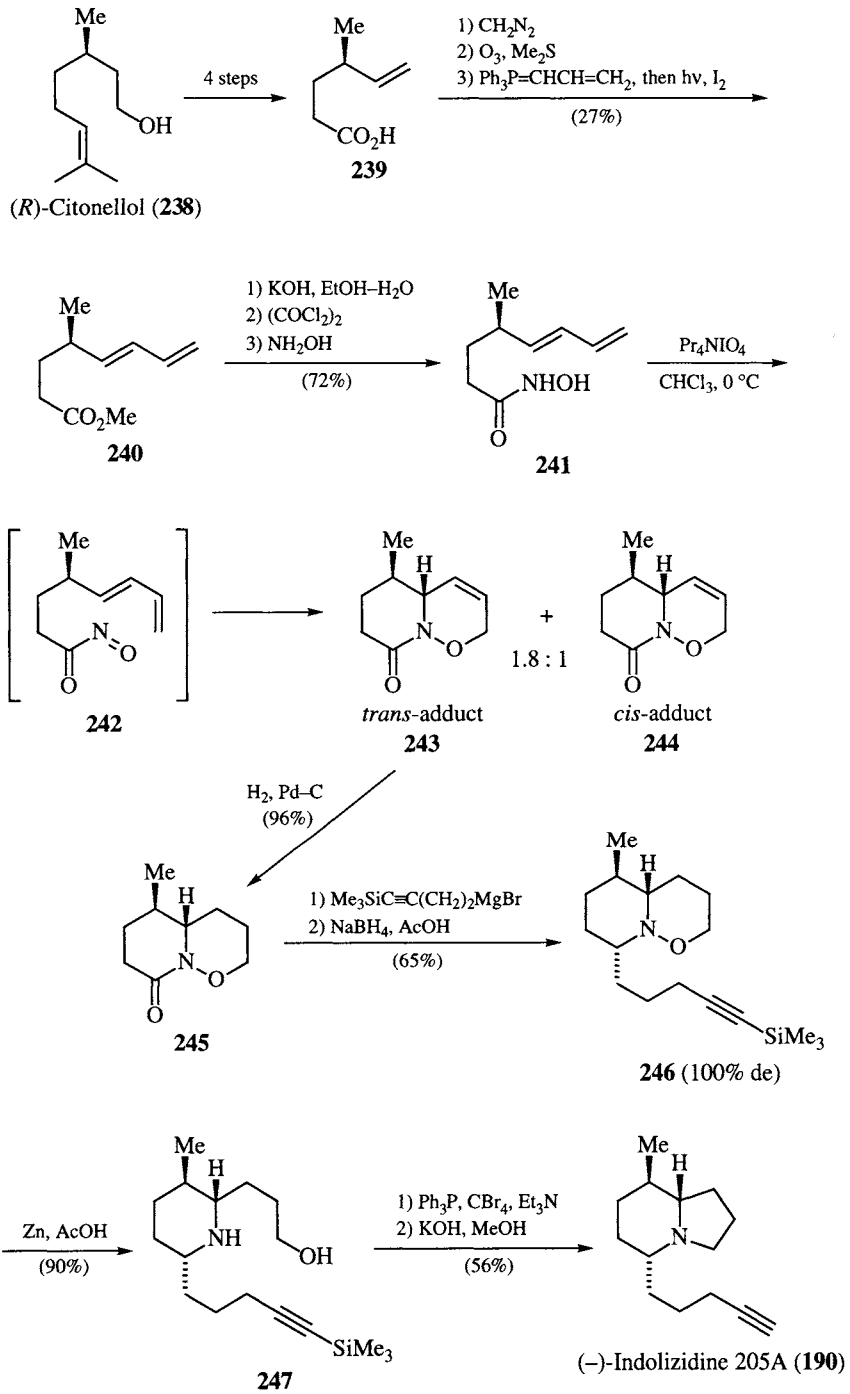
compound in situ, which spontaneously cyclized to give the *trans*- and *cis*-bicyclic oxazinolactams **243** and **244** in a 1.8:1 ratio. The *trans*-isomer **243** was utilized as a common synthetic precursor to all of the target alkaloids. Thus, after hydrogenation of the olefinic double bond, **245** was subjected to Grignard reaction, followed by $NaBH_4$ reduction under acidic conditions to give **246** as a single isomer with the correct stereocenters. Reductive cleavage of the N–O bond and subsequent intramolecular dehydrocyclization of the amino alcohol **247** followed by desilylation provided (–)-indolizidine 205A (**190**).

A key feature involved in this approach was completely stereocontrolled introduction of an appropriate alkyl or alkenyl side chain into the pyridoxazine **245** by means of the Grignard addition–reduction sequence. Thus, applying this strategy, we undertook the enantioselective synthesis of (–)-indolizidines 235B (**193**), 207A (**191**), and 209B (**192**) according to Scheme 37 (ref. 25b).

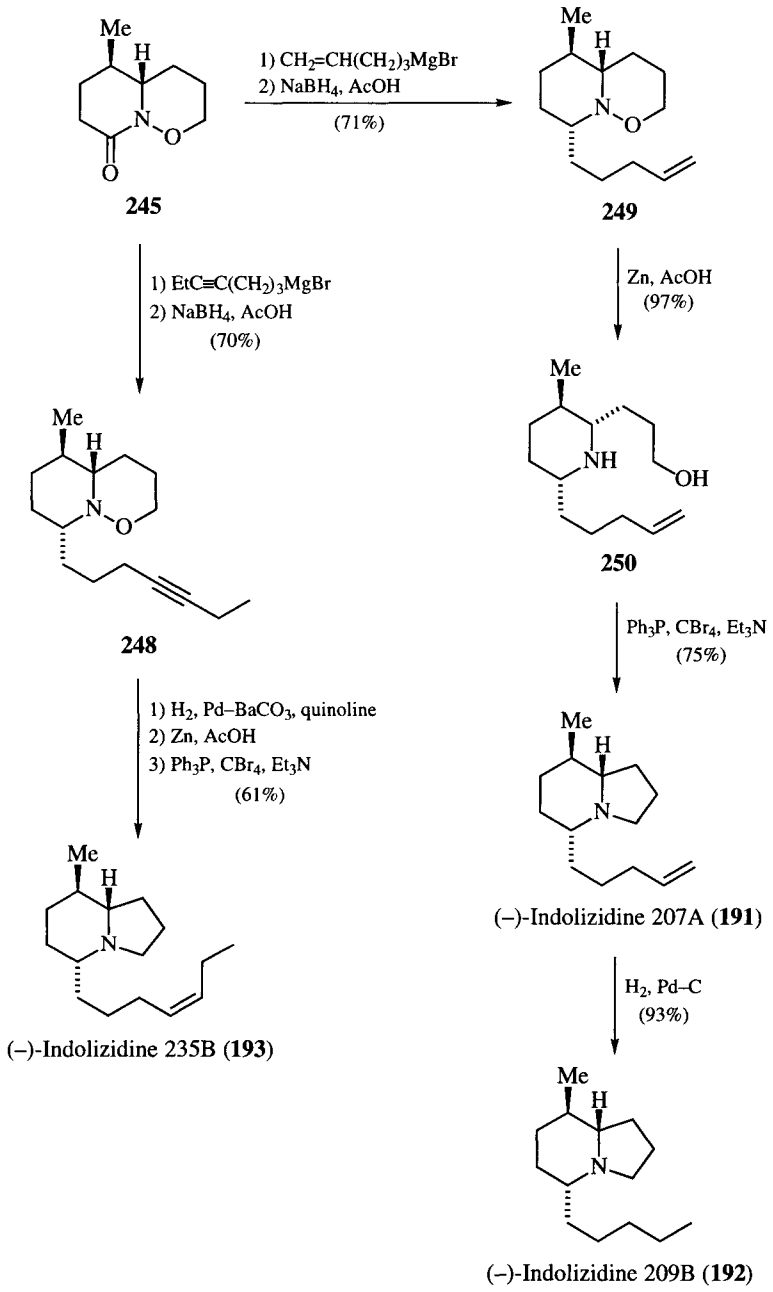
The use of the aminoalcohols, e.g. **247** and **250**, of general utility in the synthesis of (–)-indolizidine alkaloids, discussed above (see Schemes 36 and 37), has been demonstrated by Momose and Toyooka (ref. 75). Their enantioselective route to the preparation of these precursors is based on using the chiral trisubstituted piperidine **253**, which has previously been prepared by this research group (ref. 76) via enzymatic dissymmetrization of the *meso* compound **251** (Scheme 38). After protection and elimination, conjugate addition of Me_2CuLi to the resulting 2,3-didehydropiperidine **254** led to the stereocontrolled introduction of the β-methyl group. Nine additional steps including functional group manipulations and chain extensions served to convert to **257**, which underwent Grignard coupling followed by deprotection to afford the amino alcohol **250**. The transformation of **250** into (–)-indolizidines 207A (**191**) and 209B (**192**) can be achieved by the procedure discussed above. Alternatively, a similar sequence starting with **257** was applied to the first asymmetric synthesis of (–)-indolizidine 235B' (**194**).

Shimizu and Satake (ref. 77) have published a general approach to levorotatory 5-substituted 8-methylindolizidine alkaloids including 205A, 209A, and 235B, in which a chiral indolizidinone serves as a common synthetic intermediate (Scheme 39). The synthesis of this key intermediate **263** was initiated by Sharpless asymmetric epoxidation of the allylic alcohol **258** using (–)-diethyl tartrate to give the alkenyloxilane **259**, which by sequential Swern oxidation, Horner–Emmons reaction, and hydrogenolytic ring opening with a palladium catalyst afforded the homoallylic alcohol **260** with 98% ee. After tosylation and hydrogenation of the olefin, inversive azidation converted **260** to the azide **261**, hydrogenation of which led to ring closure to the piperidone **262**. Straightforward functional group manipulation of the side chain of **262** provided the iodide which on treatment with sodium hydride was cyclized to the required indolizidinone **263**. Introduction of the side chain into C-8 was accomplished by treatment with the Grignard reagents followed by reduction to produce the corresponding (–)-indolizidines 209B (**192**), 235B (**193**), and 205A (**190**) in low yields in the latter two cases.

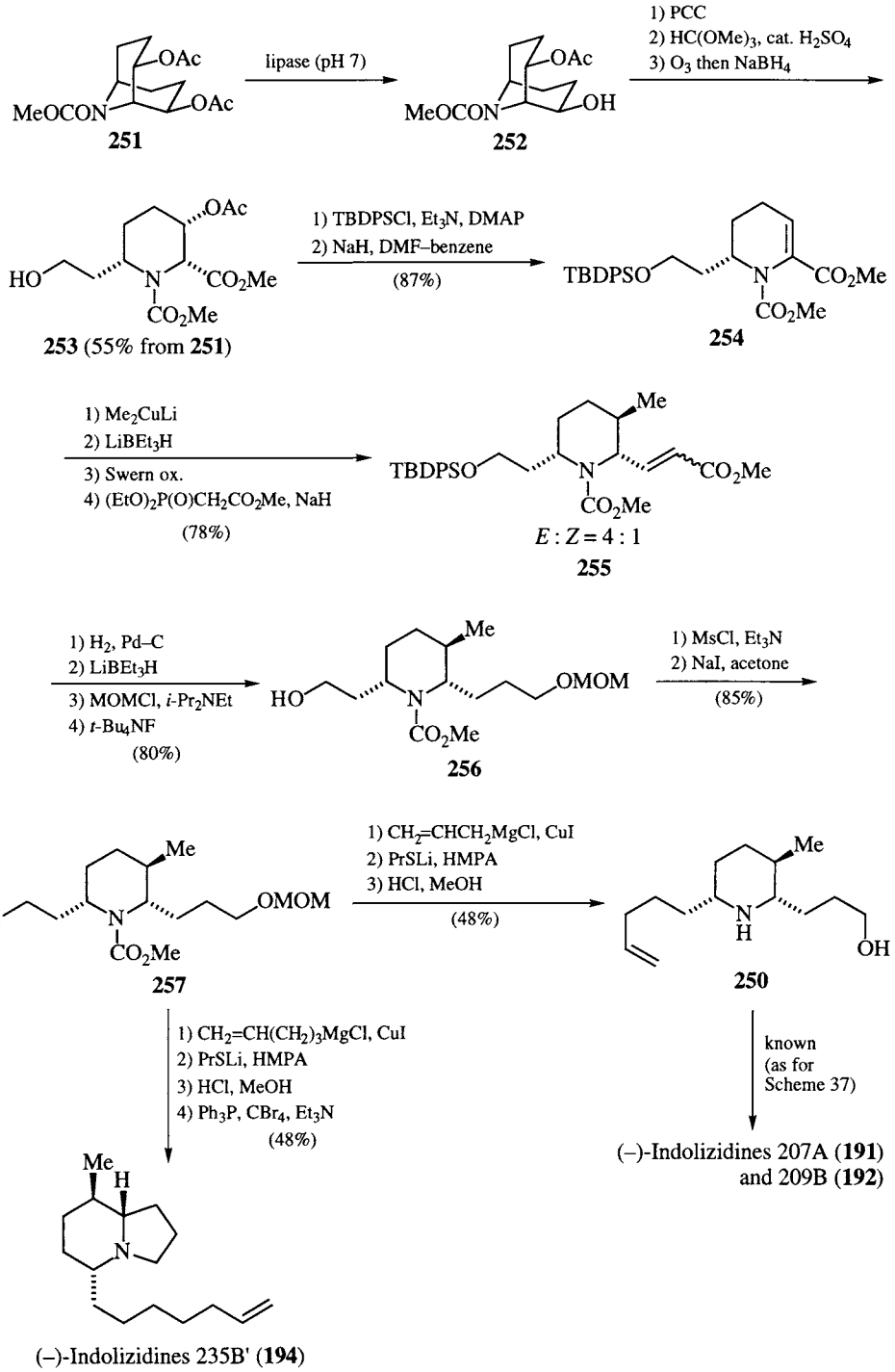
Another asymmetric synthesis of (–)-indolizidine 209B has been reported by Somfai and Åhman (ref. 39b) in a similar fashion discussed in Section 4.1 for the synthesis of (–)-indolizidine 209D (**85**) (see Scheme 17), in which the key step is the efficient aza-[2,3]-Wittig rearrangement of


Me
OH
4 steps
Me
CO2H
239
1) CH2N2
2) O3, Me2S
3) Ph3P=CHCH=CH2, then hv, I2
(27%)
(R)-Citonellol (238)
Me
CO2Me
240
1) KOH, EtOH–H2O
2) (COCl2)2
3) NH2OH
(72%)
Me
NHOH
O
241
Pr4NIO4
CHCl3, 0 °C
242
trans-adduct
243
1.8 : 1
cis-adduct
244
H2, Pd–C
(96%)
245
1) Me3SiC≡C(CH2)2MgBr
2) NaBH4, AcOH
(65%)
SiMe3
246 (100% de)
Zn, AcOH
(90%)
NH
247
1) Ph3P, CBr4, Et3N
2) KOH, MeOH
(56%)
(–)-Indolizidine 205A (190)

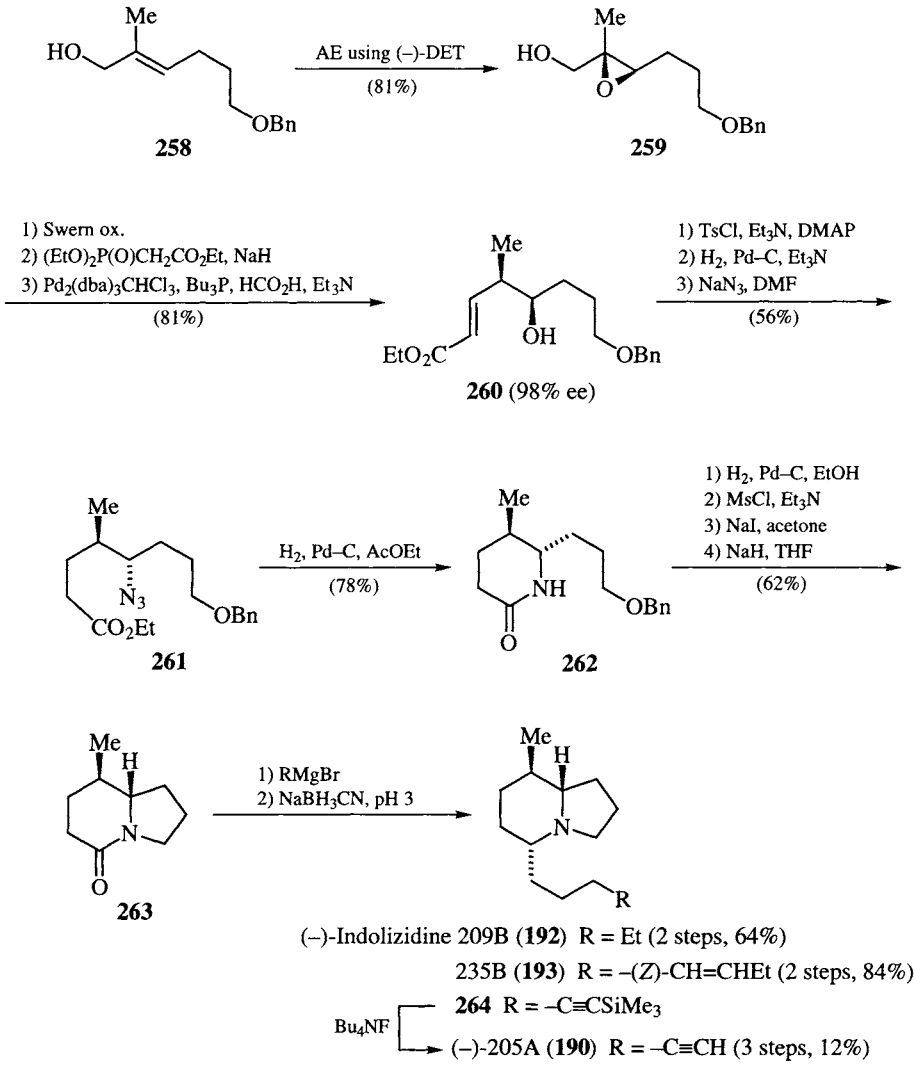
Scheme 36


Me
H
N
O
O
245
1) CH2=CH(CH2)3MgBr
2) NaBH4, AcOH
(71%)
249
1) EtC≡C(CH2)3MgBr
2) NaBH4, AcOH
(70%)
248
Zn, AcOH
(97%)
NH
OH
250
Ph3P, CBr4, Et3N
(75%)
1) H2, Pd–BaCO3, quinoline
2) Zn, AcOH
3) Ph3P, CBr4, Et3N
(61%)
(–)-Indolizidine 235B (193)
(–)-Indolizidine 207A (191)
H2, Pd–C
(93%)
(–)-Indolizidine 209B (192)

Scheme 37


OAc
MeOCON
OAc
251
lipase (pH 7)
OAc
MeOCON
OH
252
1) PCC
2) HC(OMe)3, cat. H2SO4
3) O3 then NaBH4
HO
N
CO2Me
CO2Me
253 (55% from 251)
1) TBDPSCl, Et3N, DMAP
2) NaH, DMF–benzene
(87%)
TBDPSO
N
CO2Me
CO2Me
254
1) Me2CuLi
2) LiBEt3H
3) Swern ox.
4) (EtO)2P(O)CH2CO2Me, NaH
(78%)
Me
TBDPSO
N
CO2Me
CO2Me
E : Z = 4 : 1
255
1) H2, Pd–C
2) LiBEt3H
3) MOMCl, i-Pr2NEt
4) t-Bu4NF
(80%)
Me
HO
N
OMOM
CO2Me
256
1) MsCl, Et3N
2) NaI, acetone
(85%)
Me
I
N
OMOM
CO2Me
257
1) CH2=CHCH2MgCl, CuI
2) PrSLi, HMPA
3) HCl, MeOH
(48%)
Me
N
H
OH
250
1) CH2=CH(CH2)3MgCl, CuI
2) PrSLi, HMPA
3) HCl, MeOH
4) Ph3P, CBr4, Et3N
(48%)
known
(as for
Scheme 37)
Me
H
N
(–)-Indolizidines 235B' (194)
(–)-Indolizidines 207A (191)
and 209B (192)

Scheme 38



260 (98% ee)

261

262

263

(–)-Indolizidine 209B (**192**) R = Et (2 steps, 64%)

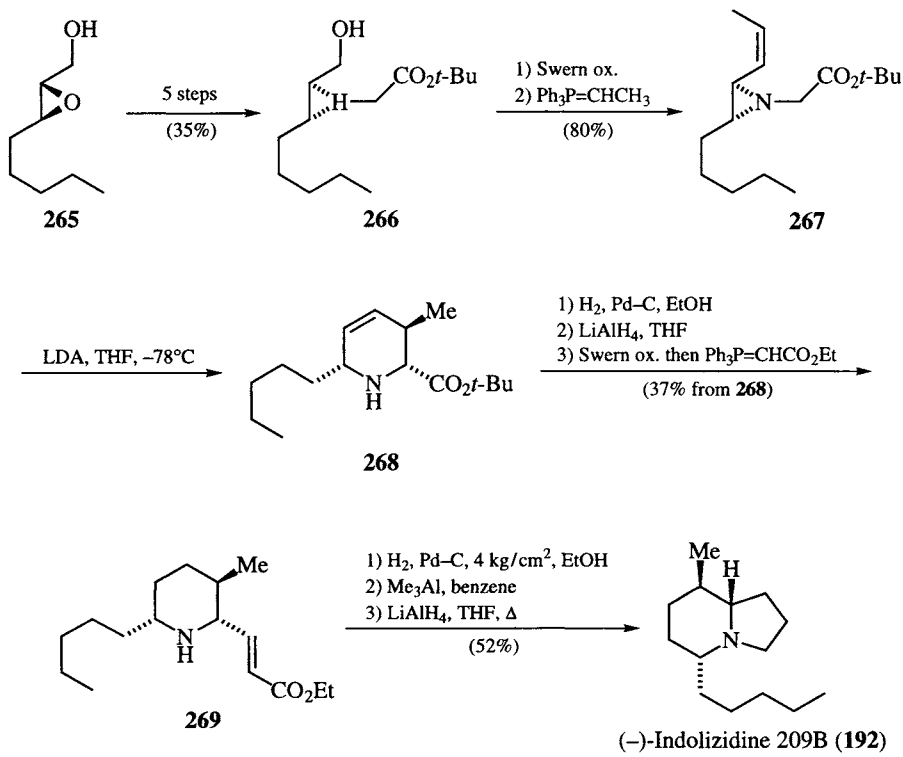
235B (**193**) R = –(Z)-CH=CHEt (2 steps, 84%)

264 R = –C≡CSiMe$_3$

(–)-205A (**190**) R = –C≡CH (3 steps, 12%)

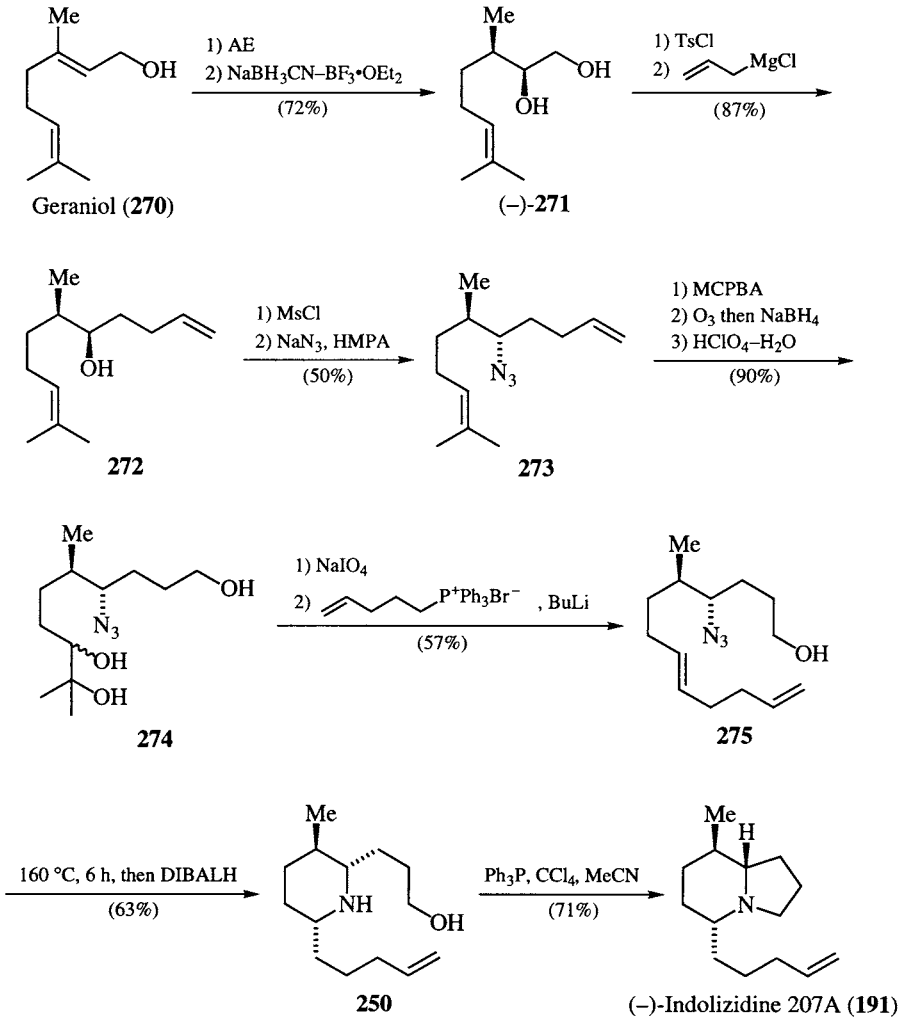
Scheme 39

vinylaziridines into tetrahydropyridines. Thus, the aziridine alcohol **266**, prepared from the known epoxy alcohol **265** in 5 steps, was converted to the vinylaziridine **267**, which underwent the aza-[2,3]-Wittig rearrangement to establish the three correct stereogenic centers present in alkaloid 209B (Scheme 40). Conversion of **268** to the target compound was then accomplished by using the same synthetic sequence discussed above, ultimately affording (–)-indolizidine 209B (**192**).



Scheme 40

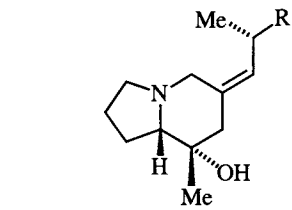
Very recently, Taber and co-workers (ref. 78) have reported the enantioselective synthesis of (–)-indolizidine 207A. The key intermediate in their synthesis is 2-hydroxycitronellol (**271**), prepared from geraniol (**270**) by the Sharpless asymmetric epoxidation followed by reduction of the epoxide with control of both relative and absolute configuration (Scheme 41). After introduction of the allyl group by tosylation followed by Grignard coupling, mesylation of **272** and subsequent azidation gave the azide **273**, which was then converted to the triol **274**. Glycol cleavage with periodate followed by the Wittig reaction provided the azide **275**, thermolysis of which proceeded via dipolar azide cycloaddition and subsequent fragmentation to provide the amino alcohol **250** previously discussed in this Section and utilized as the direct precursor to (–)-indolizidine 207A (**191**).


Me
OH
1) AE
2) NaBH3CN–BF3•OEt2
(72%)
Me
OH
OH
1) TsCl
2) MgCl
(87%)
Geraniol (270)
(–)-271
Me
OH
1) MsCl
2) NaN3, HMPA
(50%)
Me
N3
1) MCPBA
2) O3 then NaBH4
3) HClO4–H2O
(90%)
272
273
Me
N3
OH
OH
OH
1) NaIO4
2) P+Ph3Br–, BuLi
(57%)
Me
N3
OH
274
275
160 °C, 6 h, then DIBALH
(63%)
Me
NH
OH
Ph3P, CCl4, MeCN
(71%)
Me
H
N
250
(–)-Indolizidine 207A (191)

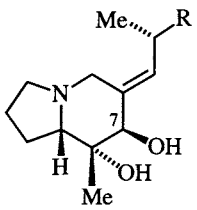
Scheme 41

5. SYNTHESES OF PUMILIOTOXIN A CLASS ALKALOIDS

The pumiliotoxin A class, one of the major classes of the alkaloids from dendrobatid frog, has been found in skin extracts from various genera of other amphibian families: Myobatrachidae (genus *Pseudophryne*), Bufonidae (genus *Melanophryniscus*), and Ranidae (genus *Mantella*) (refs. 3 and 5). The alkaloids belonging to the pumiliotoxin A class are divided into the three subclasses: pumiliotoxins (6-alkylidene-8-methyl-8-hydroxyindolizidines), allopumiliotoxins (7-hydroxy congeners of pumiliotoxins), and homopumiliotoxins which contain a quinolizidine rather than a indolizidine nucleus (Figure 3).

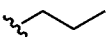


Pumiliotoxins

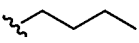


Allopumiliotoxins

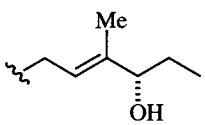
251D (**276**) R =



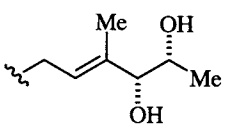
267A (**279**) R =



307A' (**277**) R =
(PTX-A)

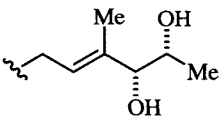


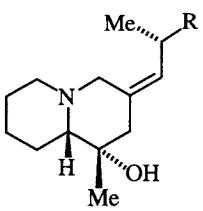
339A (**280**) R =



339B (**281**) C7-epimer of 339A

323A (**278**) R =
(PTX-B)





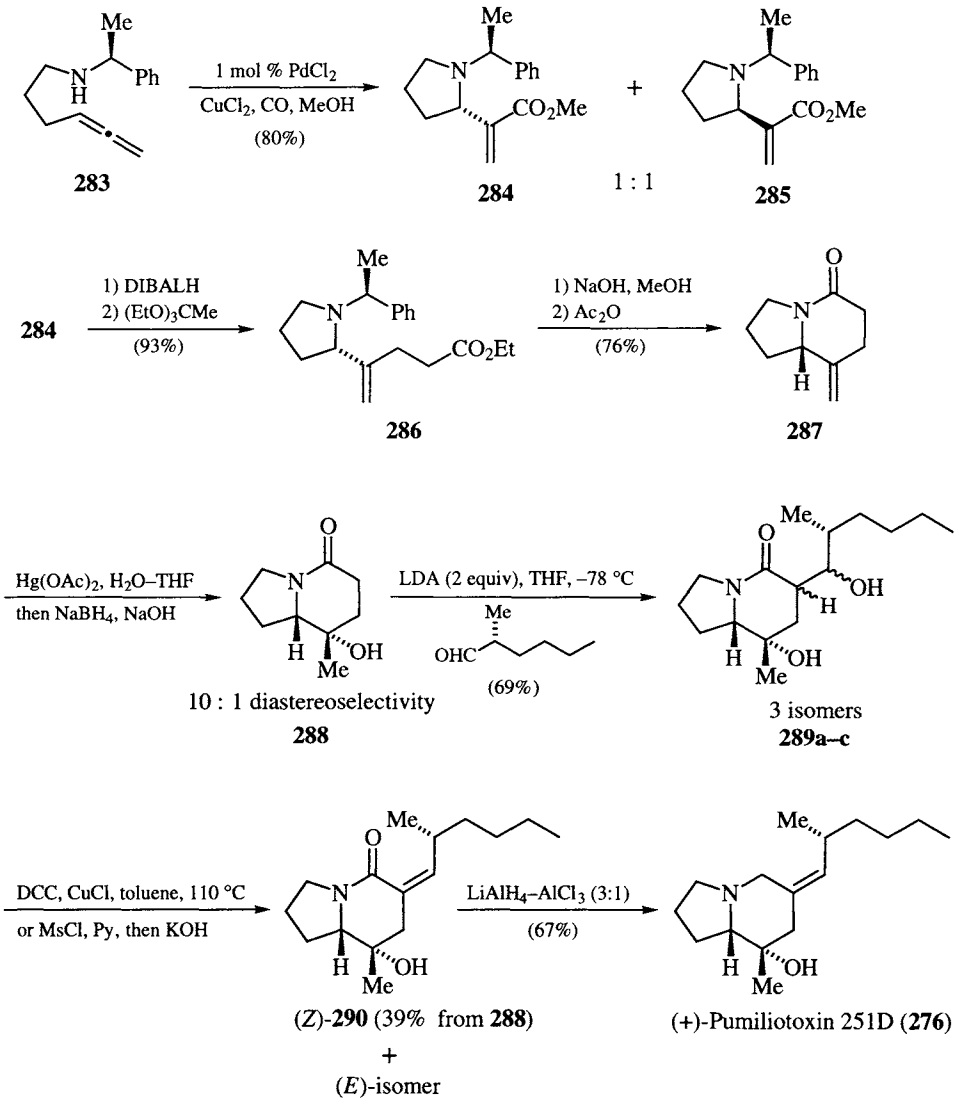
Homopumiliotoxins

223G (**282**) R = Me
(the absolute configuration
is unknown)

Figure 3. Representative pumiliotoxin A class alkaloids

The first synthetic efforts in this field were reported in 1981 by Overman and Bell (ref. 79). The enantioselective synthesis using as the key reaction the stereospecific iminium ion–vinylsilane cyclization led to pumiliotoxin 251D (**276**). This strategy was also applied to the synthesis of the

more complex pumiliotoxin A (**277**) (ref. 80) and pumiliotoxin B (**278**) (ref. 81). After that alternative approach to the enantioselective synthesis of pumiliotoxins by means of palladium(II)-mediated cyclization of the optically pure allenic amine has been presented by Gallagher and co-workers (ref. 82) (Scheme 42). Palladium(II)-mediated cyclization of the allenic amine **283** (ref. 83) under carbomethoxylation conditions provided the 2-substituted pyrrolidine **284** and its C-2 epimer **285**. Although diastereoselectivity is negligible, the desired diastereomer **284** was isolated on a multigram scale. After homologation of **284** to ester **286** in two steps, hydrolysis of **286** and treatment of the resulting carboxylate salt with acetic anhydride led directly to the enantiomerically



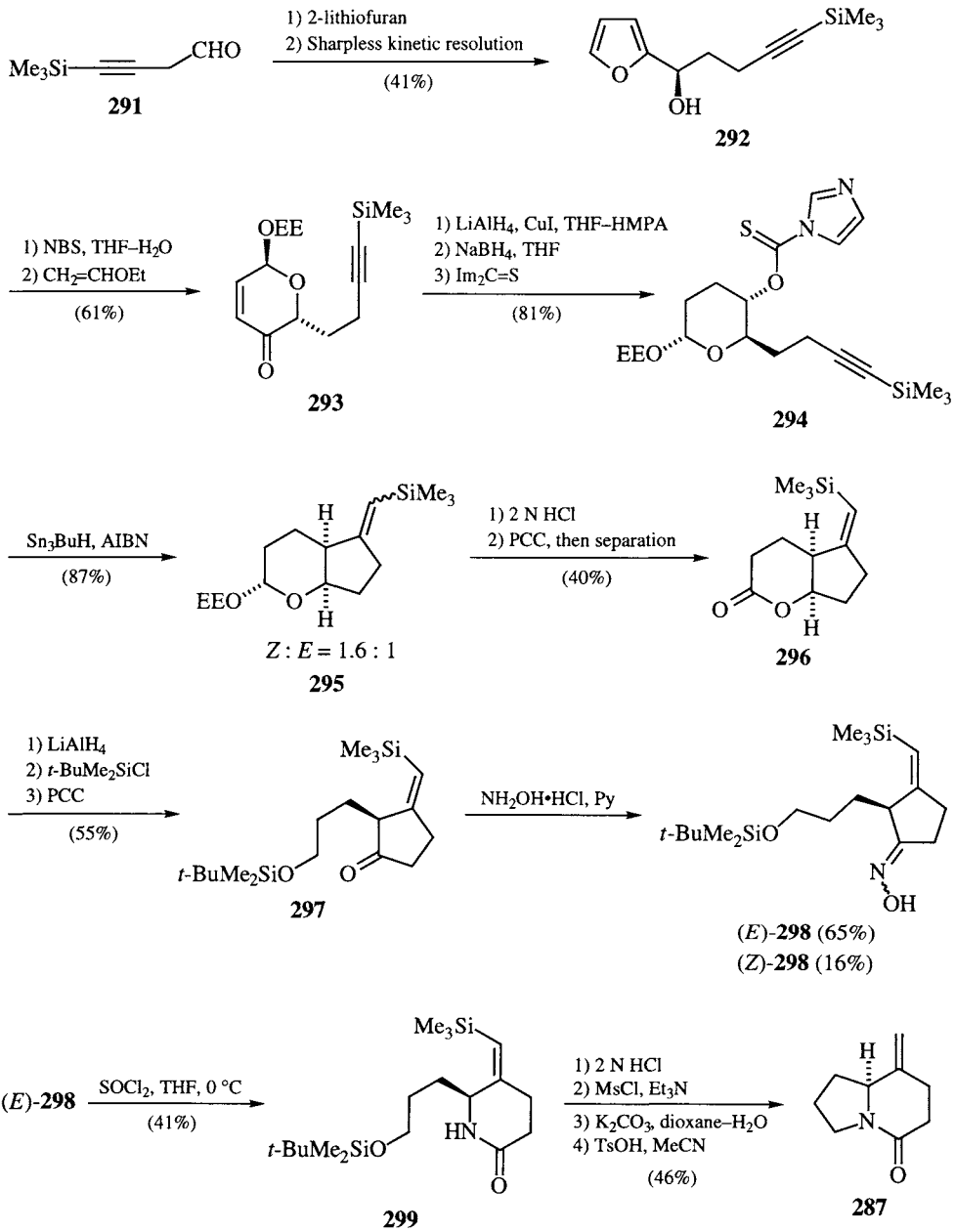
Scheme 42

pure bicyclic lactam **287**, accompanying cleavage of the α-methylbenzyl residue. Hydration of **287** by a hydroxymercuration afforded the tertiary alcohol **288** with a 10:1 stereoselectivity. Aldol condensation of the lactam **288** (10:1 mixture of diastereomers) gave a mixture of three isomers of **289a–c**. One isomer of these, **289a**, was exposed to stereospecific syn elimination using DCC and CuCl(I) to give (*Z*)-**290**. Application of an anti elimination sequence (MsCl then KOH) to the remaining inseparable mixture of **289b/c** gave a 2.6:1 mixture of the (*E*)- and (*Z*)-**290**. X-Ray crystallographic analysis of (*E*)-**290** served to establish the absolute stereochemistry of the initial adduct **284** but also confirmed both the relative configuration at C-8 and the geometry of the exocyclic alkene. Finally, 1,2-reduction of the unsaturated lactam moiety of (*Z*)-**290** by using $LiAlH_4/AlCl_3$ afforded (+)-pumiliotoxin 251D (**276**).

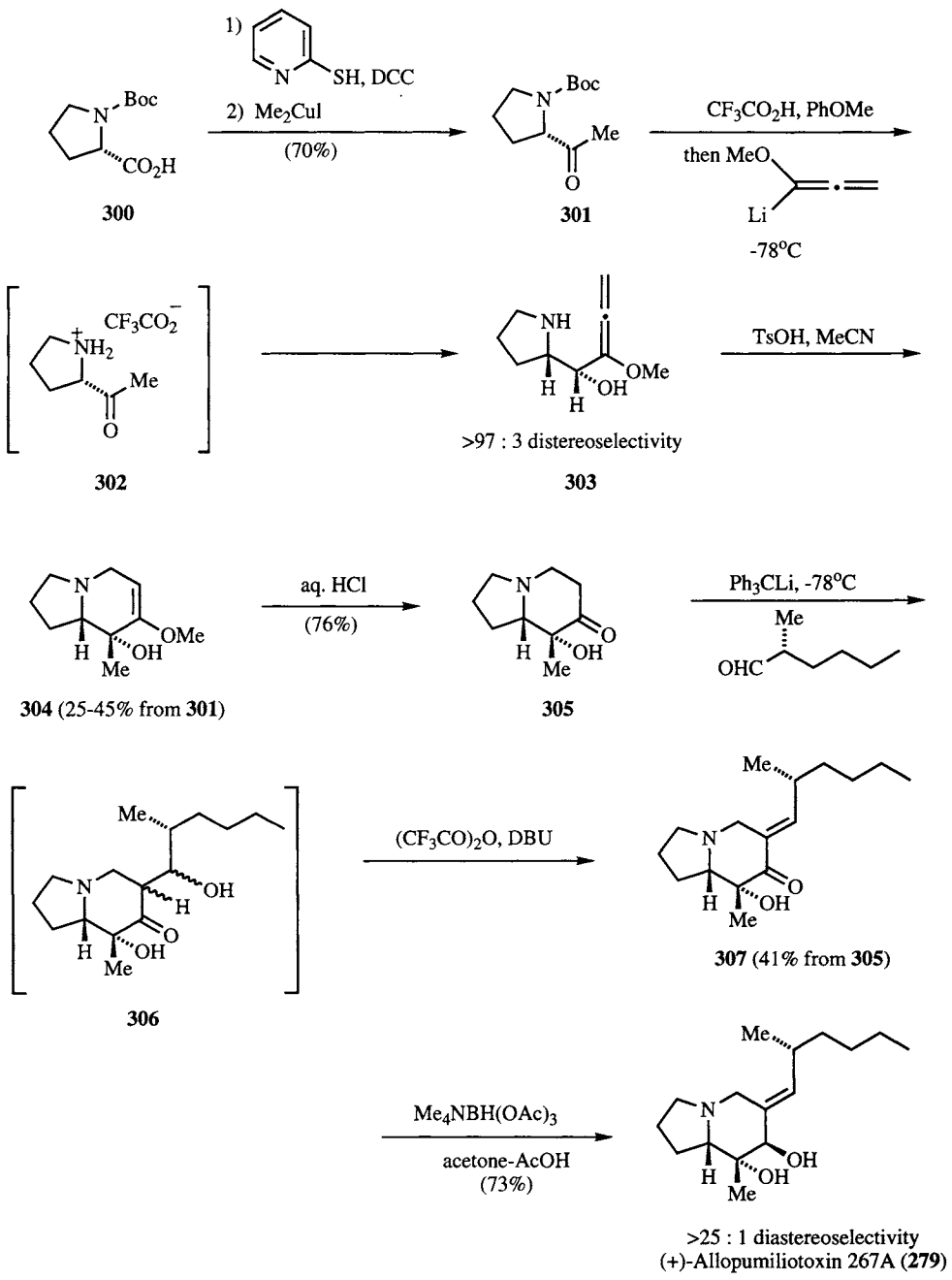
An enantioselective formal synthesis of (+)-pumiliotoxin 251D by Honda and co-workers (ref. 84) is based on the enantioselective preparation of the bicyclic lactam **287**, an intermediate in the approach of Gallagher's group (ref. 82). As summarized in Scheme 43, the key transformation was a stereoselective radical cyclization of the thiocarbonylimidazole **294** to the bicyclic compound **295**.

The most complex members of the pumiliotoxin A class, the allopumiliotoxins, contain a hydroxyl group at C-7 of the indolizidine ring. Representatives of this group are allopumiliotoxins 267A (**279**), 339A (**280**), and 339B (**281**). The first total synthesis of **279** and **281** by Overman's group, previously reported as a communication in 1984 (ref. 85), has been published with full experimental details in 1992 (ref. 86) (Scheme 44 and 45). In the first stage, 2-acetylpyrrolidine **301** prepared from *N*-Boc-proline (**300**) in two steps was converted into the enantiomerically pure 7-indolizidinone **305**. These reactions involved addition of 1-lithio-1-methoxy allene to the unprotected 3-acetylpyrrolidine **302** to give the labile intermediate **303** as a single diastereomer, which was lead to the bicyclic enol ether **304** on treatment with *p*-toluenesulfonic acid. Hydrolysis of **304** provided the indolizidinone **305**. Introduction of the side-chain into **305** was accomplished by employing an aldol–dehydration sequence. Condensation of the dianion of **305** with (*R*)-2-methylhexanal yielded a mixture of aldol products **306**, which was directly dehydrated with trifluoroacetic anhydride and DBU to give the enone **307**. Reduction of **307** with the usual reducing reagent gave predominantly the undesirable *cis*-diol, C-7 epimer of **279**. However, reduction of **307** with $Me_4NBH(OAc)_3$ in acetone–AcOH afforded the *cis*-diol **279** exclusively. The first enantioselective total synthesis of (+)-allopumiliotoxin 267A (**279**) was thus accomplished in seven steps and ~5% overall yield from **300**.

In identical fashion, the total synthesis of (+)-allopumiliotoxin 339B (**281**) was accomplished using the indolizidinone **305** (Scheme 45). The aldol condensation between the indolizidinone **305** and (*R*)-4-benzyloxy-2-methylbutanal (**308**) provided the enone **309**. Subsequent reduction of **309** with $NaBH_4$ and $CeCl_3$ gave the *cis*-diol **310** exclusively. The latter steps assembling the side chain are identical to the sequence previously employed in the synthesis of (+)-pumiliotoxin B by this group (ref. 81). Treatment of the aldehyde **311** with the ylide **312** provided the α'-silyloxy (*E*)-enone **313**. Finally, threo selective reduction of **313** with $LiAlH_4$ was accompanied by desilylation to afford (+)-allopumiliotoxin 339B (**281**).

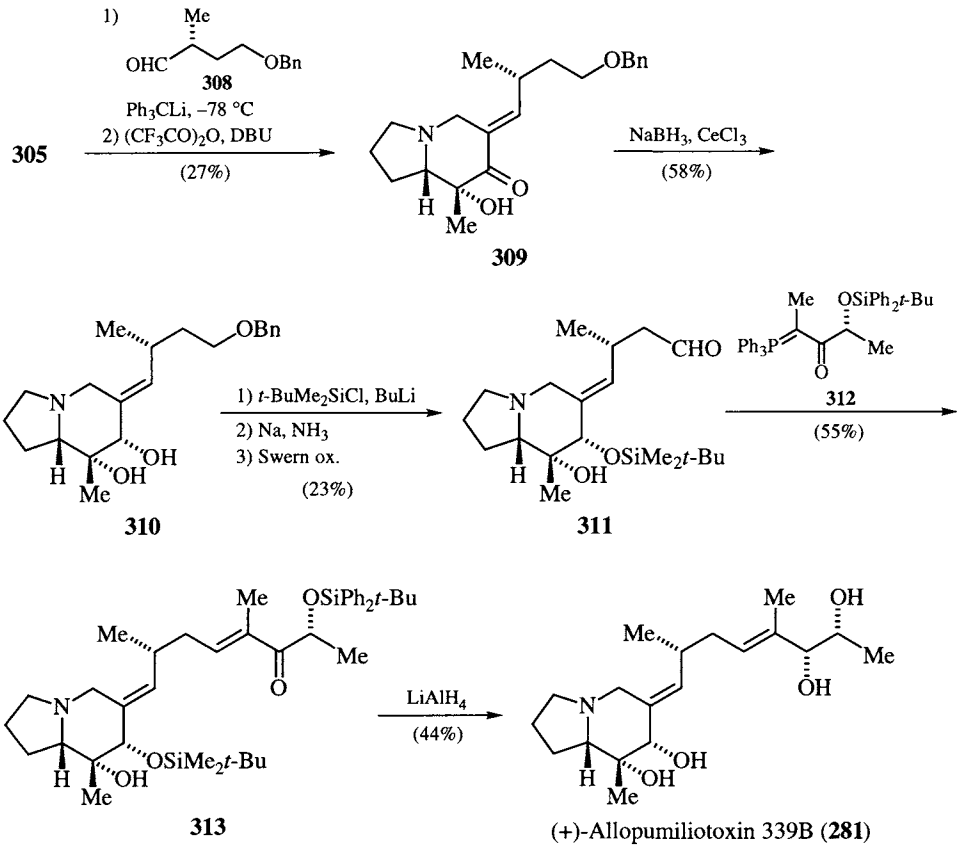

1) 2-lithiofuran
2) Sharpless kinetic resolution
(41%)
291
292
1) NBS, THF–H2O
2) CH2=CHOEt
(61%)
293
1) LiAlH4, CuI, THF–HMPA
2) NaBH4, THF
3) Im2C=S
(81%)
294
Sn3BuH, AIBN
(87%)
Z : E = 1.6 : 1
295
1) 2 N HCl
2) PCC, then separation
(40%)
296
1) LiAlH4
2) t-BuMe2SiCl
3) PCC
(55%)
297
NH2OH•HCl, Py
(E)-298 (65%)
(Z)-298 (16%)
(E)-298
SOCl2, THF, 0 °C
(41%)
299
1) 2 N HCl
2) MsCl, Et3N
3) K2CO3, dioxane–H2O
4) TsOH, MeCN
(46%)
287

Scheme 43



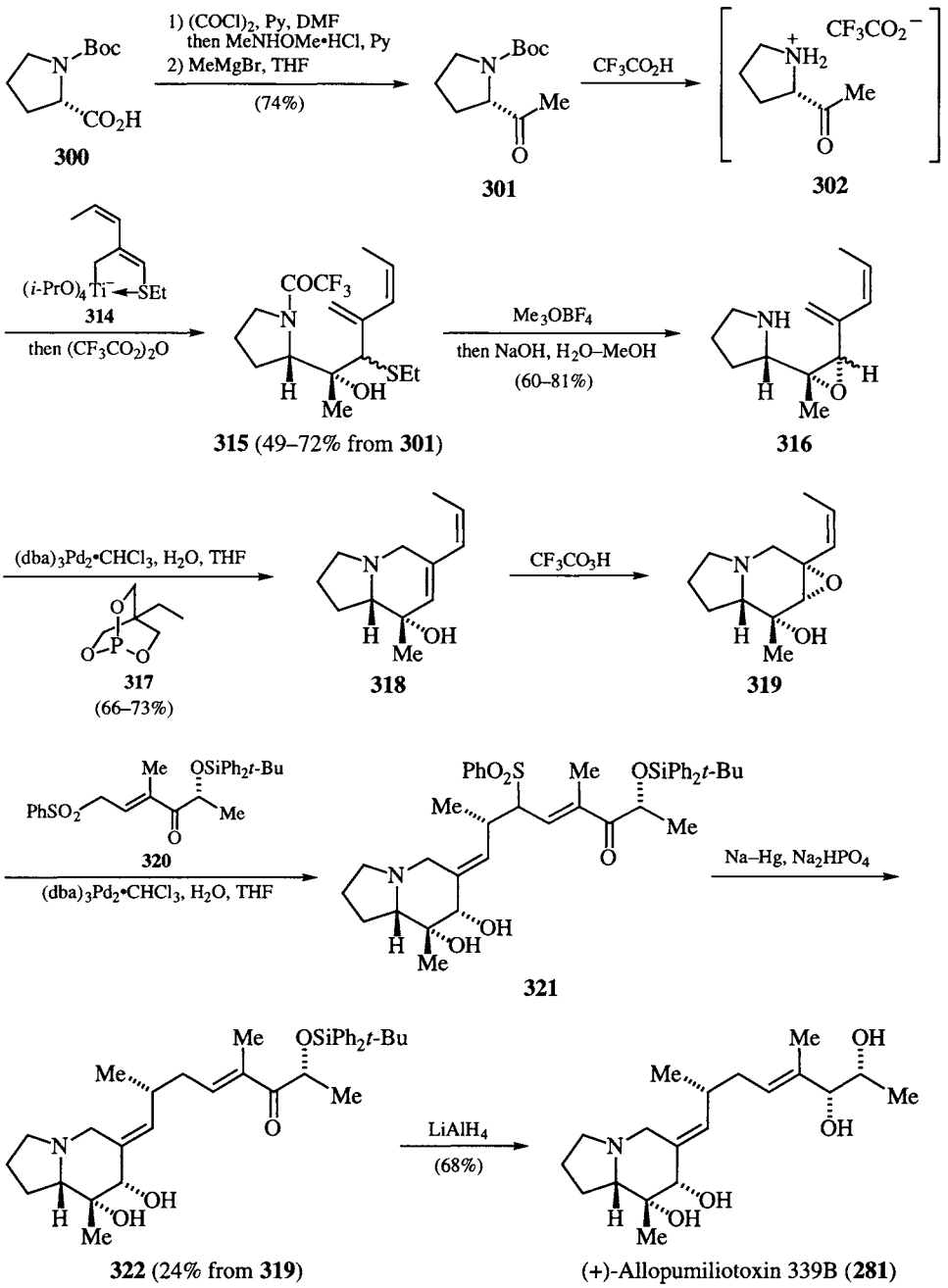
300 301 302 303 304 (25-45% from 301) 305 306 307 (41% from 305)

Scheme 44



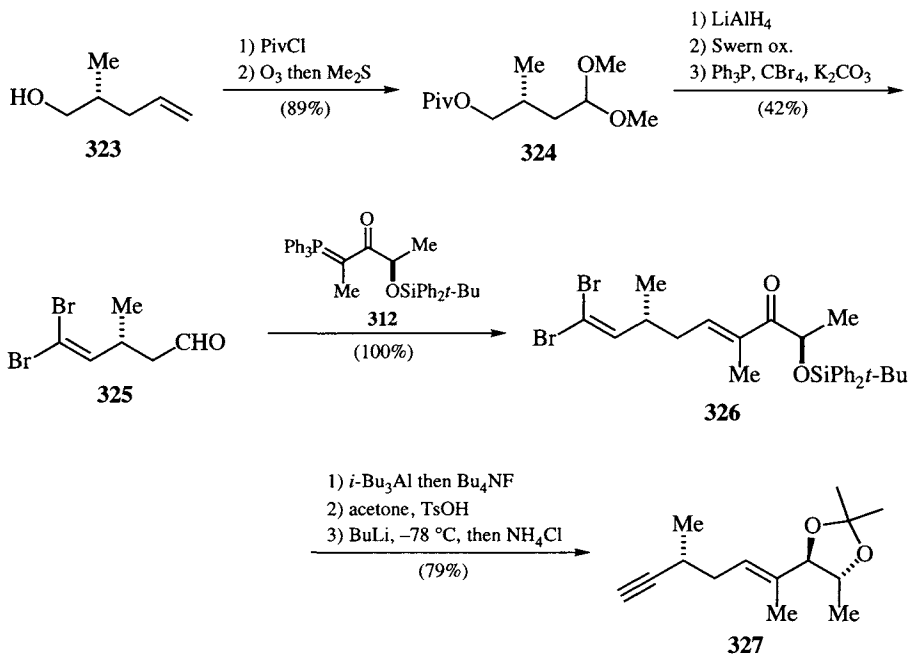
Scheme 45

Trost and Scanlan have reported novel and highly convergent synthesis of (+)-allopumiliotoxin 339B (**281**), involving, as the key step, a Pd(0)-mediated 6-endo cyclization (ref. 87) (Scheme 46). The precursor **316** for this reaction was prepared by diastereoselective addition of allytitanium reagent **314** to the 2-acetylpyrrolidine **302**, generated from the *N*-Boc-pyrrolidine **301**, followed by sequential treatment with Me_3OBF_3 and NaOH. Although the cyclization of the vinyl epoxide **316** was effected a variety of Pd(0) catalysis conditions, optimization of the reaction was achieved by employing a catalyst system consisting of $(dba)_3Pd_2{\bullet}CHCl_3$ and ligand **317** and adding water as a proton source to provide the indolizidine **318**. The indolizidine **318** was then converted into the vinyl epoxide **319** via hydroxy-directed epoxidation of its trifluoroacetate salt (ref. 88) with trifluoroperacetic acid. Palladium(0)-catalyzed condensation of the vinyl epoxide **319** and allylsulfone **320**, which was derived from the known Wittig reagent **312** (ref. 81), followed by reductive desulfonylation of the crude product **321** provided the homogeneous ketone **322**. This alkylation also benefited from the addition of water. Threo-selected reduction of **322** with $LiAlH_4$ was accompanied by concomitant desilylation to give (+)-allopumiliotoxin 339B (**281**).


1) (COCl)2, Py, DMF
then MeNHOMe•HCl, Py
2) MeMgBr, THF
(74%)
CF3CO2H
300
301
302
(i-PrO)4Ti–SEt
314
then (CF3CO2)2O
Me3OBF4
then NaOH, H2O–MeOH
(60–81%)
315 (49–72% from 301)
316
(dba)3Pd2•CHCl3, H2O, THF
317
(66–73%)
CF3CO3H
318
319
320
(dba)3Pd2•CHCl3, H2O, THF
Na–Hg, Na2HPO4
321
LiAlH4
(68%)
322 (24% from 319)
(+)-Allopumiliotoxin 339B (281)

Scheme 46

(+)-Allopumiliotoxin 339A (**280**) is C-7 hydroxyl isomer of (+)-allopumiliotoxin 339B (**281**) and is more effective than (+)-allopumiliotoxin 339B in stimulating both sodium influx and phosphoinositide breakdown in guinea pig cerebral cortical synaptoneurosomer (ref. 89). Overman and co-workers have published the first total synthesis of (+)-allopumiliotoxin 339A (**280**) (ref. 90). This practical entry to the allopumiliotoxin alkaloids used a nucleophile-promoted iminium ion–alkyne cyclization (ref. 91) for elaboration of the alkylideneindolizidine ring. The required alkyne **327** was prepared from (*R*)-2-methyl-4-pentenol (**323**) (Scheme 47). Thus, **324** was converted to the dibromopentanal **325** via the acetal **324** in five steps. Wittig condensation of **326** with the phosphorane **312** gave α'-silyloxy (*E*)-enone **326**. Reduction of **326** with *i*-Bu_3Al and removal of the silyl protecting group provided the *syn*-diol, which was converted to the acetonide derivative. This intermediate was subsequently treated with excess butyllithium followed by protonolysis to provide the (–)-alkyne **327**.

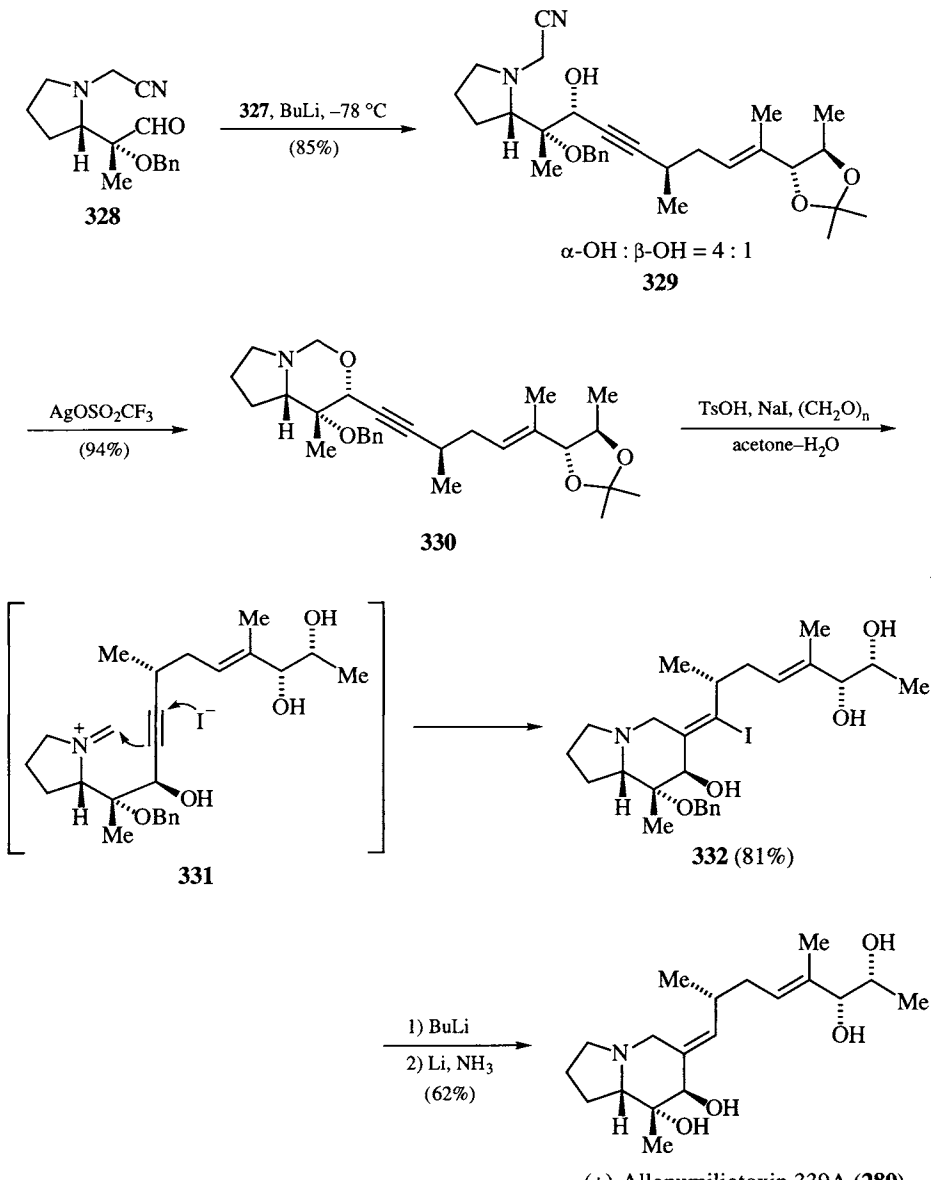


323 **324** **325** **326** **327**

Scheme 47

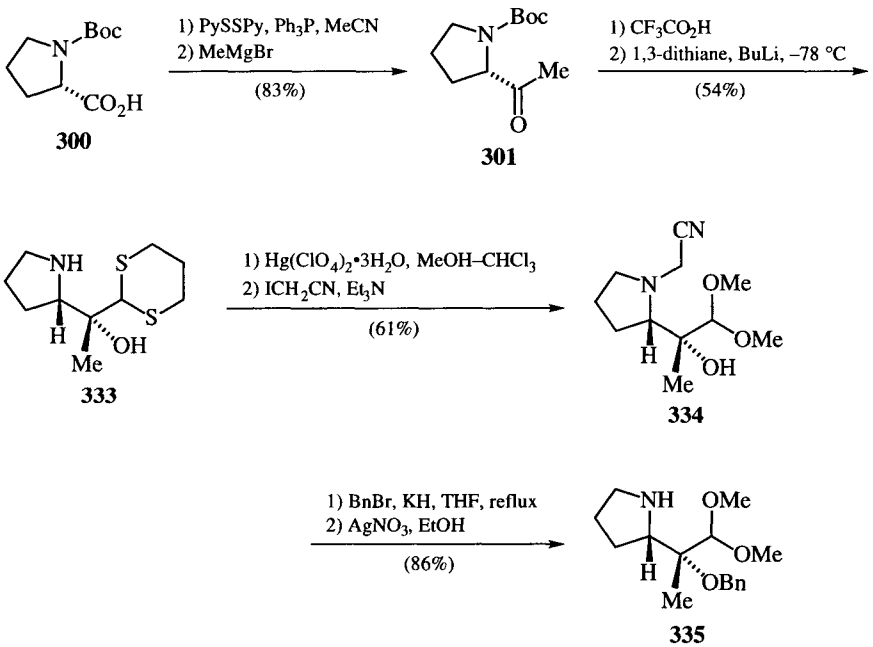
Addition of the alkynyllithium derivative of **327** to the α-benzyloxy aldehyde **328**, previously used by these workers (ref. 92) in model studies for entry into the allopumiliotoxin class alkaloid, gave the alcohol **329** with 4:1 diastereoselectivity (Scheme 48). Although the employment of the corresponding alkynyldiisopropoxytitanium nucleophile derived from **327** for the reaction with **329** improved the facial selectivity (>10:1), the yield of this reaction was unacceptably low. After chromatographic separation, treatment of the α-OH isomer **329** with AgOTf provided the

cyclopentaoxazine **330**. Iodine-promoted cyclization of **330** proceeded with loss of the isopropylidene group, via the formaldiminium ion **331**, to afford the alkylideneindolizidine **332** as a single stereoisomer. Deionization of **332** followed by cleavage of the benzyl ether with Li–NH_3 provided (+)-allopumiliotoxin 339A (**280**). This highly convergent synthesis of **280** was thus attained in 16 steps and 15% overall yield from **323**.



Scheme 48

Our group (ref. 94) have developed a new, highly regio- and stereocontrolled approach to the synthesis of (+)-allopumiliotoxin 267A (**279**) and 339A (**280**), utilizing an intramolecular nickel(II)/chromium(II)-mediated coupling reaction developed by Nozaki (ref. 95) and Kishi (ref. 96) groups. This pivotal cyclization step led to both formation of 6(*E*)-alkylideneindolizidine framework and introduction of the axial 7β-hydroxy group at the same time in a single operation. These synthesis commenced with the enantioselective synthesis of the pyrrolidine fragment **335** (Scheme 49). Deprotection of *N*-Boc acetylpyrrolidine **301**, prepared from **300**, with trifluoroacetic acid by the known procedure (refs. 86 and 87), and subsequent treatment of the resulting salt with 2-lithio-1,3-dithiane to produce the tertiary alcohol **333** with complete diastereoselection. Transformation of the cyclic dithioacetal of **333** into the corresponding dimethylacetal with methanol and $Hg(ClO_4)_2$, followed by N-protection with the cyanomethyl group (ref. 92) gave **334**. O-benzylation of the *tert*-hydroxyl group in **334** followed by deblocking of the cyanomethyl group gave the desired pyrrolidine **335**.



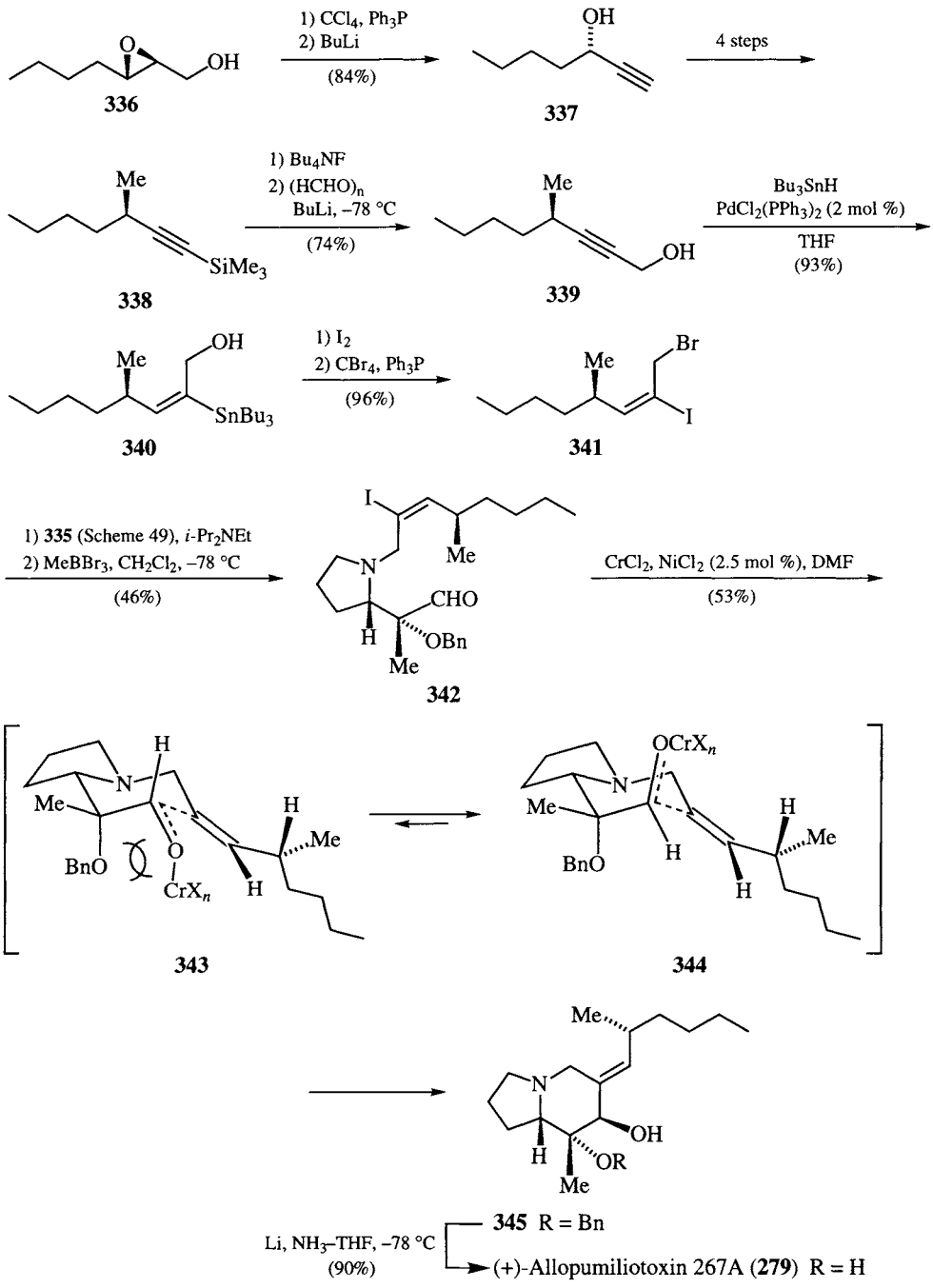
Scheme 49

The alkene side-chain segment, (*E*)-allyl bromide **341**, was prepared from the optically active epoxy alcohol (ref. 96) (Scheme 50) obtained by Sharpless asymmetric epoxidation of 2-heptenol. Chlorination of **336** followed by treatment with butyllithium (ref. 97) provided (*S*)-1-heptyn-3-ol (**337**), which was converted to the (*R*)-silylalkyne **338** using Overman's method (ref. 81). Desilylation of **338** followed by hydroxymethylation (paraformaldehyde, butyllithium) of the

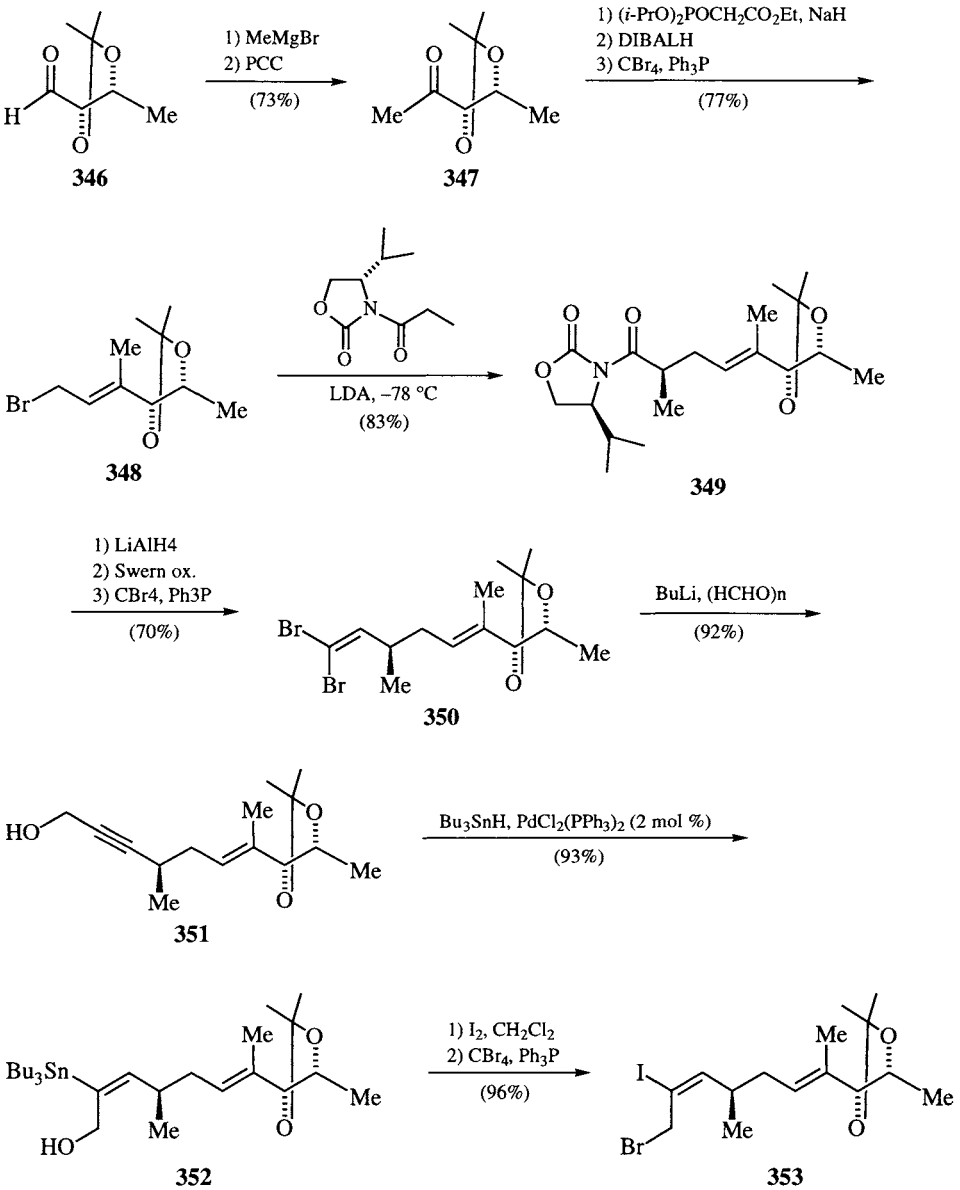
resulting alkyne gave (*R*)-4-methyloctynol (**339**). Palladium-catalyzed syn hydrostannation (ref. 98) of **339** with Bu_3SnH in the presence of catalytic $PdCl_2(PPh_3)_2$ proceeded regioselectively to give the 2-(tributylstannyl)alkene **340** with correct (*E*)-olefin geometry. Iododestannation of **340** with iodine gave the corresponding (*E*)-vinyl iodide which was converted into the (*E*)-allyl bromide **341**. Coupling of the two segments **341** and **335** in the presence of Hünig base followed by cleavage of the dimethyl acetal by treatment with Me_2BBr afforded the (*E*)-iodoalkenylaldehyde **342**. Intramolecular cyclization of **342** proceeded under the mild conditions ($CrCl_2$, catalytic $NiCl_2$, DMF, room temperature) with virtually complete stereocontrol, giving rise to **345**. The extremely high degree of diastereoselectivity in this process can be explained by examination of the two chair-like transition states, **343** and **344**, the former of which would be destabilized owing to an allylic 1,3-strain (ref. 26) between the equatorial chromium alkoxide and the olefin and, more importantly, steric hindrance/electrostatic repulsion between the benzyloxy and the chromium alkoxide groups. The preferred transition conformer **344**, leading to **345**, is quite free from these interactions. Reductive cleavage of the benzyl group of **345** provided (+)-allopumiliotoxin 267A (**279**).

Total synthesis of the more complex (+)-allopumiliotoxin 339A (**280**) was completed by employing the strategy developed for **279**. The side-chain segment, (*E*)-vinyl iodide **353**, was elaborated in 13 steps from D-4-deoxythreose derivative **346** (Scheme 51). Grignard reaction and subsequent PCC oxidation provided the methyl ketone **347**. Horner–Emmons reaction of **347**, followed by DIBALH reduction of the resulting unsaturated ester gave an allylic alcohol which was teated with CBr_4/PPh_3 to afford the allylbromide **348**. C_2 homologation of **348** by using Evans alkylation (ref. 99) provided **349** with virtually complete diastereoface selection. Reductive removal of the oxazolidinone auxiliary on **349** with $LiAlH_4$, followed by Swern oxidation and treatment of the resulting aldehyde with CBr_4/PPh_3 furnished the dibromide **350**, which was converted to the propargyl alcohol **351** by treatment with butyllithium and paraformaldehyde. Stereospecific and highly regioselective syn hydrostannation of **351** was effected by applying palladium-catalyzed hydrostannation under conditions previously described, giving the (*E*)-2-(tributylstannyl)alkenyl alcohol **352**. Iododestannation and subsequent bromination gave the allylbromide **353**.

The preparation of the pyrrolidine segment **355** was attained from the foregoing pyrrolidine **335** (Scheme 49) as outlined in Scheme 52. Compound **335** was successively converted to the alcohol **354** through N-protection by Cbz group, acetal hydrolysis, and $NaBH_4$ reduction of the resulting aldehyde. Silylation of **354** and hydrogenolytic removal of the Cbz group resulted in **355**, which was alkylated with **353** to provide **356**. Removal of the silyl protecting group followed by Swern oxidation gave the aldehyde **357**. On the subsequent treatment with nickel(II)/chromium(II), intramolecular coupling reaction of **357** proceeded to give exclusively **358**. Sequential removal of the isopropylidene protecting group under the acidic conditions and the benzyl group by treatment with Li/NH_3 provided (+)-allopumiliotoxin 339A (**280**).

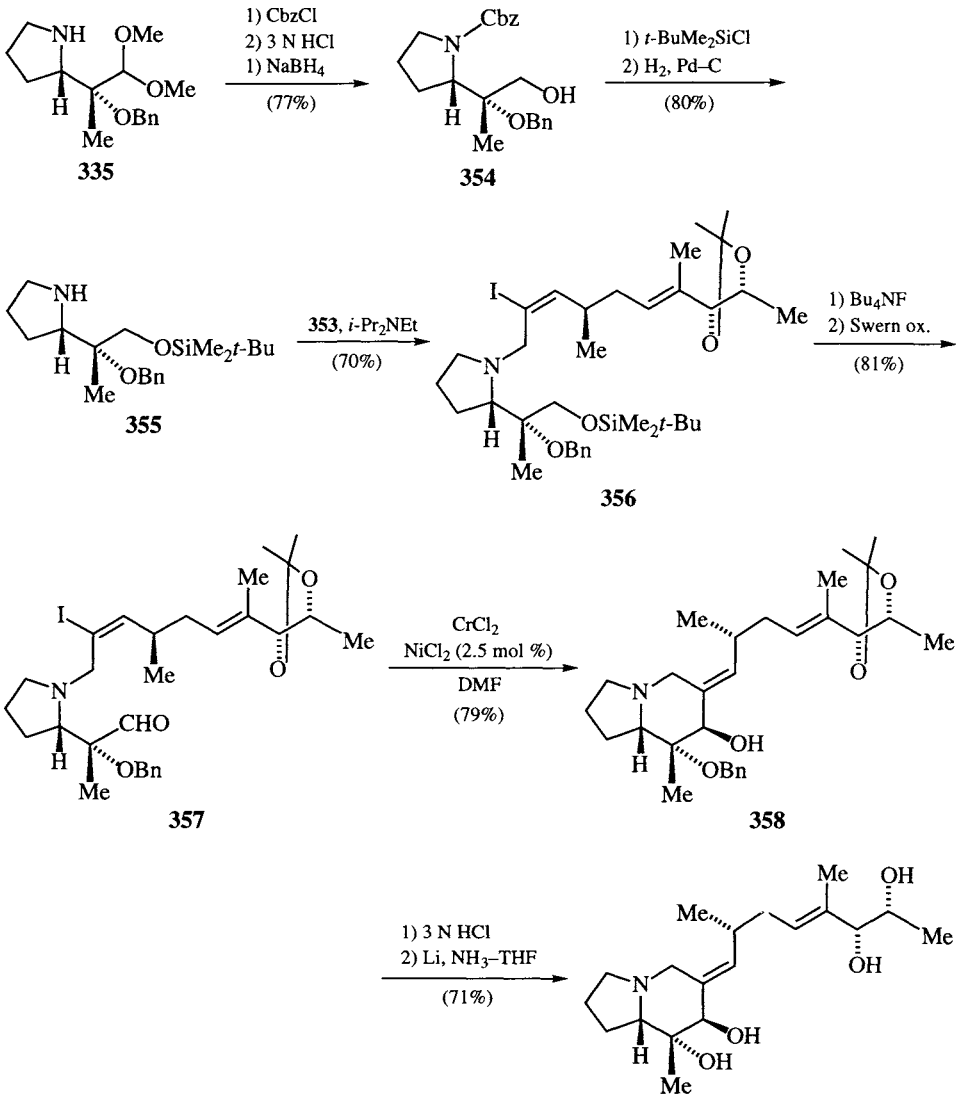

1) CCl_4, Ph_3P
2) BuLi
(84%)
4 steps
1) Bu_4NF
2) $(HCHO)_n$
BuLi, –78 °C
(74%)
Bu_3SnH
$PdCl_2(PPh_3)_2$ (2 mol %)
THF
(93%)
1) I_2
2) CBr_4, Ph_3P
(96%)
1) 335 (Scheme 49), i-Pr_2NEt
2) $MeBBr_3$, CH_2Cl_2, –78 °C
(46%)
$CrCl_2$, $NiCl_2$ (2.5 mol %), DMF
(53%)
Li, NH_3–THF, –78 °C
(90%)
345 R = Bn
(+)-Allopumiliotoxin 267A (279) R = H

Scheme 50



346 **347** **348** **349** **350** **351** **352** **353**

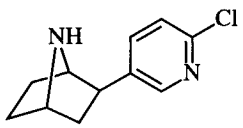
Scheme 51


1) CbzCl
2) 3 N HCl
1) NaBH4
(77%)
1) t-BuMe2SiCl
2) H2, Pd–C
(80%)
335
354
353, i-Pr2NEt
(70%)
1) Bu4NF
2) Swern ox.
(81%)
355
356
CrCl2
NiCl2 (2.5 mol %)
DMF
(79%)
357
358
1) 3 N HCl
2) Li, NH3–THF
(71%)
(+)-Allopumiliotoxin 339A (280)

Scheme 52

6. SYNTHESES OF EPIBATIDINE

A new class of amphibian alkaloid epibatidine (**359**), isolated by Daly and co-workers in a trace amount from the skin of the Ecuadorian poison frog, *Epipedobates tricolor*, of the family Dendrobatidae proved to be a potent analgesic with a non-opioid mechanism of action (ref. 100). The preliminary tests showed that epibatidine has about 200 to 500 times the potency of morphine in the hot plate and Straub-tail analgesic assays, respectively. The relative stereochemistry of epibatidine has been deduced as *exo*-2-(6-chloro-3-pyridyl)-7-azabicyclo[2.2.1]heptane on the basis of MS, IR, and ^{1}H NMR spectra, and later confirmed by Broka's first synthesis (ref. 101) of (±)-epibatidine. The absolute configuration of the natural epibatidine was determined by comparison of synthetic material with authentic material by using chiral HPLC and by using X-ray crystallography in 1994 by the Merck group (refs. 102 and 103).

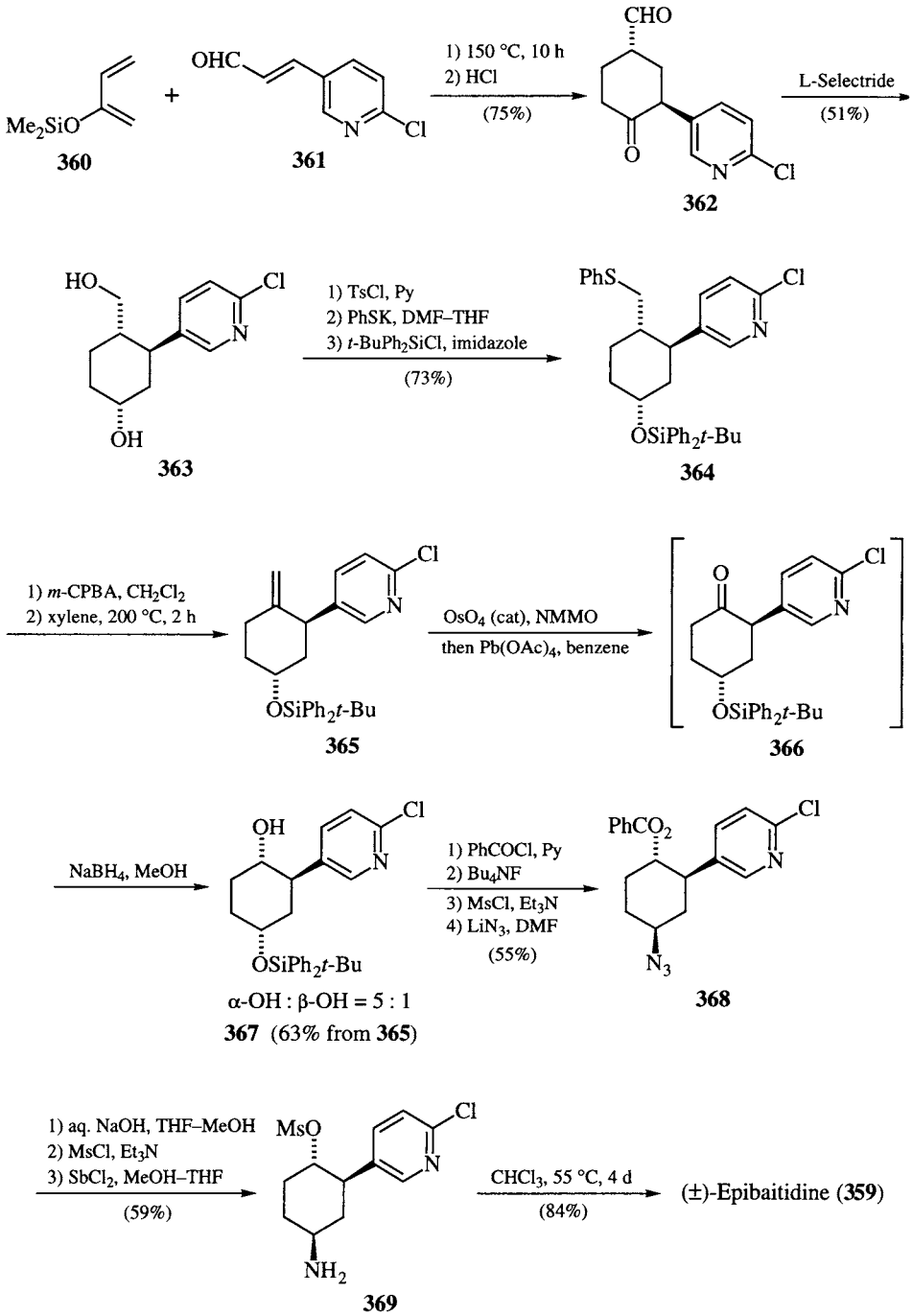


(+)-Epibatidine (**359**)

Owing to its intriguing biological activity and interesting structural features as well as its scarcity in nature, a number of total syntheses have been reported for the last few years. The synthetic approaches to epibatidine may be divided into three classes according to the procedures for the construction of 7-azabicyclo[2.2.1]heptane ring system: (1) synthesis involves a transannular reaction of appropriately 4-substituted cyclohexylamines, (2) synthesis in which Diels–Alder cycloaddition of N-protected pyrroles with acetylenic dienophiles is used, (3) synthesis in which [3 + 2] cycloaddition of non-stabilized azomethin ylide and substituted vinylpyridine is used.

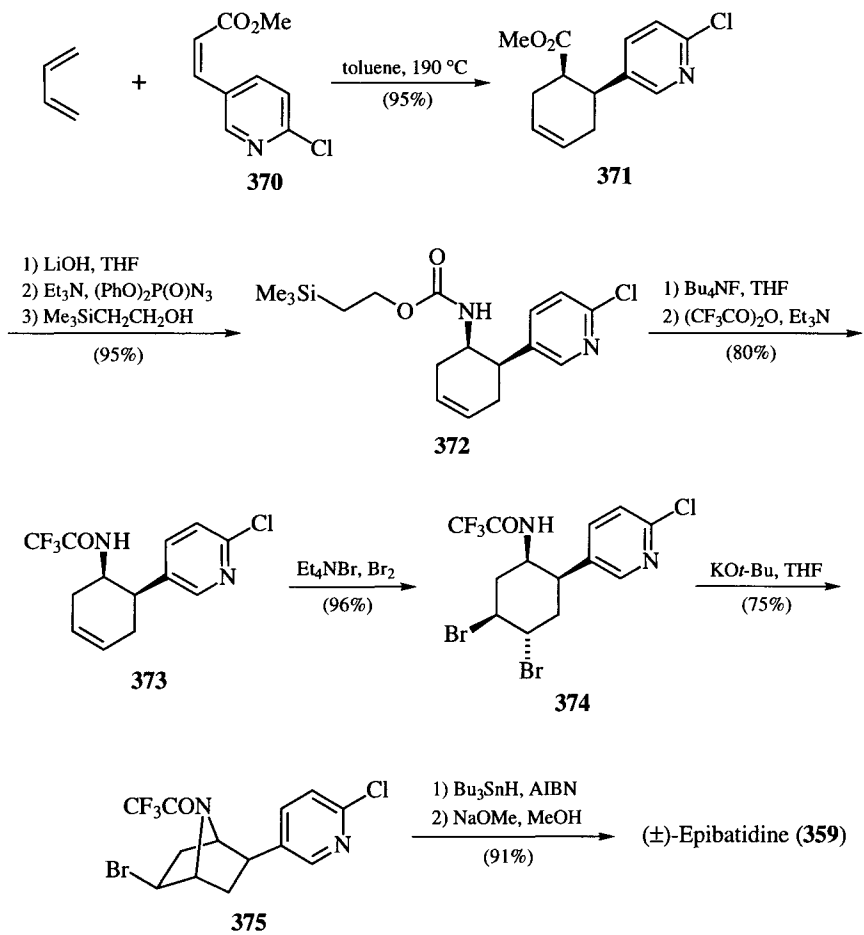
6.1 Approaches via a Transannular Reaction

Broka (ref. 101) has reported the first total synthesis of epibatidine (**359**) in racemic form (Scheme 53). Diels–Alder reaction of the enal **361** with 2-(trimethylsiloxy)-1,3-butadiene (**360**) followed by treatment of the crude adduct with HCl gave the ketoaldehyde **362** as a single stereoisomer which was converted to the diol **363**. Sequential tosylation of the primary hydroxy group, displacement with thiophenoxide, and protection of the resulting hydroxy group gave the sulfide **364**. Oxidation of **364**, followed by sulfoxide elimination gave the olefin **365**. Cleavage of the methylene unit and borohydride reduction of the resulting ketone **366** produced the equatorial cyclohexanol along with its axial epimer in a ratio of 5:1. The separated **367** was then transformed through four steps into the azide **368**. After conversion of **368** into the aminotosylate **369**, intramolecular displacement of mesyloxy group occurred on heating **369** in $CHCl_3$ to complete the synthesis of (±)-**359**.


Me2SiO
360
OHC
N
Cl
361
1) 150 °C, 10 h
2) HCl
(75%)
CHO
O
N
Cl
362
L-Selectride
(51%)
HO
Cl
N
OH
363
1) TsCl, Py
2) PhSK, DMF–THF
3) t-BuPh2SiCl, imidazole
(73%)
PhS
Cl
N
OSiPh2t-Bu
364
1) m-CPBA, CH2Cl2
2) xylene, 200 °C, 2 h
Cl
N
OSiPh2t-Bu
365
OsO4 (cat), NMMO
then Pb(OAc)4, benzene
O
Cl
N
OSiPh2t-Bu
366
NaBH4, MeOH
OH
Cl
N
OSiPh2t-Bu
α-OH : β-OH = 5 : 1
367 (63% from 365)
1) PhCOCl, Py
2) Bu4NF
3) MsCl, Et3N
4) LiN3, DMF
(55%)
PhCO2
Cl
N
N3
368
1) aq. NaOH, THF–MeOH
2) MsCl, Et3N
3) SbCl2, MeOH–THF
(59%)
MsO
Cl
N
NH2
369
CHCl3, 55 °C, 4 d
(84%)
(±)-Epibaitidine (359)

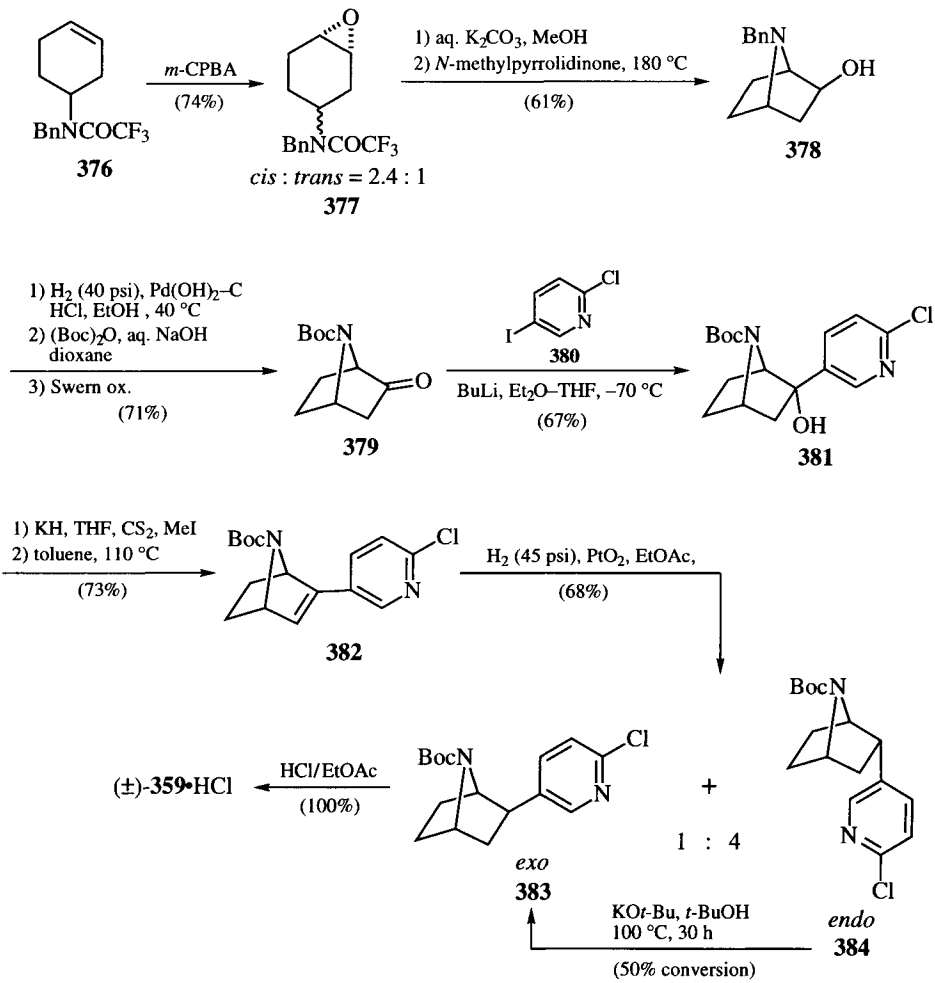
Scheme 53

Corey and co-workers (ref. 104) have carried out the stereocontolled total synthesis of (±)-epibatidine (**359**) in 40% overall yield from (*Z*)-α,β-unsaturated ester **370** (Scheme 54). Diels–Alder addition of 1,3-butadiene to **370** gave the *cis*-ester **371**. After elaboration of **371** into the *cis*-carbamate **372** via Curtius rearrangement in the presence of 2-(trimethylsilyl)ethanol, carbamate cleavage followed by acylation gave the *cis*-trifluoroacetamide **373**. This amide **373** underwent stereospecific bromination to form the dibromide **374**. The stereochemical outcome from the bromination was rationalized in terms of kinetically favored formation of the bromonium ion with cis CF_3CONH (axial) and bromine substituents followed by diaxial opening of that bromonium ion by bromide ion attack. Base-promoted internal nucleophilic displacement using sodium *tert*-butoxide transformed the dibromide **374** into the bridged monobromide **375**. Sequential debromination of **375** and deacylation of the intermediate amide completed the synthesis of (±)-**359**.



Scheme 54

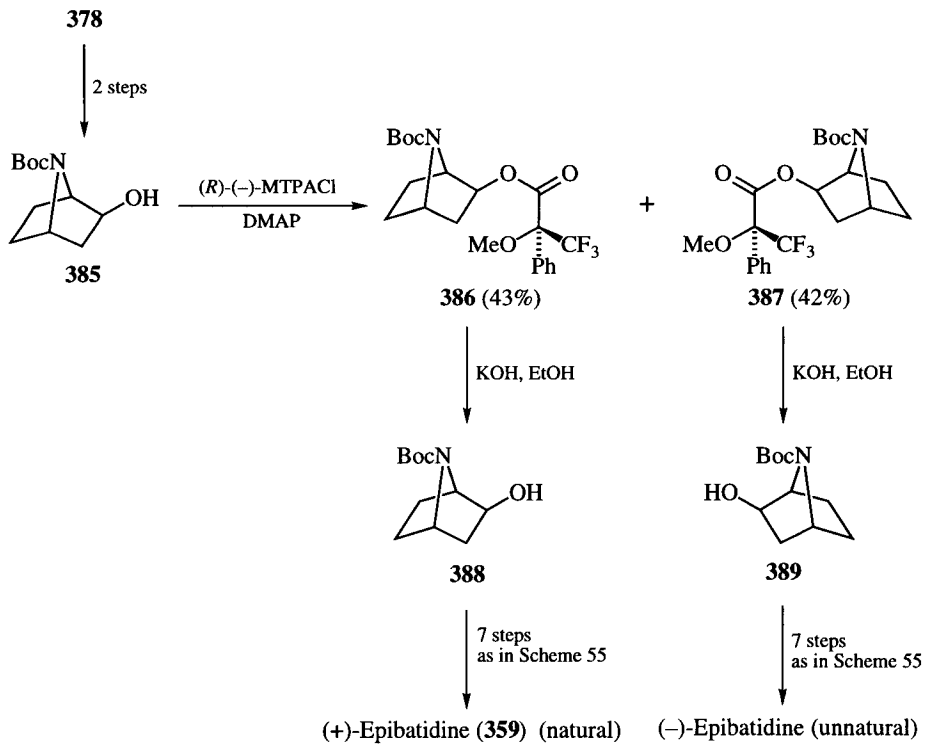
The total synthesis of (+)- and (–)-epibatidine (**359**) involving the coupling reaction of 5-lithio-2-chloropyrididine with *N*-Boc-7-azabicyclo[2.2.1]heptan-2-one (**379**) as the critical step was accomplished by the Merck group (refs. 102 and 105) as shown in Scheme 55 and 56. Synthesis of the required ketone **379** was prepared from the aminocyclohexene derivative **376**. Epoxidation of **376** afforded a 2.4:1 (*cis:trans*) mixture of the epoxides **377**. Basic hydrolysis of **377** and subsequent heating the hydrolysis products in *N*-methyl-2-pyrrolidinone at 180 °C afforded the cyclized product, the unexpected *endo*-alcohol **378**. Removal of *N*-benzyl group in **378** by hydrogenolysis, followed by *N*-Boc protection of the resulting amine and Swern oxidation of the resulting carbamate provided the *N*-Boc ketone **379** required for the key coupling reaction. Treatment of 2-chloro-5-pyridine (**381**) with butyllithium followed by reaction with the ketone **379**



Scheme 55

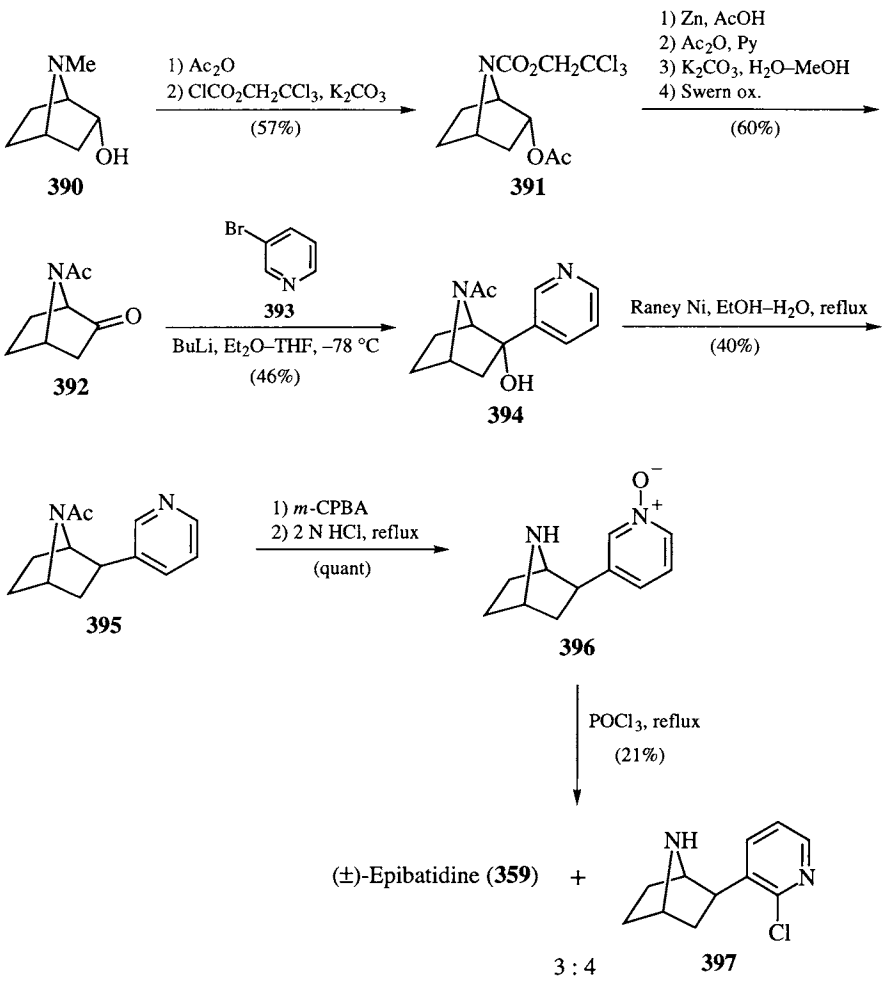
afforded the *tert*-alcohol **381** as a single product. Compound **381** was converted to the *S*-methyl xanthate and subsequent thermolysis resulted in elimination of the xanthate to give the olefin **382**. Hydrogenation of **382** using Adam's catalyst produced a 4:1 mixture of *endo*- and *exo*-isomers, of which the undesired *endo*-isomer **384** was epimerized using potassium *tert*-butoxide to afford the *exo*-isomer **383**. Removal of the Boc group on treatment with hydrogen chloride in ethyl acetate produced (±)-**359**.

For preparation of the (+)- and (–)-enantiomers of epibatidine, resolution of the *exo*-alcohol **385** derived from compound **378** was achieved by formation of diastereomeric esters with (*R*)-(–)-Mosher's acid chloride (Scheme 56). Crystallization from hexane and chromatographic purification of the mother liquors afforded **386** and **387**, respectively. Hydrolysis of the esters **386** and **387** with KOH gave the enantiomers **388** and **389**, respectively. Each enantiomer was taken through the reaction sequence developed for the racemate to afforded the (+)- and (–)-enantiomers of epibatidine (**359**). An X-ray structure determination of the Mosher's ester **387** and comparison of synthetic material with authentic epibatidine by high performance liquid chromatography (ref. 103) using chiral stationary phases established the absolute configuration of the natural product to be 1*R*,2*R*,4*S*.



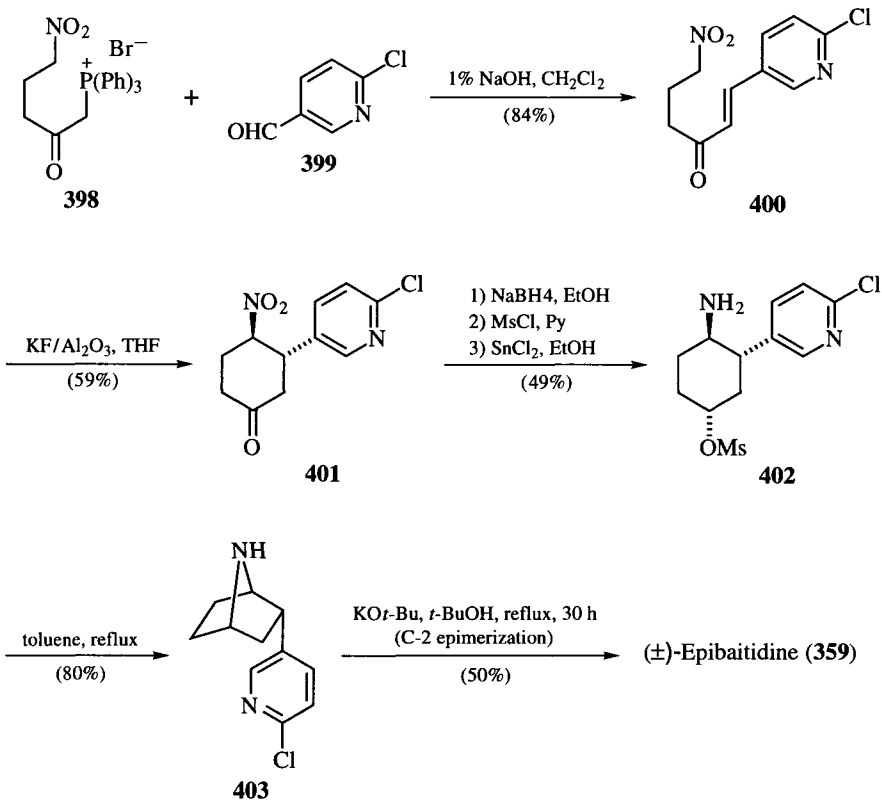
Scheme 56

The closely related coupling reaction has been applied to the synthesis of (±)-epibatidine (**359**) by Nakai and co-workers (ref. 106) (Scheme 57). The azabicyclo[2.2.1]heptanone **392**, which was prepared in six steps from the known *endo*-7-azabicyclo[2.2.1]heptanol **390**, served as a key intermediate in their synthesis. The stereoselective exo addition of pyridine nucleus to the ketone **392** afforded **394**. Subsequent reductive elimination of hydroxy function in **394** using Raney nickel proceeded with retention of the configuration to afford **395**. Oxidation of **395** with *m*-CPBA , followed by removal of the acetyl group by acidic hydrolysis gave the *N*-oxide **396**, which on treatment with phosphorus oxychloride produced racemic **359** and its isomer **397** (3:4 ratio).



Scheme 57

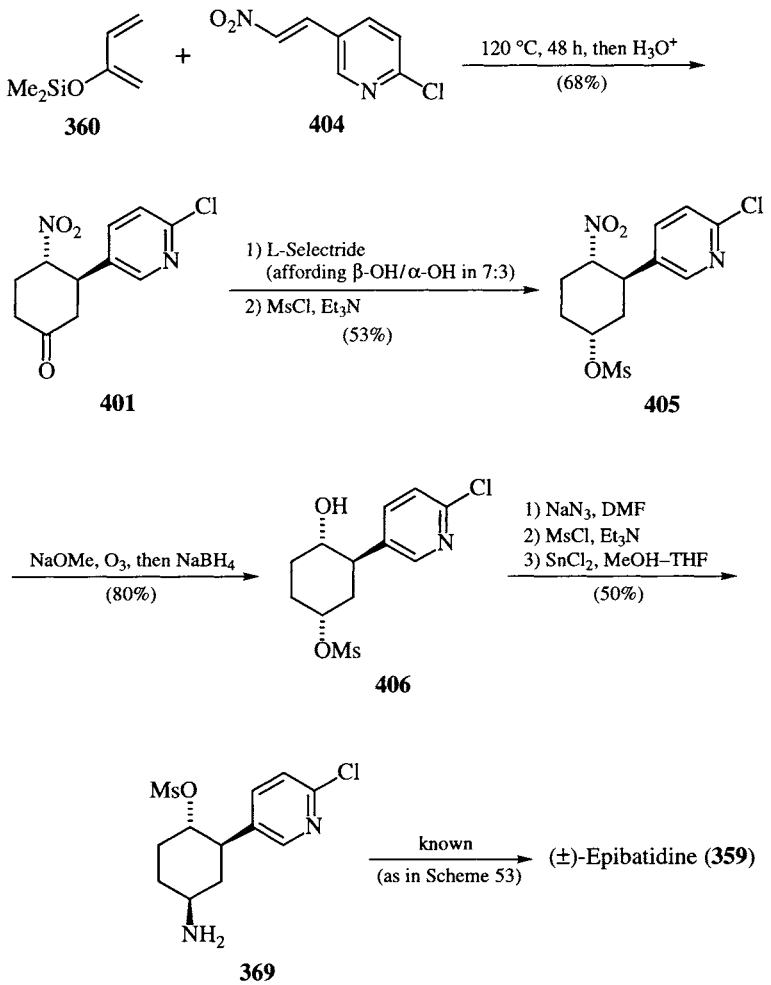
Szántay and co-workers (ref. 107) have developed a practical synthesis of (±)-epibatidine (**359**) based on a intramolecular Michael addition of the nitro compound (Scheme 58). Wittig reaction of the phosphorane **398** with the chloropyridine aldehyde **399** gave rise to **400**. Treatment of **402** with KF/alumina furnished the cyclohexane derivative **401**. Reduction of the keto group in **401** followed by mesylation and subsequent reduction of the nitro group gave the amine **402**, which on heating in refluxing toluene resulted in 2-*epi*-epibatidine (**403**). Epimerization of **403** by heating with potassium *tert*-butoxide led to racemic **359**.



Scheme 58

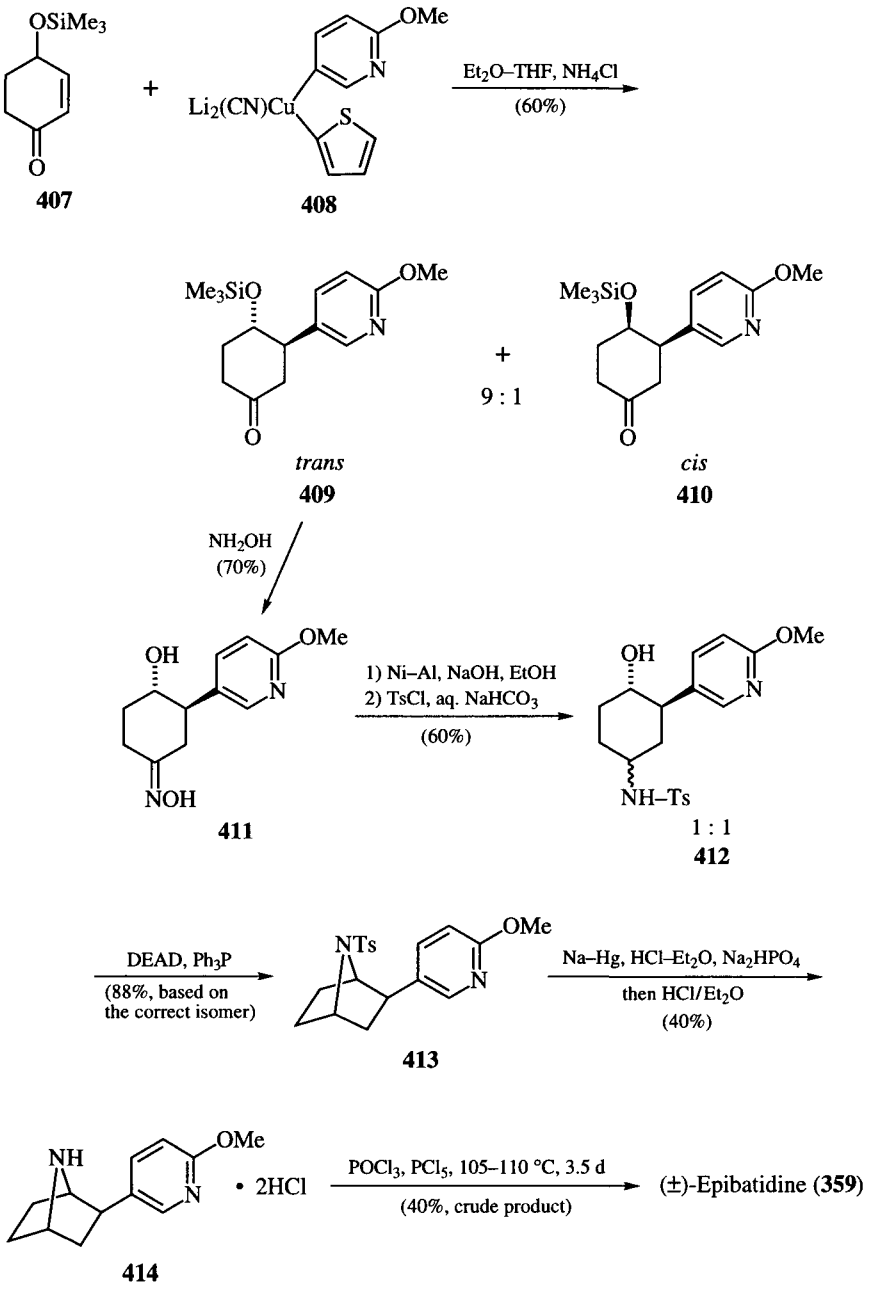
Another approach to (±)-epibatidine (**359**) using the nitrocyclohexanone **401** has been reported by Albertini and co-workers (ref. 108) (Scheme 59). The known **401** was prepared from Diels–Alder reaction between 5-(2-nitrovinyl)-2-chloropypyridine (**404**) and 1,3-diene **360**, followed by treatment of the crude cycloadduct with aqueous acid. In order to avoid the tedious C-2 epimerization step in the synthesis by Szántay and co-workers (see Scheme 58), the device to secure the proper anti-stereochemistry of the functionalities involved in the nitrogen bridge formation was adopted. Thus, the ketone **401** was reduced with L-Selectride, giving a 7:3 mixture of the corresponding β- and α-alcohols which was mesylated to afford **405** and its C-4 epimer. The

separated nitromesylate **405** was then submitted to oxidative Nef conditions, and the reaction mixture was quenched with $NaBH_4$ to afford the mesyloxyalcohol **406**. Treatment of **406** with NaN_3, followed by mesylation of the hydroxyl group and subsequent reduction of the nitro group with an excess of $SnCl_2$ in MeOH–THF solution gave the aminomesylate **369**, the intermediate in Broka's synthesis (see Scheme 53). Compound **369** was heated in chloroform to produce (±)-**359**.



Scheme 59

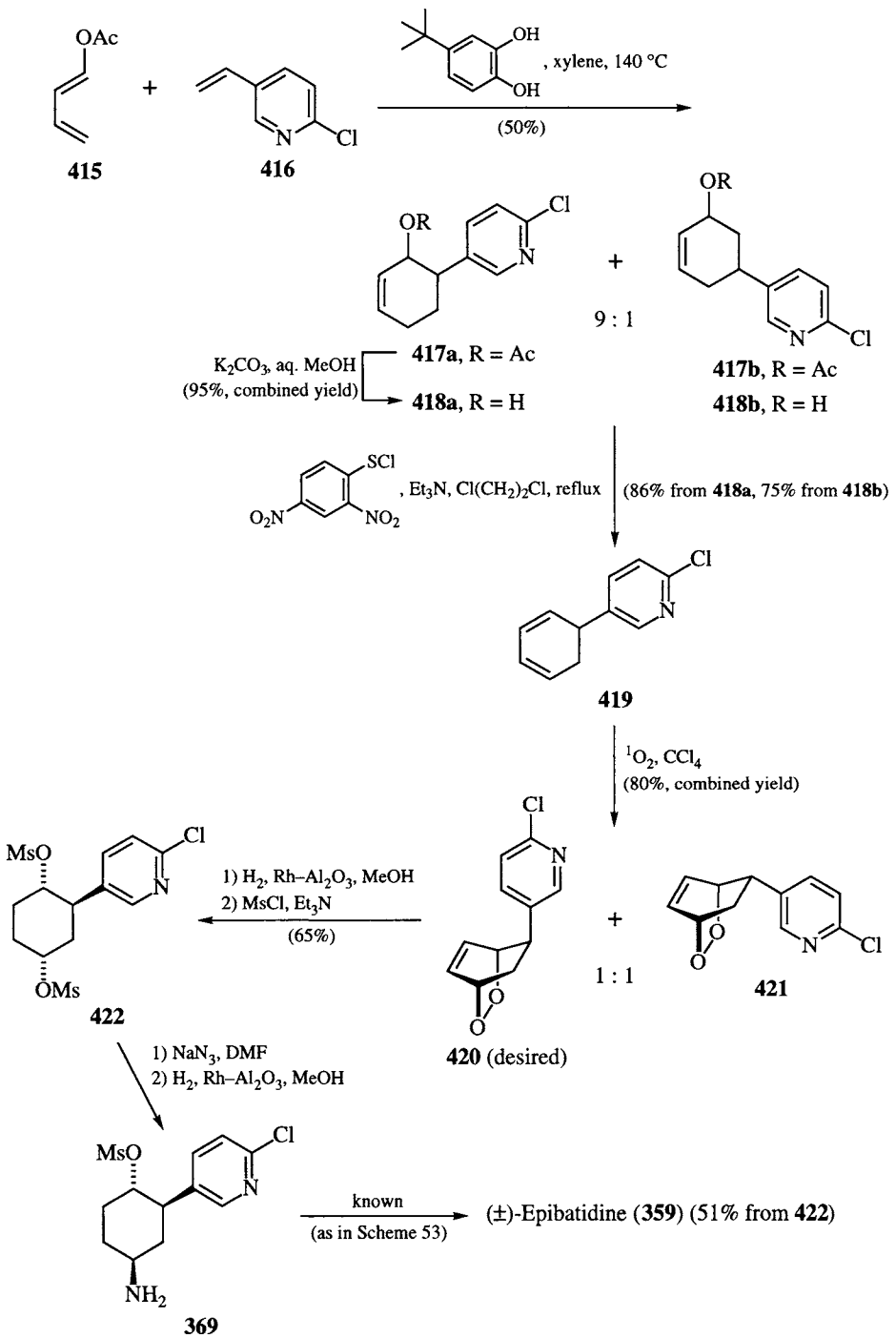
Sestanj and co-workers (ref. 109) exploited the conjugated addition of a higher order lithiocuprate reagent to the 2-cyclohexenone derivative **407** as one of the key steps in their synthesis of (±)-epibatidine (**359**) (Scheme 60). A conjugate addition of the pyridyl lithiocuprate **408** to **407** gave a 9:1 mixture of the *trans*- and *cis*-ketones **409** and **410**, thereby establishing two of three stereogenic centers. The *trans*-ketone **409** was then converted to a mixture of the tosylamino-


OSiMe3
OMe
N
Li2(CN)Cu
S
O
Et2O–THF, NH4Cl
(60%)
407
408
Me3SiO
OMe
N
O
trans
409
+
9 : 1
cis
410
NH2OH
(70%)
OH
NOH
411
1) Ni–Al, NaOH, EtOH
2) TsCl, aq. NaHCO3
(60%)
NH–Ts
1 : 1
412
DEAD, Ph3P
(88%, based on
the correct isomer)
NTs
413
Na–Hg, HCl–Et2O, Na2HPO4
then HCl/Et2O
(40%)
NH
• 2HCl
414
POCl3, PCl5, 105–110 °C, 3.5 d
(40%, crude product)
(±)-Epibatidine (359)

Scheme 60

isomers **412** via its oxime **411**. Without separation of the stereoisomers, **412** was subjected to Mitsunobu conditions giving the 7-azabicyclo[2.2.1]heptane ring system **413**. Removal of the tosyl group in **413** followed by transformation of the methoxy group to the chloro-substituent by heating **414** with $POCl_3/PCl_5$ completed the synthesis of racemic **359**.

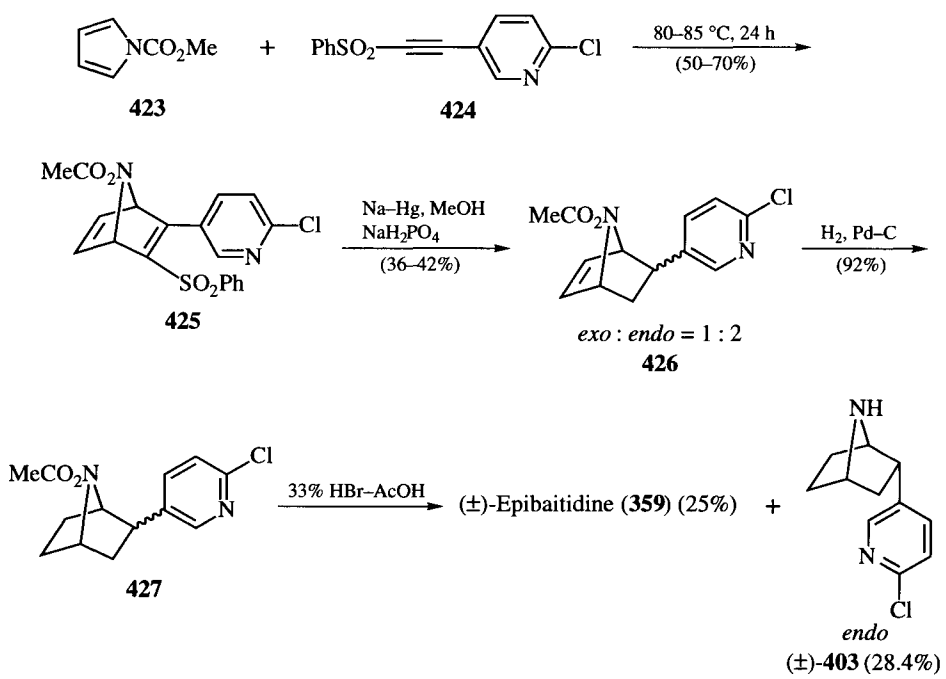
Ko and co-workers (ref. 110) at the Sandoz institute applied a singlet oxygen reaction as the key step in the preparation of (±)-epibatidine (**359**) (Scheme 61). The Diels–Alder reaction of the pyridyl dienophile **416**, prepared from commercially available 6-chloronicotinic acid in two steps, with 1- acetoxybuta-1,3-diene (**415**) in the presence of 4-*tert*-butylcatechol afforded a 9:1 mixture of **417a** and **417b**, which was hydrolyzed in alkaline conditions and separated. Treatment of each allylic alcohol with 2,4-dinitrobenzenesulfonyl chloride and triethylamine in refluxing 1,2-dichloroethane resulted in the rearrangement–elimination reaction, producing the same diene **419**. Irradiation of **419** in the presence of a photosensitizer under an oxygen atmosphere produced two isomeric products, **420** and **421**, in equal amounts. Treatment of the *trans*-isomer **420**, separated by chromatography, with hydrogen over rhodium on alumina gave a diol which was converted to the bismesylate **422**. Reaction of **422** with a single equivalent of sodium azide followed by hydrogenolysis using rhodium on alumina furnished the aminomesylate **369**. Compound **369** was then converted into racemic **359** by the procedure of Broka (see Scheme 53).


OAc
+
N
Cl
415
416
OH
OH
, xylene, 140 °C
(50%)
OR
Cl
N
+
9 : 1
OR
N
Cl
417a, R = Ac
417b, R = Ac
K2CO3, aq. MeOH
(95%, combined yield)
418a, R = H
418b, R = H
SCl
O2N
NO2
, Et3N, Cl(CH2)2Cl, reflux
(86% from 418a, 75% from 418b)
Cl
N
419
1O2, CCl4
(80%, combined yield)
Cl
N
MsO
OMs
1) H2, Rh–Al2O3, MeOH
2) MsCl, Et3N
(65%)
422
O
O
420 (desired)
+
1 : 1
421
1) NaN3, DMF
2) H2, Rh–Al2O3, MeOH
MsO
NH2
369
known
(as in Scheme 53)
(±)-Epibatidine (359) (51% from 422)

Scheme 61

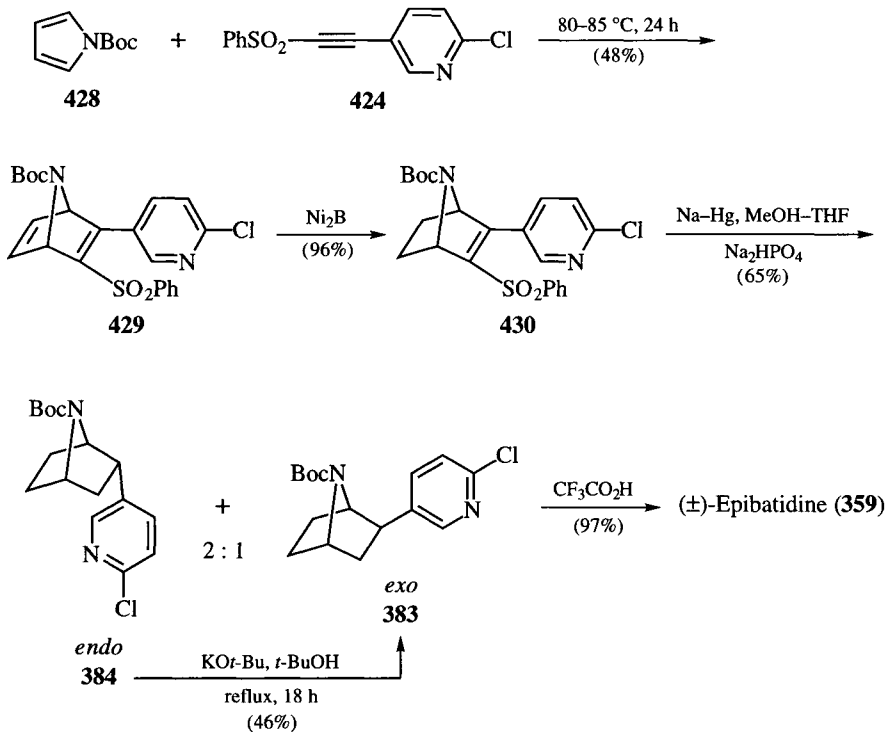
6.2 Approaches via Diels–Alder Cycloaddition of *N*-Substituted Pyrroles

One of the shortest strategy for the synthesis of (±)-epibatidine (**359**) has been reported by Huang and Shen (ref. 111) who utilized the Diels–Alder reaction of *N*-carbomethoxy pyrrole (**423**) and phenylsulfonyl 6-chloro-3-pyridyl acetylene (**424**) (Scheme 62). The Diels–Alder reaction was performed at 80–85 °C for 24 h with excess **424** as the solvent to give the 7-azanorbornadiene **425**, whose structure was confirmed by X-ray crystallographic analysis. Desulfonation of the adduct **425** with sodium amalgam in methanol containing sodium dihydrogenphosphate occurred with concomitant reduction of the conjugated double bond, giving a 1:2 mixture of *exo*- and *endo*-isomers **426**. Catalytic reduction of the double bond in **426** gave the carbamate **427**. Deblocking of **427** by treatment with hydrobromic acid produced a mixture of **359** and the corresponding *endo*-isomer **403**, from which racemic epibatidine (**359**) could be isolated.



Scheme 62

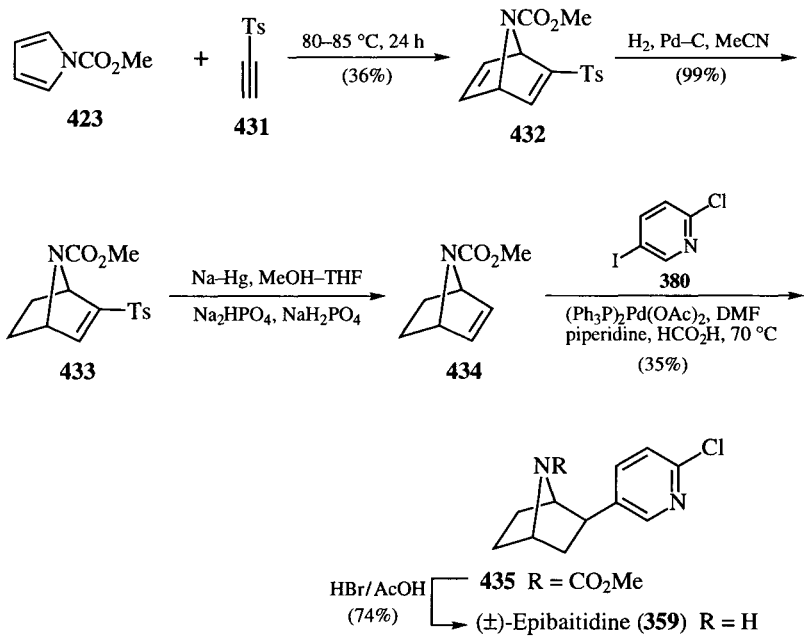
The strategy proposed by Kotian and Carroll (ref. 112) is very similar to that of Huang and Shen; it converts the cycloadduct **429**, prepared via the Diels–Alder reaction between *N*-Boc pyrrole (**428**) and **424**, to (±)-epibatidine (**359**) (Scheme 63). In this approach, the least substituted double bond of the cycloadduct **429** was selectively reduced using nickel bromide to give **430**.



Scheme 63

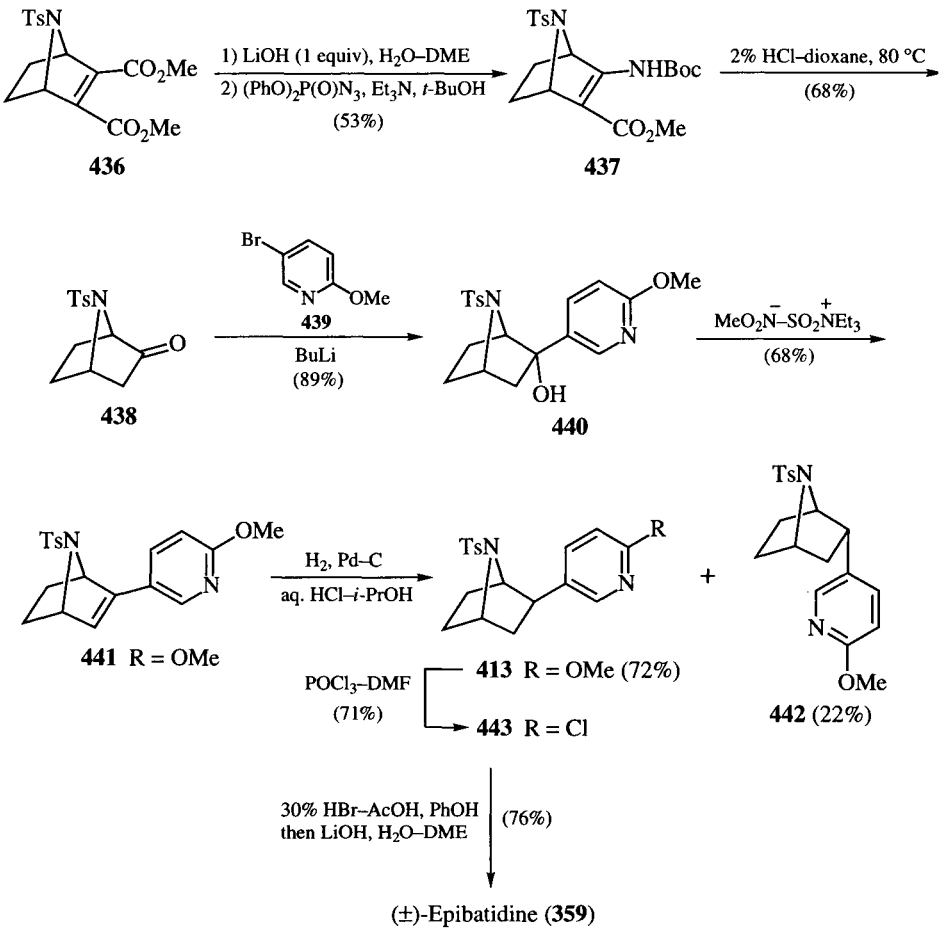
Desulfonation and concomitant reduction of the double bond in **430** gave a 2:1 mixture of the *endo*- and *exo*-isomers **384** and **383**, which were led to racemic **359** in a manner identical with that of the Merck group (see Scheme 55).

Clayton and Regan (ref. 113) applied a reductive palladium-catalyzed Heck-type coupling as a key step in the synthesis of epibatidine (**359**) in racemic form (Scheme 64). The known *N*-methoxycarbonyl-7-azabicyclo[2.2.1]heptane (**434**) required for the coupling steps was synthesized following the route of Altenbach et al. (ref. 114) from *N*-carbomethoxy pyrrole (**423**). Thus, the Diels–Alder reaction between **423** between *p*-toluenesulfonylacetylene (**431**) afforded the bicyclic compound **432**. Selective catalytic hydrogenation of **432** and reductive cleavage of the tosyl group using sodium amalgam gave **434**. The crucial reductive coupling reaction (ref. 115) of **434** and 2-chloro-5-iodopyridine (**380**) was carried out at 70 °C in solution in DMF containing piperidine, formic acid, and 8 mol % of the palladium catalyst formed in situ from palladium(II) acetate and triphenylphosphine, forming the desired *exo*-product **435** with complete stereoselectivity. The resulting protected epibatidine **435** was finally treated with hydrogen bromide in acetic acid to afford racemic **359**.



Scheme 64

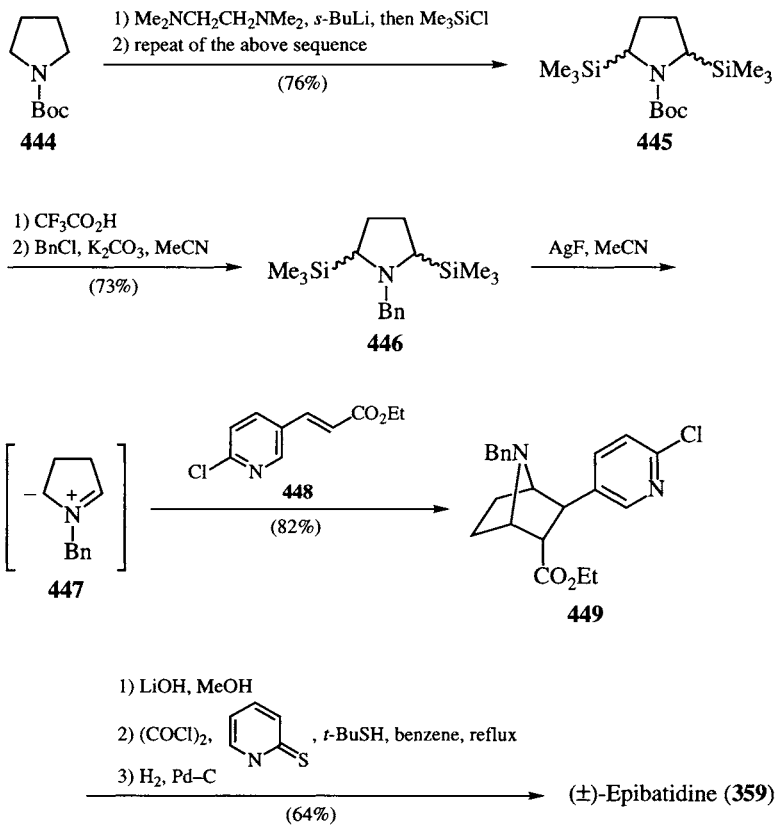
Okabe and Natsume (ref. 116) have synthesized (±)-epibatidine (**359**) starting from the known compound **436** (ref. 117) derived from the Diels–Alder adduct (ref. 118) between *N*-tosylpyrrole and dimethyl acetylenedicarboxylate (Scheme 65). Hydrolysis (1 molar equiv of LiOH) of the one ester group of **436** having σ symmetry gave the corresponding monoester which was subjected to a Curtius rearrangement sequence (diphenylphosphoryl azide, Et_3N, *tert*-BuOH) affording the carbamate **437**. Heating a solution of **437** in a mixture of hydrogen chloride-containing dioxane and water gave the ketone **438**. Compound **438** was then condensed with a lithium salt prepared from 5-bromo-2-methoxypyridine (**439**) and butyllithium to produce the *exo*-product **440**, which was converted into **441** by treatment with Burgess reagent (ref. 119). The palladium-catalyzed hydrogenation of **441** in a solution of 2-propanol and water containing hydrochloric acid afforded the *exo*-product **413** together with the *endo*-product **442**. Conversion of **413** into the chloropyridine using the Vilsmeier reagent (ref. 120), followed by deprotection of the tosyl group on treatment with hydrobromic acid and subsequently with lithium hydroxide of the resulting crude product completed the synthesis of (±)-**359**.



Scheme 65

6.3 Approach via [3 + 2] Cycloaddition of Azomethin Ylide

Pandey and co-worker (ref. 121) have proposed an efficient synthesis of (±)-epibatidine (**359**) based on a [3 + 2] cycloaddition reaction (ref. 122) of non-stabilized azomethine ylide and substituted 6-chloro-3-vinyl pyridine (Scheme 66). Metalation of *N*-(*tert*-butoxycarbonyl)pyrrolidine (**444**) with *sec*-butyllithium followed by addition of chlorotrimethylsilane, and repetition of this sequence gave the N-protected 2,4-di(trimethylsilyl)pyrrolidine **445**. Deprotection of **445** with trifluoroacetic acid and subsequent N-benzylation afforded **446**. The [3 + 2] cycloaddition of the azomethine ylide **447**, generated by the double desilylation of **446** using Ag(I)F, with the α,β-unsaturated ester **448** gave the cycloadduct **449**. The conversion of **449** to (±)-**359** was achieved by radical decarboxylation of the corresponding thiohydroxamate ester using Barton's method (ref. 123), followed by reductive N-benzylation carried out by palladium-catalyzed hydrogenation.



Scheme 66

7. MISCELLANEOUS

In 1992 Daly and Spande (ref. 124) reported the isolation and structural elucidation of alkaloid 251F (**450**) from the skin exudate of the dendrobatid poison from *Minyobates bombetes* of Colombia. Unlike most of the dendrobatid alkaloids, which are apparently acetogenins, **450** is clearly terpene-derived. Taber and You (ref. 125) have recently reported the first total synthesis, and thus structural confirmation, of **450**. Their convergent assembly to succeed needed to prepare both the enantiopure cyclopentane and piperidine moieties. Thus, the synthesis began with the preparation of the highly substituted cyclopentane as **456** based on the approach via Rh-mediated cyclization of

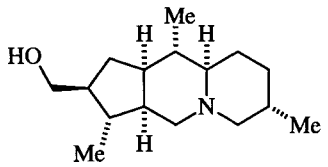
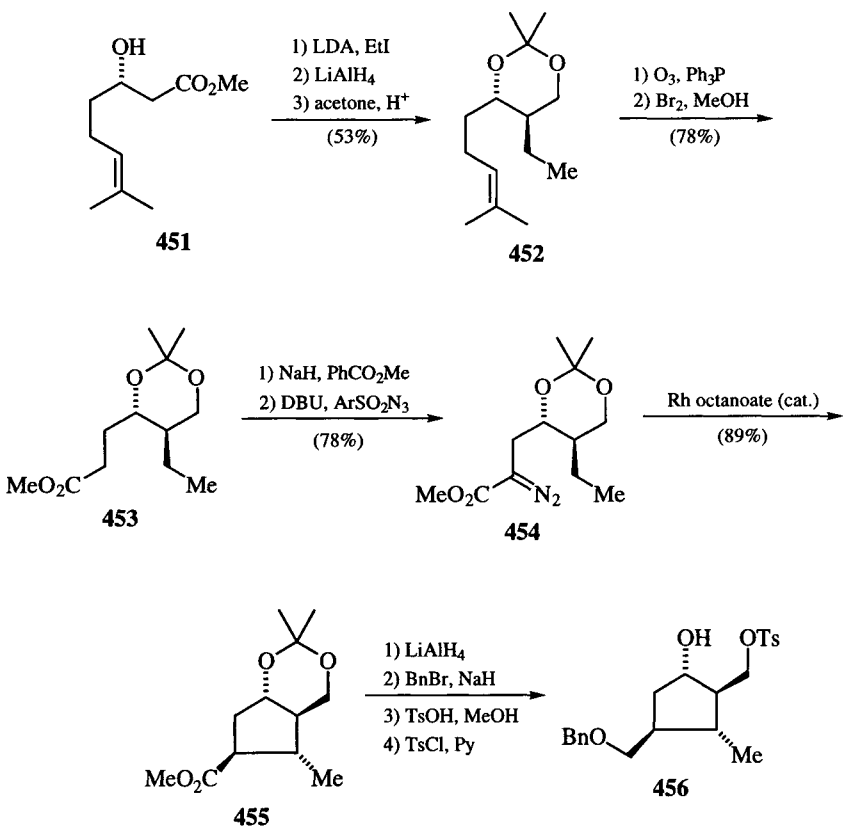


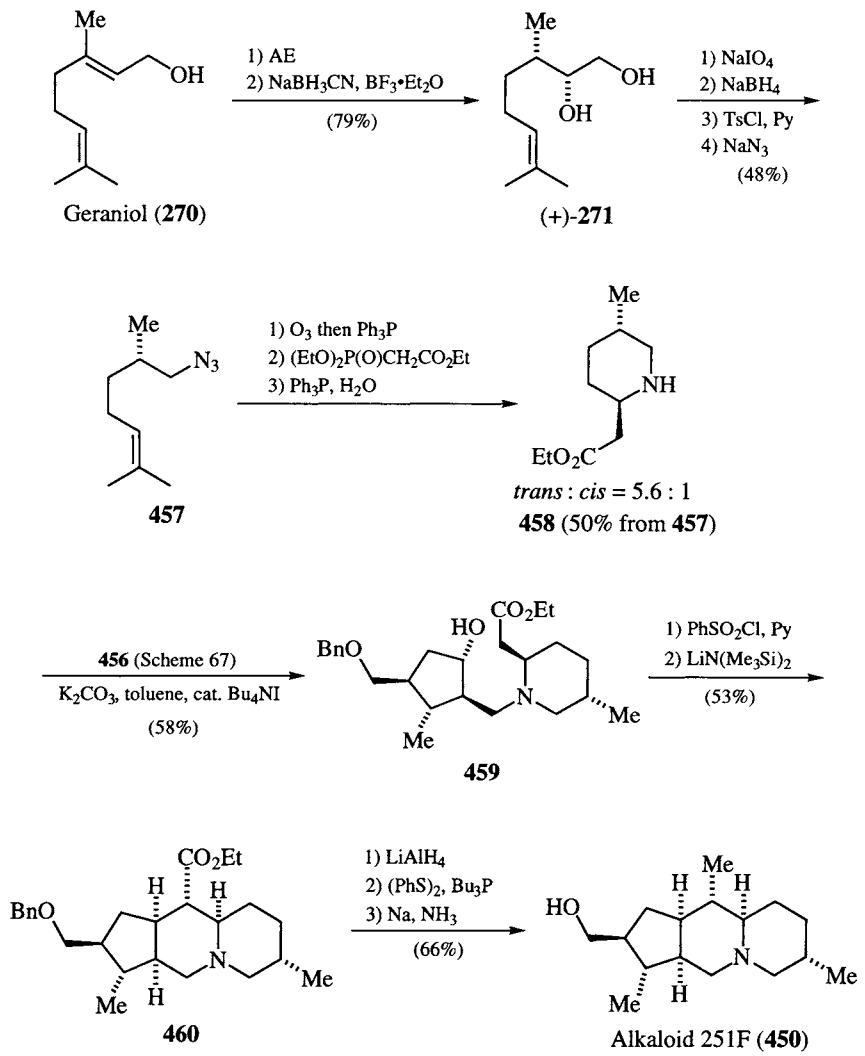
Figure 4. Dendrobatid alkaloid 251F (**450**)

the diazoester (Scheme 67). The enantiomerically pure β-hydroxy ester **451** (ref. 126) was alkylated through the dianion to give the expected anti product, which was reduced and protected to afford **452**. Subsequent ozonolysis of the alkene followed by oxidation gave the ester **453**, which was converted to the azide **454** by initial benzoylation of the ester enolate followed by diazo transfer. Rh-mediated cyclization of **454** proceeded stereoselectively to form **455** as a single diastereomer and subsequent transformations provided **456**.



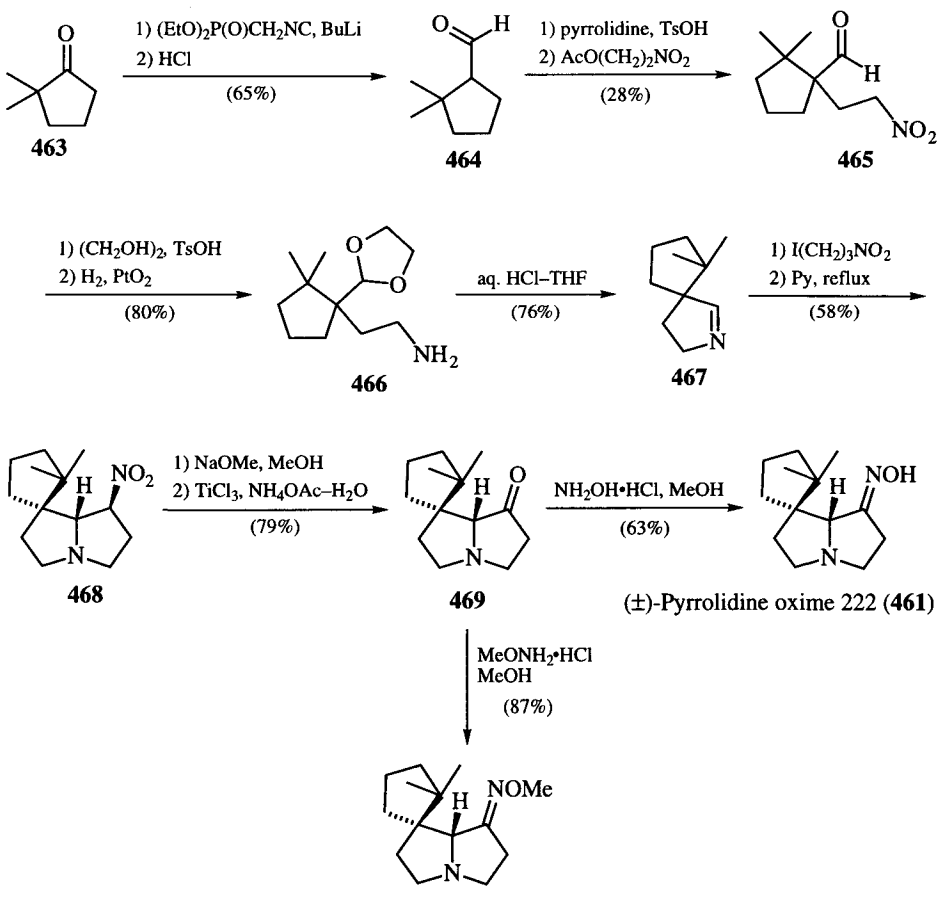
Scheme 67

For the preparation of the enantiomerically pure piperidine moiety, 2-hydroxycitronellol [(+)-**271**], conveniently available from geraniol (**270**) as discusses above in Section 4.3 (see Scheme 41), underwent periodate cleavage followed by reductive workup to give norcitronellol, which was then converted to the azide **457** (Scheme 68). Ozonolysis of **457** followed by phosphonate condensation and reduction of the azide afforded the *trans*-piperidines **458** in 5.6:1 diastereoselectivity. N-alkylation of **458** with **456** gave **459**, which was cyclized via the benzenesulfonate to afford the tricyclic amine **460**. Conversion of the ester to a methyl group and deprotection resulted in the synthesis of alkaloid 251F (**450**).



Scheme 68

Unique minor alkaloids pyrrolidine oximes 222 (**461**) and 236 (**462**), isolated from skin extracts of the Panamanian poison-frog *Dendrobates pumilio* (ref. 127), have been synthesized in a racemic form by Hutchinson et al. (ref. 128). Their synthesis is based on the route developed by Meinwald (ref. 129) for the synthesis of spiropentanopyrrolizidines, and started with the preparation of the aldehyde **464** from 2,2-dimethylcyclopentanone (**463**) utilizing the Wittig–Hornner reagent diethyl isocyanomethylphosphonate (Scheme 69). The pyrrolidine enamine of the aldehyde **464** underwent Michael reaction with nitroethylene, generated in situ from 2-nitroethyl acetate, to form **465**, which was protected as the ethylene acetal and then reduced to give the amine **466**. Polyzonimine **467**, generated by acid treatment of **466**, was converted to nitropolyzonamine **468**, an alkaloid isolated from the defense secretions of the North American millipede (ref. 129), via the quaternary salt. Treatment of **468** with sodium methoxide and buffered, aqueous $TiCl_3$ provided the ketone **469**, which on treatment of hydroxylamine or methoxylamine afforded (±)-pyrrolidine oximes 222 (**461**) and 236 (**462**), respectively.



Scheme 69

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Synthesis of Some Aspidosperma and Related Alkaloids

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1. Introduction

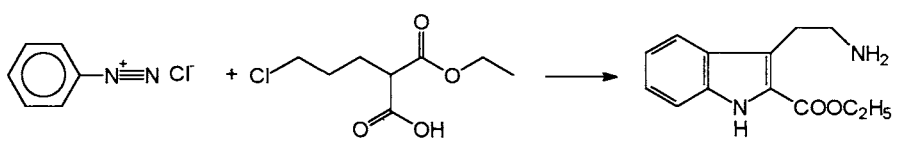
The *Aspidosperma* alkaloids constitute the largest group of indole alkaloids and number well over 220 entities. Dealing with these bases is not only important in order to gain more knowledge about their nature, but some members are also of commercial value, e.g. the transformation of vincadifformine to the pharmaceuticals vincamine, vincamone, and Cavinton® is carried out on a large scale. Vindoline is a characteristic part of the anti-cancer bis-indole alkaloids vincristine and vinblastine.

Extensive and excellent reviews have been published about this alkaloid family by Cordell (1), and Saxton (2). In the following therefore we confine ourselves exclusively to the synthetic work which has been done in our institute, put it in a historic perspective to show how the approach developed during the years.

All the synthetic compounds discussed below are racemic.

2. Early synthetic approaches

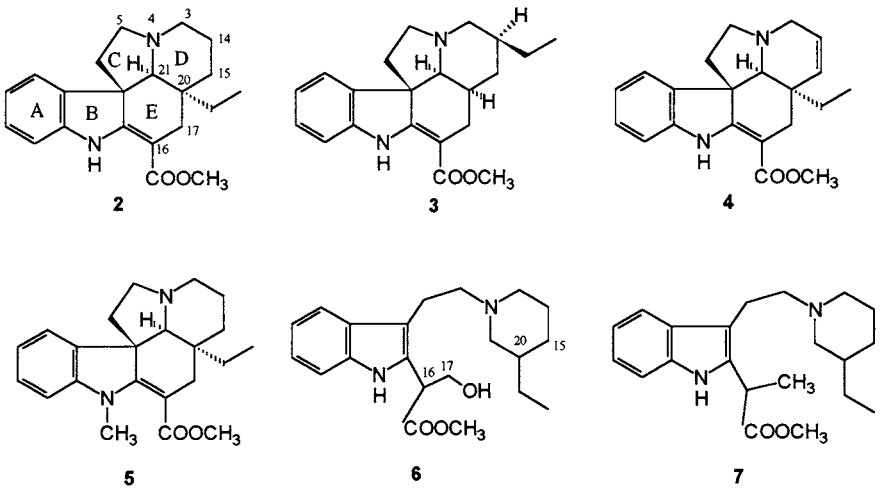
In the course of our work with indole derivatives we found a short and efficient route to 2-(ethoxycarbonyl)-tryptamine **1** by reacting diazotized aniline with chloropropyl malonic ester acid. The reaction is carried out in one step (3) (Scheme 1).



1

Scheme 1

As compound **1** was easily accessible, we wanted to use it for the synthesis of the aspidosperma and pseudoaspidosperma alkaloids, e.g. vincadifformine **2** (racemic form from *Vinca difformis*), Ψ-vincadifformine **3** (from *Pandaca caducifolia*), tabersonine **4** (from *Amsonia tabernaemontana*), minovine **5** (from *Vinca minor*), tetrahidrosecodine-17-ol **6** (from *Rhazya orientalis*), tetrahyd-rosecodine **7** (from *Rhazya stricta*), and some epimers as well (Scheme 2).



Scheme 2

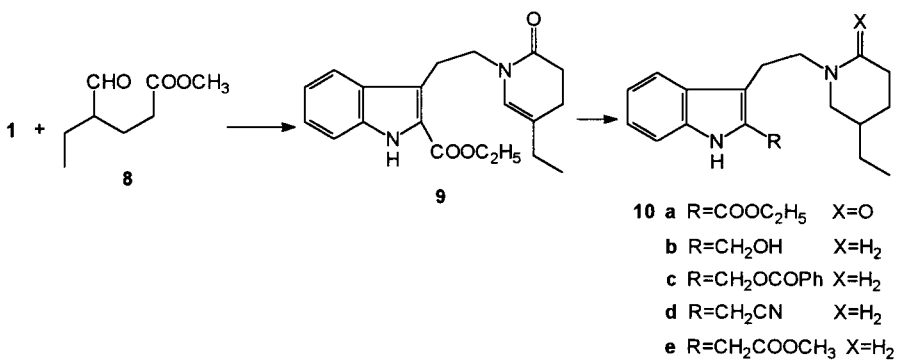
In Kuehne's studies on biomimetic alkaloid syntheses (2) the proposed biogenetic secodine intermediate **13** (Scheme 5) plays an important role. Thus at the outset we aimed at the preparation of transient key intermediates **13** and **14**. We envisaged that **6** (an alkaloid in itself) could serve as an intermediate source for producing dihydrosecodine **11** (Scheme 4) and tetrahydrosecodine **7**.

For preparing precursor **6**, our starting material **1** was allowed to react with the ester aldehyde **8**. For the preparation of **8** the reaction of various enamines of *n*-butyraldehyde with acrylic ester was first investigated (4). It is well known that, depending on the reaction conditions, the respective pyrrolidine enamine can react with either one or two mole equivalents of acrylic ester. The instability of pyrrolidine enamine prompted us to study the behavior of other enamines. It was found that the much more stable morpholine enamine reacts even with an excess of acrylic ester exclusively with one mol equivalent of the partner, and yields the aldehyde **8** after hydrolysis, thus providing a convenient route for its preparation.

The Pictet-Spengler type reaction of tryptamines substituted at position C-2, e.g. 2,3-dihidro-2-oxo-tryptamine often results in "spiro" coupling (5) at position C-3. However, compound **1** behaved differently with aldehydes. On boiling the hydrochloride of **1** in glacial acetic acid with monoester **8**, the enamide **9** was isolated in 55% yield, and no "spiro" compound was formed.

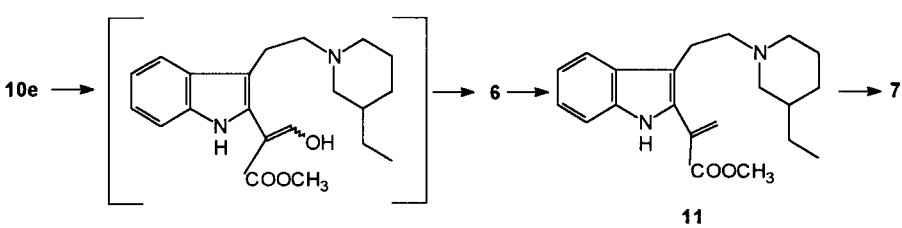
The enamide was reduced catalytically to **10a**, which upon further reduction with LAH provided **10b**. Using the method described by Kutney **10b** was transformed to **10e** by the following steps (6).

Benzoylation of **10b** gave **10c**, which was allowed to react with potassium cyanide in DMSO furnishing **10d**. When treated with methanol/HCl in the presence of trace of water, **10d** gave rise to **10e** (Scheme 3).



Scheme 3

For continuation of the synthetic sequence the ester **10e** was formylated in benzene with methyl formate in the presence of sodium hydride, and the obtained enol was immediately reduced ($NaBH_4$/methanol) to the diastereomers of 16,17,15,20-tetrahydrosecodin-17-ol **6**. Water elimination from the latter compound yielded 15,20-dihydrosecodine **11**. Subsequent catalytic reduction of **11** furnished a mixture of racemic 16,17,15,20-tetrahydrosecodine **7** and its diastereomer.

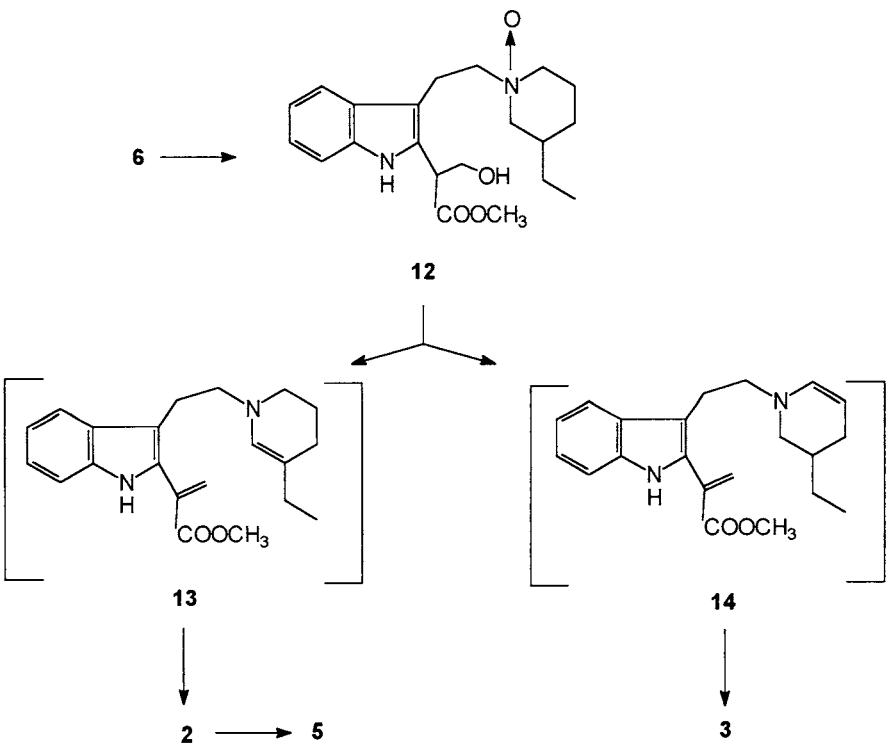


Scheme 4

Attempted chemoselective methylation of **10e** to **7** was unsuccessful, the indole N-methyl derivative of **7** being obtained.

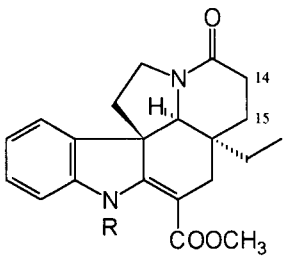
A synthesis of the key secodine intermediate **13** and its isomer **14** could now be projected through transformation of **6** into its N-oxide **12**, and subsequent treatment of **12** with acetic anhydride. As a result of the latter reaction **13** and **14** were formed and were immediately cyclized into a 2:1 mixture of vincadifformine **2** and Ψ-vincadifformine **3** in a rather low (12.3%) yield. After chromatographic separation **2** was obtained in a crystalline form, while **3** was isolated as an oil.

Transformation of **2** into minovine **5** was carried out by methylation of **2** with methyl iodide (Scheme 5).



Scheme 5

Compound **9** proved to be a useful starting material also for the synthesis of 3-oxovincadifformine **15** and 3-oxominovine **16** (Scheme 6).



15 R=H

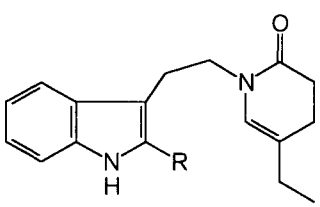
16 R=CH_3

Scheme 6

Le Men et al. (5) reported the synthesis of **15** as an intermediate in their total synthesis of vincadifformine. It is noteworthy that the 14,15-unsaturated derivative of **15** is a natural product isolated from *Amsonia elliptica* (7).

In our case the repetition of the same reaction sequence on the unsaturated compound **9** as described above with the saturated derivative **10a**, gave somewhat different results (8).

The ester group in **9** was reduced with LAH in THF at -40°C to yield **17a** in 60% yield. Subsequent benzoylation (87%) followed by reaction with potassium cyanide afforded compound **17c** in 87% yield. The product was treated at room temperature with methanol saturated with hydrogen chloride. Surprisingly instead of the expected substance **17e** the product proved to be a new compound **18** of an unusual structure, formed in 65% yield. Therefore we had to find a detour to prepare **17e** (Scheme 7).



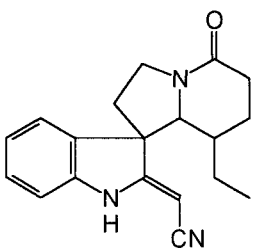
17 **a** R=CH_2OH

b R=CH_2OCOPh

c R=CH_2CN

d R=CH_2COOH

e R=CH_2COOCH_3



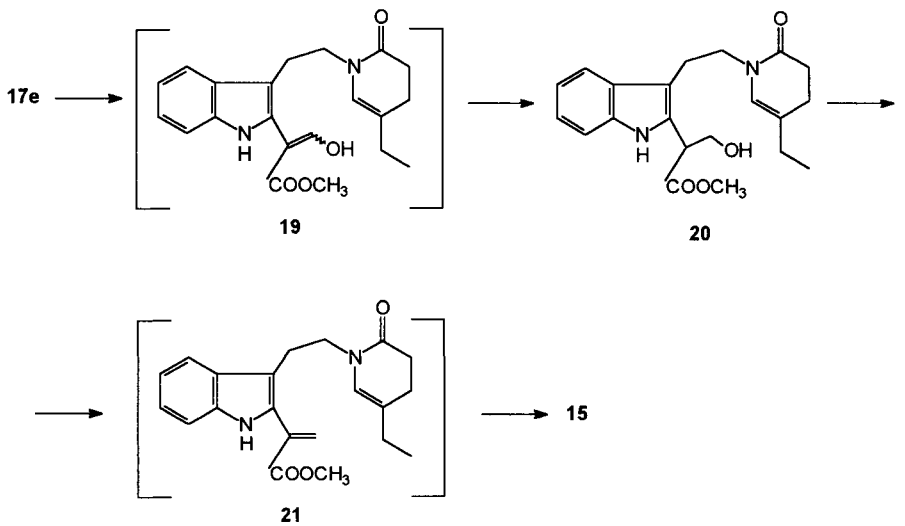
18

Scheme 7

When compound **17c** was refluxed in 2M sodium hydroxide solution, acidification of the mixture gave **17d** in 80% yield. Treatment of this product with diazomethane furnished **17e** in 93% yield.

As a continuation of the synthesis, the ester **17e** was dissolved in benzene and formylated as above. The resulting enol **19** was immediately reduced to give **20** in 49% yield.

As an alternative synthesis, compound **20** was also prepared by treatment of **17e** with gaseous formaldehyde in THF in the presence of lithium diisopropylamide, the yield being 23%. Elimination of the elements of water from **20** gave, through the secodine-type intermediate **21**, the required product, i.e. 3-oxovincadifformine **15**, in 48% yield.



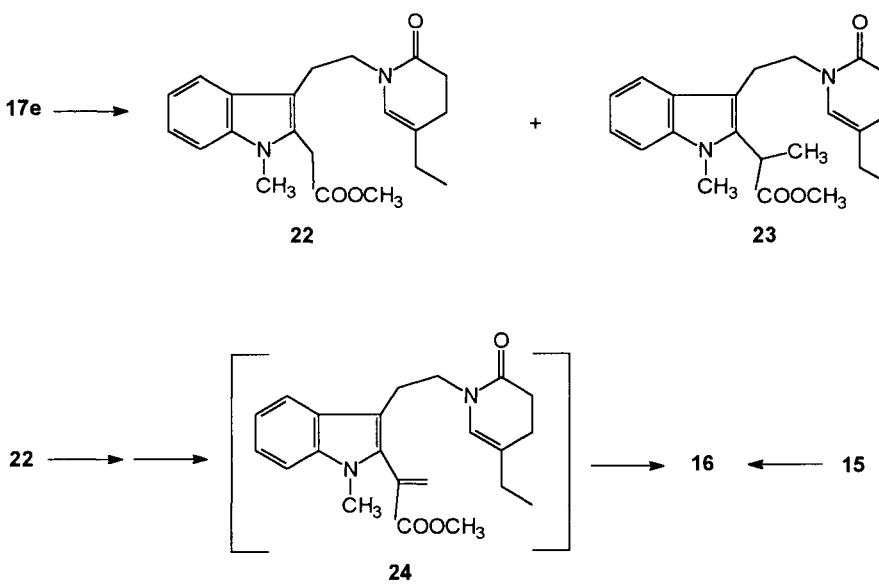
Scheme 8

The reaction sequence **17e→21→15** was also realized by the use of Eschenmoser's salt through the following steps: a.) THF/*n*-BuLi, $CH_2N^+(CH_3)_2I^-$, -75°C, b.) MeI/MeOH, r.t., c.) $NaHCO_3/H_2O$, r.t., d.) heating in toluene, in 13.6% combined yield (Scheme 8).

When compound **17e** was methylated with methyl iodide in DMSO in the presence of sodium hydride, two products were obtained: **22** in 44% yield and **23** in 5.5% yield.

The carbanion from **22** was generated with lithium diisopropylamide, and it was allowed to react with Eschenmoser's salt. The resulting basic material was treated with methyl iodide, the mixture was then made alkaline and the product was refluxed in toluene. The final product, formed *via* the secodine-type

intermediate **24**, was 3-oxominovine **16**. The latter compound was also prepared by the direct methylation of 3-oxovincadifformine **15** (Scheme 9).

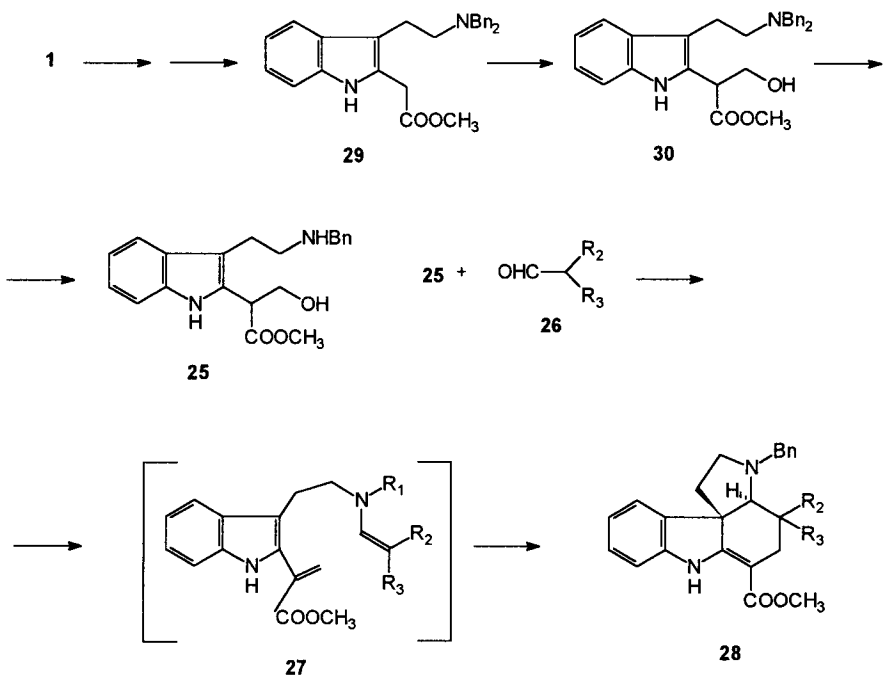


Scheme 9

3. Improved methods

Further objective of our work was to develop a convergent synthetic pathway that is suitable for the more convenient and efficient preparation of a number of compounds having the aspidospermane skeleton (10). The strategy we planned to use was as follows: from indole derivative **1** we wanted to prepare compound **25**, which, on reaction with suitably substituted aldehydes **26**, would give - via the unisolated intermediates **27** (R_1=Bn) - tetracyclic compounds of the general formula **28** (Scheme 10).

Formation of the fifth ring would afford the target compounds. As the aldehyde component could be varied, this strategy would allow the preparation of many analogous compounds; thus, a convenient synthesis of aspidosperma alkaloids and related compounds would become possible.



Scheme 10

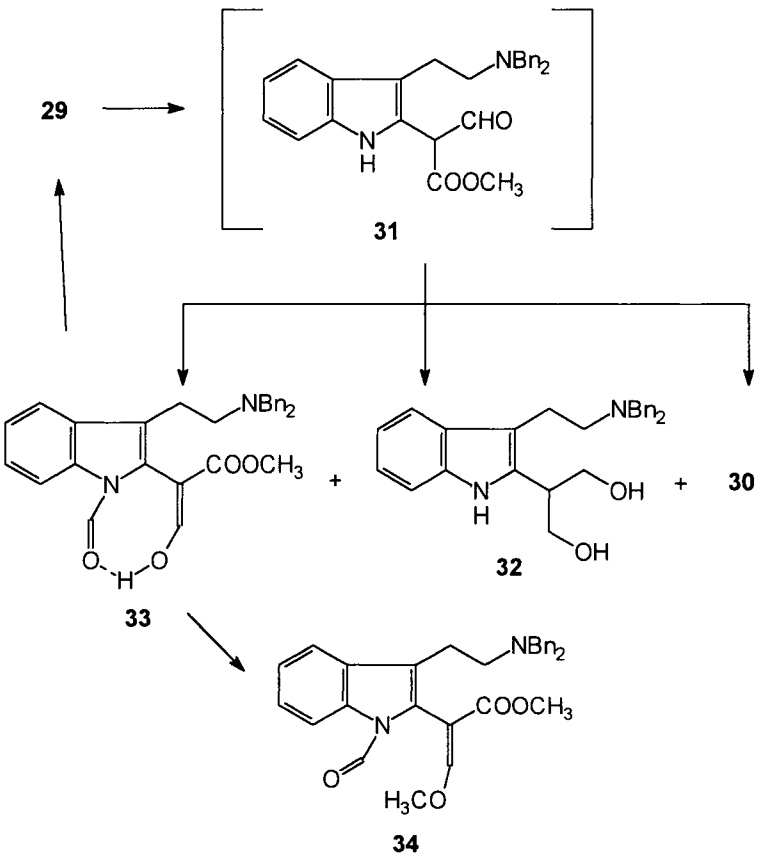
Dibenzylamino ester **29** was prepared from **1** through N-dibenzylation and subsequently using the carbon chain extension reaction described above (Schemes 3 and 7). The overall yield of this reaction sequence was 41% from **1**.

Compound **29** was subjected to Battersby's method (9) for hydroxymethylation to afford compound **30**, from which the required diene structure could be formed by the simple elimination of water. After hydroxymethylation, the ester **30** was partially debenzylated to give the secondary amine **25**, the structural unit required for the preparation of the enamine function.

The reaction, in which the hydroxymethyl group was introduced, was thoroughly studied, and some interesting observations were made. The reaction was first effected by the treatment of the ester **29** with sodium hydride and methyl formate in benzene. The reaction mixture was then worked up in the usual way, and the crude intermediate **31** was dissolved in methanol and reduced with sodium borohydride at -25° to afford **30** (62%). If the reaction was done at a higher temperature, a considerable amount of by-product, diol **32,** was isolated from the reaction mixture (Scheme 11).

Several attempts were made to find a way to eliminate this undesired side reaction and to improve this poor yield. The best result was obtained when the

formylation was carried out using methyl formate as the solvent; without further processing, the reaction mixture was diluted with cold methanol, and the temperature of the resulting solution was strictly controlled during reduction with sodium borohydride. These modifications of the procedure eliminated the need for chromatographic purification of the crude product, and a higher yield of the crystalline dibenzyl ester **30** was obtained.



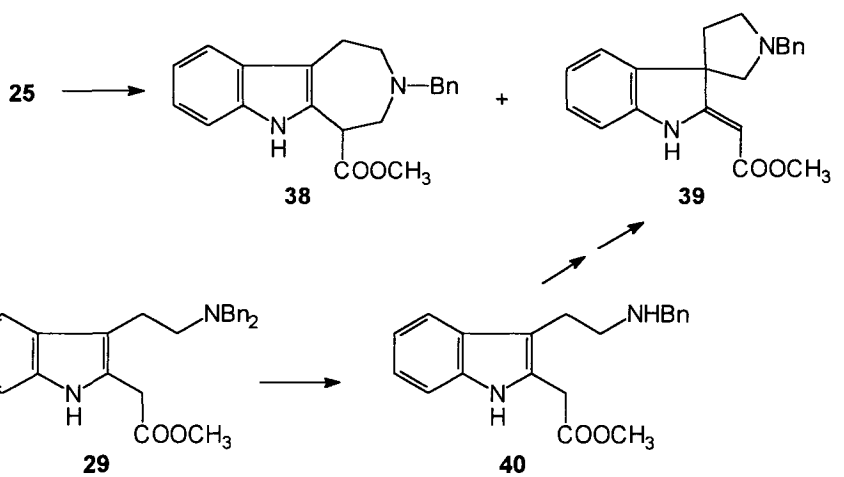
Scheme 11

The reaction mixture, however, still contained small amounts of two byproducts, the diols **32** and **33**. In order to confirm the structure of the latter, the enol ether **34** was also prepared by alkylation of **33** with diazomethane. As suggested by the NMR data, the structure of **34** confirmed that compound **33** exists in the enol form stabilized by a hydrogen bridge.

When the enol **33** was treated with a saturated solution of hydrogen chloride in methanol, the starting ester **29** was recovered. After we elucidated the structure of the byproduct **33,** the surprising fact that in the sodium borohydride reduction the potential formyl group was not converted to the expected alcohol became understandable.

The first aldehyde to be used in the ring closure reaction was methyl-4-formylhexanoate **35**. We first attempted to effect the reaction of **25** and **35** by refluxing the components in benzene; however, the isolated products corresponded not to the two expected tetracyclic epimers **36, 37**, but to the esters formed by the elimination of water **38** and **39** (Scheme 12). The former product was a benzylazepin derivative, that had been prepared earlier by Kuehene.

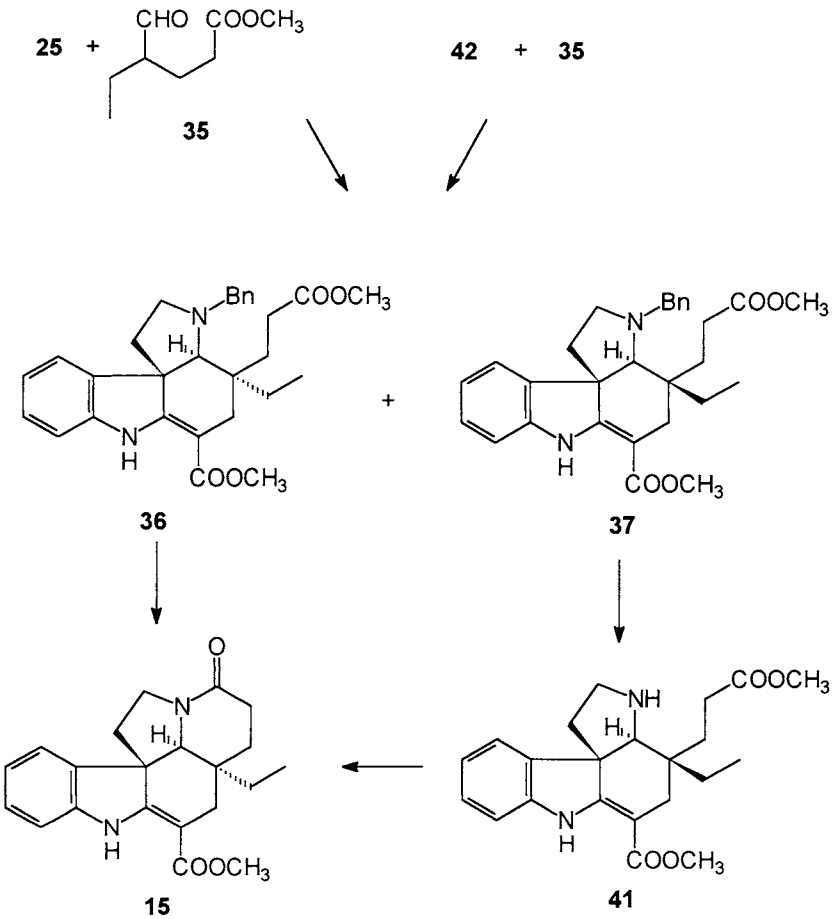
Compound **39** had been previously synthesized in our laboratory in another way, from the secondary amine **40**. The latter compound had been allowed to react with formaldehyde in an aqueous acidic medium to afford the hydrochloride of **39**. We suggest that compound **39** may have been formed from the ester **25** by a competing reaction in which the hydroxymethyl group migrated from the carbon to the nitrogen atom to form an α-amino alcohol intermediate and ultimately the spiro compound **39**.



Scheme 12

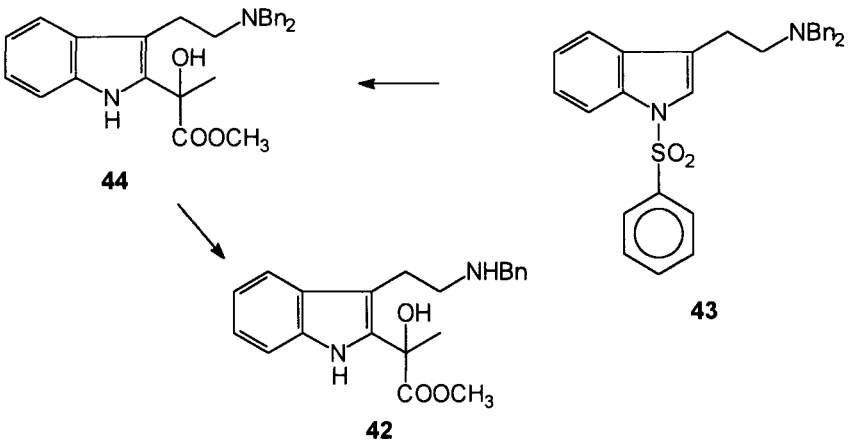
Subjecting compound **25** and the aldehyde **35** to rather vigorous reaction conditions, such as refluxing toluene for 24 h, afforded two products **36** and **37** previously reported by Kuehne. By previously described method these

compounds could be converted to 3-oxovincadifformine **15** in one or two steps, in the latter case via the secondary amine **41** (Scheme 13).



Scheme 13

Experiments were also conducted to find out which of the two byproducts **38** and **39** would react with the aldehyde component. Therefore **38** and **39** were separately caused to react with the aldehyde **35**. It was found that the azepine ester **38** could be converted into a mixture of the desired products **36** and **37**, but the spiro compound **39**, after identical treatment, remained unchanged.



Scheme 14

The indole derivative **42**, which fitted well into our strategic plan, was also prepared to test its utility in the cyclization reaction. From tryptamine the hydrochloride of the N_b-dibenzyl derivative was prepared, and the N_a nitrogen atom was protected with benzenesulfonyl group, which was introduced by means of a catalyzed phase-transfer reaction. When the base and methyl pyruvate were used in excess in the addition reaction, this protective group was split off as the sulphonic ester immediately after the addition. Accordingly, the trisubstituted tryptamine derivative **43** was treated with 2.3 molar equiv of *n*-butyllithium, and the resulting dicarbanion was treated with an excess of methyl pyruvate. After workup, the expected 2-hydroxypropionic ester **44** was isolated. The ester **44** was subjected to partial debenzylation to give the secondary amine **42** in good yield (Scheme 14).

A comparison of the overall yields of the two secondary amines **25** and **42** showed that the yield of **25** was 12-15% from **1** and the yield of **42** was 30-35% from tryptamine.

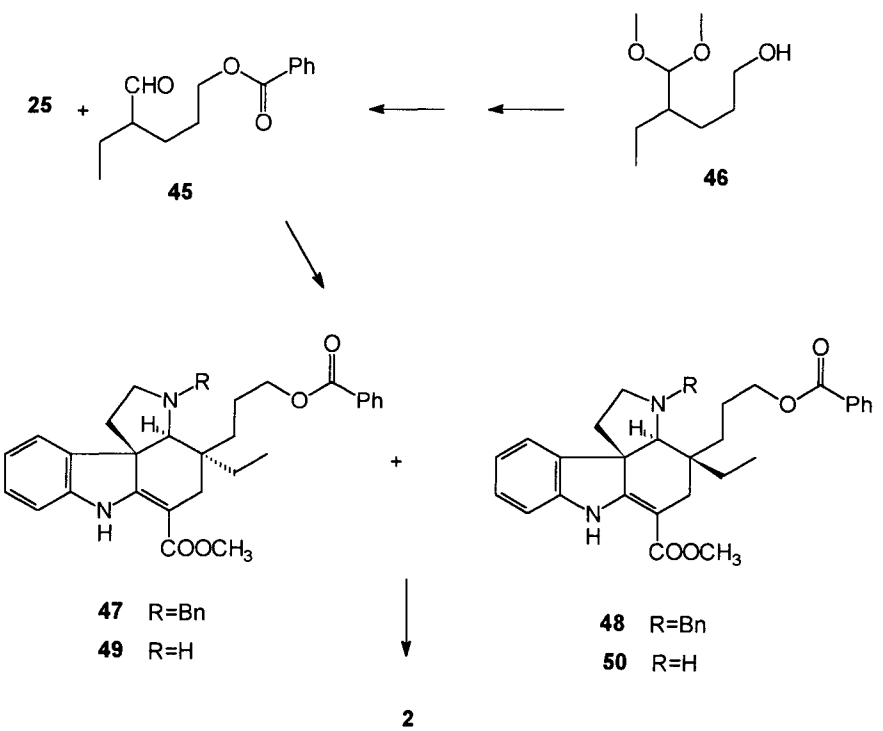
The reaction sequence was concluded by effecting the reaction of the secondary amine **42** and the aldehyde **35** under conditions similar to those used for the reaction of **25** and **35**. Since refluxing **42** and **35** in toluene gave only very small quantities of the expected products, the reaction was carried out at higher temperature, in refluxing xylene. At higher temperature, the intermediates **36** and **37** were isolated in about 20% combined yields.

Comparison of the two alternative synthetic pathways shows that the overall yield of the tetracyclic esters **36** and **37** are almost the same. However, the process starting from tryptamine has an inconvenient step: the formation of the anion by means of *n*-butyllithium and the subsequent addition of the anion to

the carbonyl; in addition, the elimination of water from **42** requires much more drastic conditions than the elimination of water from the primary alcohol **25**. In later experiments, we wanted to allow the indole key compound to react with heat-sensitive aldehydes. For this reason, the pathway using **25** was used for the remainder of the syntheses.

As continuation of our work, we attempted to develop a synthetic pathway involving a suitable aldehyde that would allow the preparation of vincadifformine **2** in fewer steps and more directly than before. The first task was to choose an appropriate aldehyde.

The aldehyde had to have a leaving group that would not be so reactive as to take part in the cycloaddition but would, at the required moment, and under suitable conditions, be prone to form the fifth ring. We discovered that these conditions were met by the benzyloxy group. Thus, the first task was the synthesis of the aldehyde **45**. Compound **46** was acylated with benzoyl chloride, and subsequent acid hydrolysis afforded the desired aldehyde **45**.

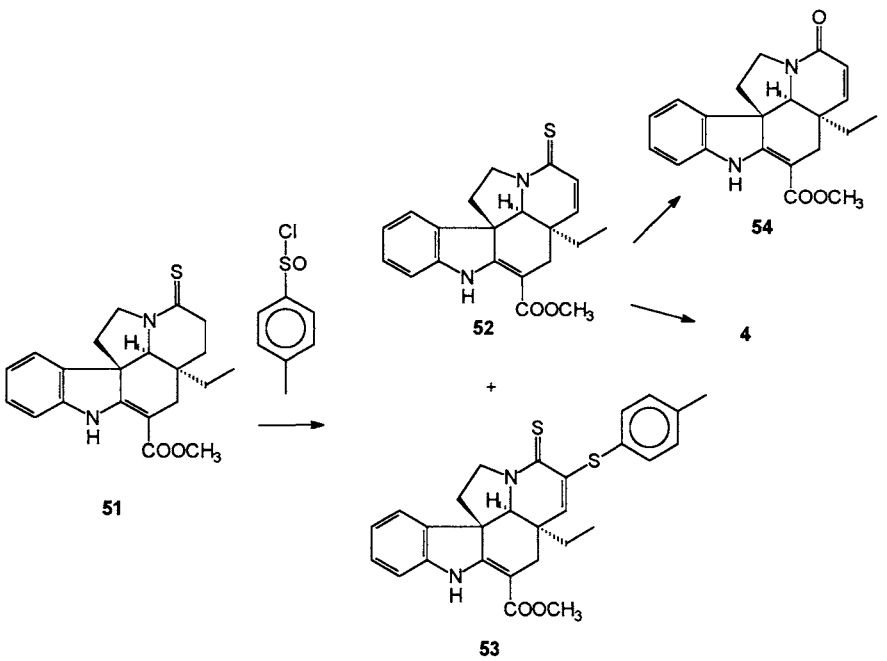


Scheme 15

The aldehyde **45** and the secondary amine **25** were refluxed in toluene, as described previously. A mixture of isomers containing the expected tetracyclic esters **47** and **48** was formed in this reaction. The next step of the reaction sequence was effected in the mixture of **47** and **48**. The protective benzyl group was removed by hydrogenolysis on glacial acetic acid. The products of the debenzylation were the secondary amines **49** and **50** (Scheme 15).

Next, the internal N-alkylation reaction leading to the formation of ring D was accomplished by refluxing the mixture of **49** and **50** in dimethylformamide. Both isomers afforded vincadifformine **2**. The formation of **2** from **50** can be explained in the same way as the formation of 3-oxovincadifformine, i.e. by epimerization at C-21.

The two other alkaloids that were synthesized were tabersonine **4** and 3-oxotabersonine **54**. Owing to the presence of a double bond in ring D, we modified our strategy for the synthesis of these compounds. It was deemed impractical to prepare or use an aldehyde partner already containing the double bond required in the product. Since an appropriately masked form could only be prepared with difficulty, we decided to employ the literature method (see in ref. 10) for forming the double bond via the thiolactam.



Scheme 16

The synthesis of the target compounds was simply achieved by starting from 3-thioxovincadifformine **51**, which was readily prepared from 3-oxovincadifformine **15**, without protection of the indole nitrogen. Accordingly, 3-thioxovincadifformine **51** was made to react with *p*-toluenesulfonyl chloride, in the presence of diisopropylethylamine, by refluxing the mixture in dichloromethane. The isolated main product was the unsaturated thiolactam **52**, which appeared to be suitable for the preparation of both target compounds. A byproduct characterized by formula **53** was also isolated from the reaction mixture (Scheme 16).

A general method was used to prepare tabersonine **4** from the thiolactam **52**. The thio-compound was first refluxed in methyl iodide. Then the excess reagent was removed by distillation, and the residue was dissolved in methanol and reduced with sodium borohydride.

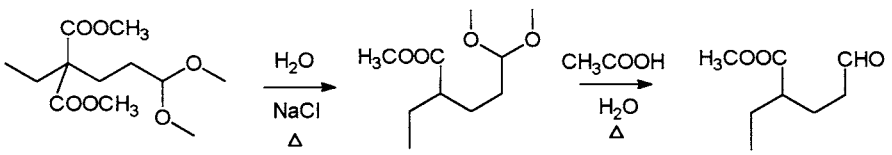
In the preparation of 3-oxotabersonine **54**, the sulphur-oxygen exchange was effected with 3-chloroperoxybenzoic acid. When compound **52** was allowed to react with 1.2 equiv of this reagent at -24° in dichloromethane, the alkaloid 3-oxotabersonine **54** was obtained.

4. Pseudoaspidosperma alkaloids

The above discussed strategy has been extended to the synthesis of compounds with the pseudoaspidospermane structure (11). Our aims included the synthesis of pseudovincadifformine **3** and 20-epipseudovincadifformine **55** (Scheme 19). Also the tried and tested method of introducing a double bond, in our synthesis of tabersonine **4**, was extended to compounds having the pseudoaspidospermane skeleton.

A characteristic feature of the pseudoaspidospermane skeleton is that, in contrast to aspidospermane compounds, where only *cis* D/E ring fusion is possible, in the pseudoskeleton *trans* fusion is also known.

As it is evident from the foregoing discussion, the primary task was the synthesis of an aldehyde component which would react with the indole ring containing the secondary amine **25** to give a product that could become the precursor of compounds having the pseudoaspidospermane skeleton. This requirement is satisfied by methyl-2-ethyl-5-oxopentanoate **56**, which was prepared as follows: dimethyl ethylmalonate was allowed to react with acrolein in the presence of base. The resulting aldehyde was treated with trimethyl orthoformate to obtain the acetal, and the latter was demethoxycarbonylated in neutral medium. The protecting group of the resulting ester was removed by acid hydrolysis (Scheme 17).



56

Scheme 17

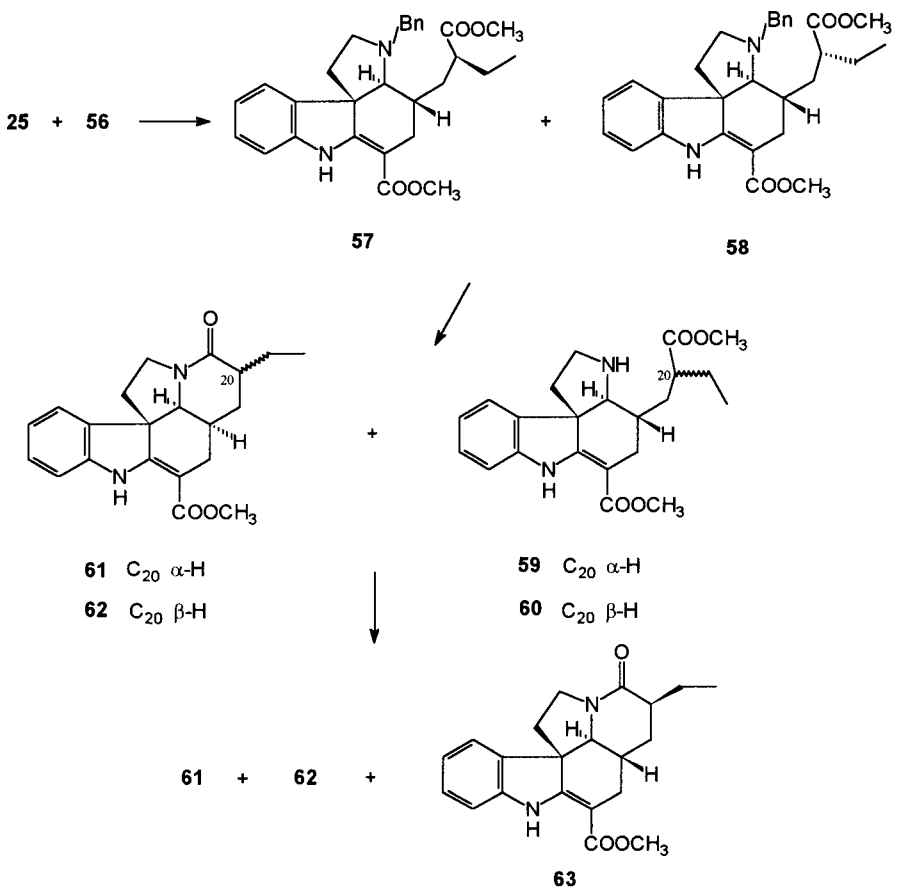
The formyl group of the product suffered oxidation on standing when exposed to air. Therefore, in later experiments the protective group was not removed until just before further conversion or before recording spectra.

The reaction between the secondary amine **25** and the aldehyde **56** was effected according to the strategy developed earlier, the product consisting of about 3:2 mixture of esters **57** and **58**. It was described earlier that when the α-carbon atom of the aldehyde component carried two hydrogen atoms, then on forming the *seco*-pseudoaspidospermane skeleton, the hydrogen atoms connected to the carbon atoms in the α- and β-positions relative to the nitrogen always had a *trans* stereochemical arrangement. This result has also been found to hold true in our present case. Therefore, it is reasonable that the reactions described in the literature and those used by us to prepare these compounds, although starting from substances of different structure, proceed via the same mechanism.

The epimers **57** and **58** were debenzylated without separation, since under the hydrogenolysis conditions in acidic medium, epimerization would have occurred resulting in a mixture of isomers. Workup of the reaction mixture revealed the presence of four components **59-62**. Thus the product mixture contained the pentacyclic derivatives, **61** and **62** with the *cis* geometry of the D/E rings, which according to earlier experience, were formed under the debenzylation conditions. The two other products, isolated as a mixture, were the secondary amines **59** and **60** having a *trans* configuration of the hydrogen atoms. In these compounds intramolecular acylation is sterically hindered. Components of the mixture were separated only for identification; further conversion was effected using the mixture.

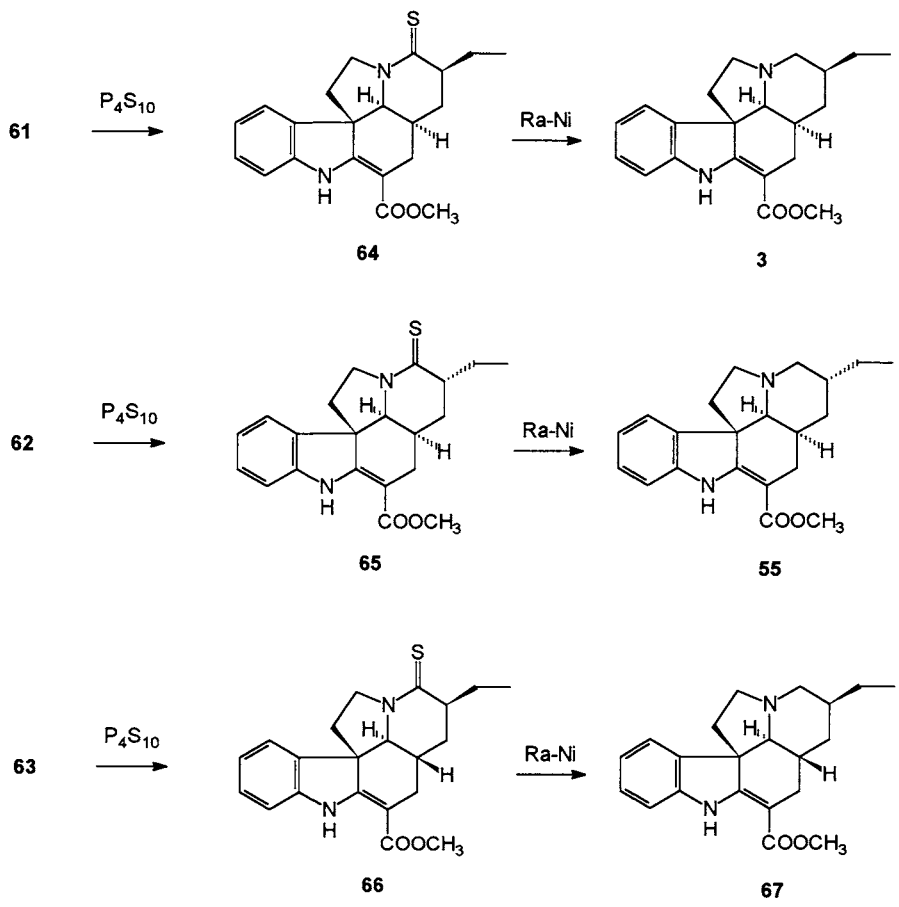
The mixture of four compounds **59-62** was refluxed in toluene in the presence of *p*-toluenesulphuric acid giving compounds **61, 62** and **63** in a ratio of about 2:3:1. Two of these substances, **61** and **62** were the above mentioned "*cis*" lactams, while the third compound **63** was the pentacyclic lactam with the *trans* D/E ring fusion (Scheme 18). No trace of the fourth possible isomer could be detected in the reaction mixture. It is worthy of note that the *trans* D/E ring fusion present in compound **63** has been found only in some non-natural

molecules, but the naturally occurring pseudoaspidospermanes all have *cis* D/E ring fusion.



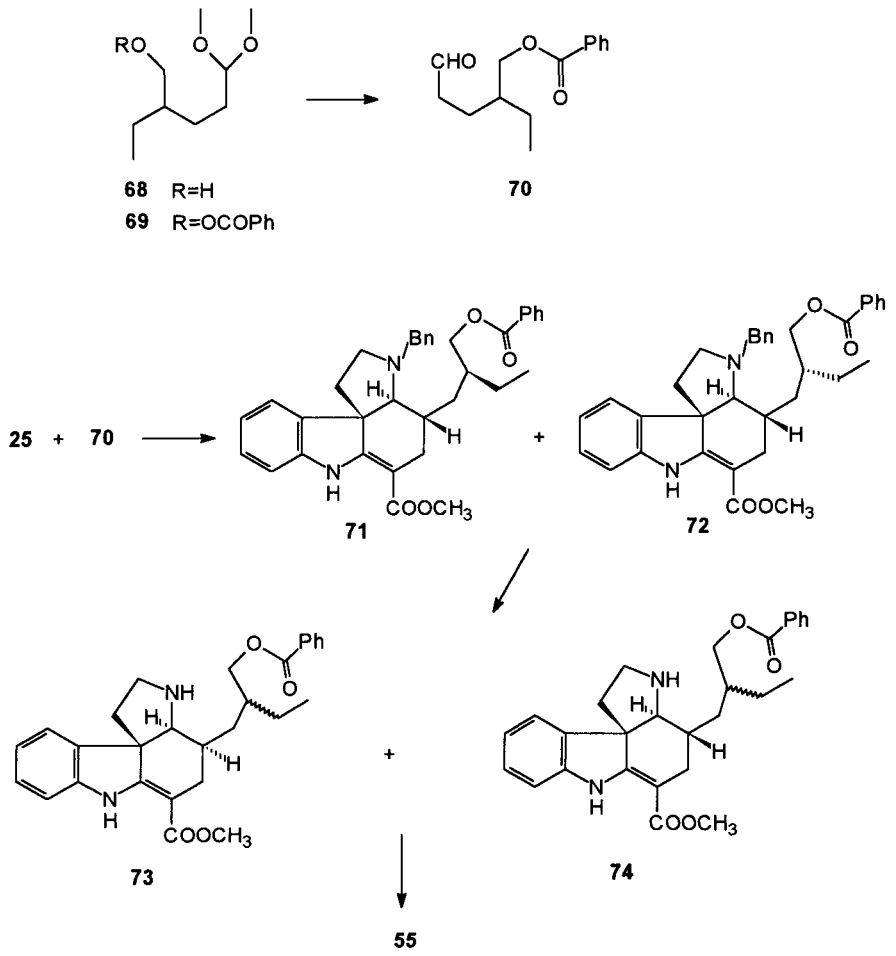
Scheme 18

In the next step all three lactams **61-63** were selectively reduced to afford the corresponding alkaloids or alkaloid-like molecules (Scheme 19) via the thiolactams, a synthetic route of proven value in our earlier work. First, the thio derivatives **64-66** were prepared by using phosphorous pentasulfide, and these products were desulfurized with Raney nickel to yield the two alkaloids, pseudovincadifformine **3** and 20-epipseudovincadifformine **55**, along with 14-epipseudovincadifformine **67** having the *trans* D/E ring fusion.



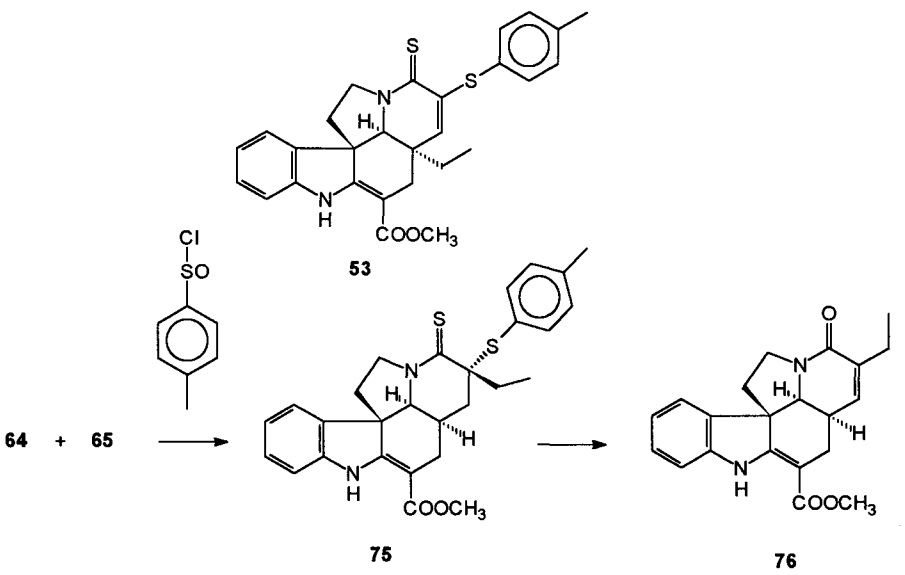
Scheme 19

A process was also developed for the direct construction of the pentacyclic pseudoaspidospermane skeleton (Scheme 20). First an aldehyde component was synthesized in which the leaving group is the benzoyloxy substituent, found suitable for this purpose earlier. In order to prepare the target compound, the ester group of acetal was initially reduced, the resulting alcohol **68** was benzoylated giving **69**, and finally the aldehyde function was regenerated. The secondary amine **25** was then allowed to react with aldehyde **70** under the conditions used in earlier analogous reactions. As expected, the reaction gave a mixture of two tetracyclic esters **71** an **72**. On the basis of considerations described above, the isomers were not separated.



Scheme 20

The mixture of **71** and **72** was hydrogenated in acetic acid to give a mixture of the secondary amines **73** and **74** (Scheme 20). Since epimerization can also occur in the subsequent alkylation step, it was not deemed worthwhile to separate the mixture of isomers. The closure of ring D could only be accomplished in DMSO. Surprisingly, the material isolated from the reaction mixture did not consist of the expected mixture of the two alkaloids (i.e. pseudovincadifformine **3** and 20-epipseudovincadifformine **55**) but was only the latter compound.



Scheme 21

An attempt was made to introduce an α,β-double bond in ring D of pseudoaspidospermane using methodology which was successful in the case of compounds having an aspidospermane skeleton. A mixture of two epimers **64** and **65** (Scheme 21) was treated with *p*-toluenesulfinyl chloride in the presence of N,N-diisopropylethylamine. Workup of the reaction mixture gave one product, which was not the expected compound but a substance characterized as structure **75**. The formation of this compound presumably takes place by analogy with the thioether **53** prepared earlier. It is surprising, however, that the molecule containing a double bond is not formed in the reaction. The required elimination reaction failed in the case of compound **75**. Therefore, this compound was further converted to 21-oxopseudotabersonine **76** under oxidative conditions. In this case **76** was obtained as the final product via a multistep reaction sequence.

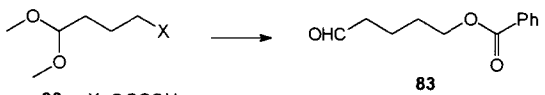
5. Deethylaspidospermane Derivatives

In the course of the above discussed syntheses we observed stereoselective [4+2] cycloadditions and epimerization reactions of compounds

containing a D-*seco*- or aspidospermane skeleton. As a continuation of that work, our new target compounds were 20-deethylvincadifformine **77** and 20-deethyl-20-epivincadifformine **78** (Scheme 25) since these molecules and the intermediates of their syntheses seemed to be promising for a thorough study of the mentioned points of stereochemical interest (12).

The strategic step of our syntheses was the reaction of the secondary amine **25** with methyl 4-formylbutanoate **79** or with 5-benzoyloxypentanal **83**. According to literature data and our own experience the stereochemistry of the resulting D-*seco* compounds is determined by the nature of the α-substituent in the aldehyde used as the reaction partner.

The intermediate **80** of the synthesis of **79** was reduced with LAH. The resulting alcohol **81** was acylated with benzoyl chloride to give the acetal **82**. Subsequent hydrolysis yielded the aldehyde **83** (Scheme 22).

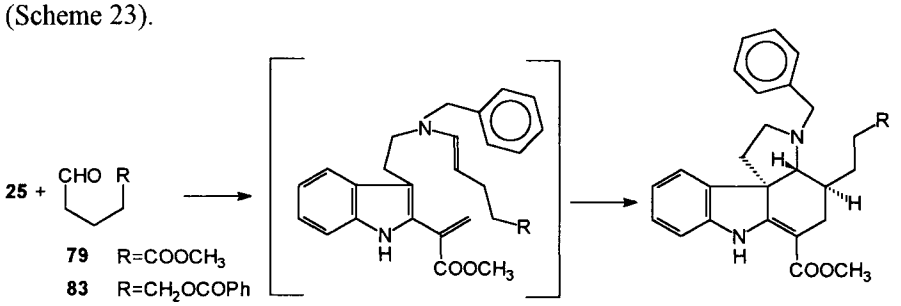


80 X=$COOCH_3$
81 X=CH_2OH
82 X=CH_2OCOPh

83

Scheme 22

The secondary amine **25** was allowed to react with 4-formylbutanoate **79** or with 5-benzoyloxypentanal **83** in boiling toluene and in the presence of *p*-toluenesulfonic acid. In both cases - as expected - only one product, **86** or **87** was obtained. This high stereoselectivity can be readily explained by the exclusive *E* geometry of the intermediates **84** and **85** of the [4+2] cycloadditions (Scheme 23).



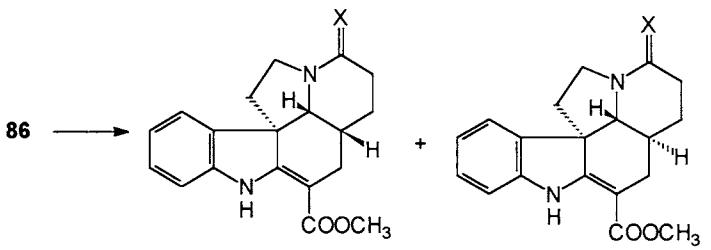
79 R=$COOCH_3$
83 R=CH_2OCOPh

84 R=$COOCH_3$
85 R=CH_2OCOPh

86 R=$COOCH_3$
87 R=CH_2OCOPh

Scheme 23

The catalytic debenzylation of the tetracyclic compound **86** in acetic acid at room temperature did not give the expected results. In spite of the mild conditions used no secondary amine, or amines, could be isolated since spontaneous intramolecular acylation occurred, completed by refluxing of the mixture in toluene, affording 20-deethyl-3-oxovincadifformine **88** and 20-deethyl-3-oxo-20-epivincadifformine **89** (Scheme 24).

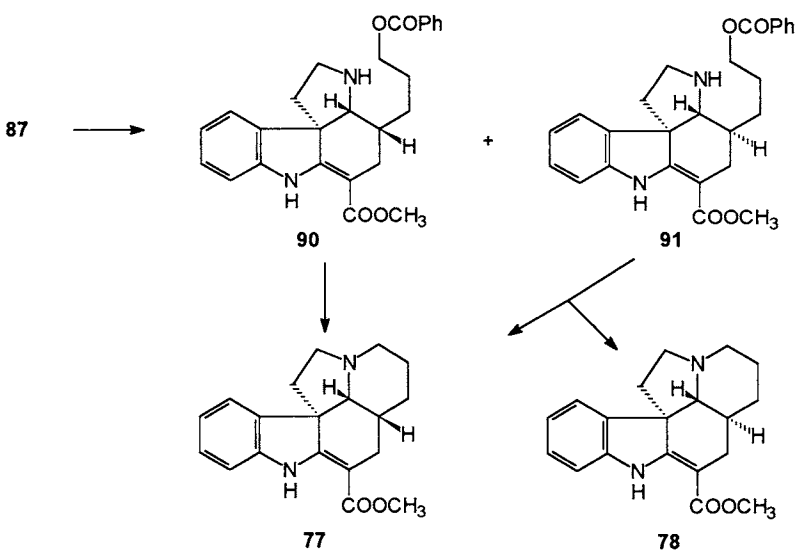


88 X=O **89** X=O

92 X=S **93** X=S

Scheme 24

Catalytic debenzylation of the benzoate ester **87** at room temperature gave a mixture of the secondary amines **90** and **91** (Scheme 25).



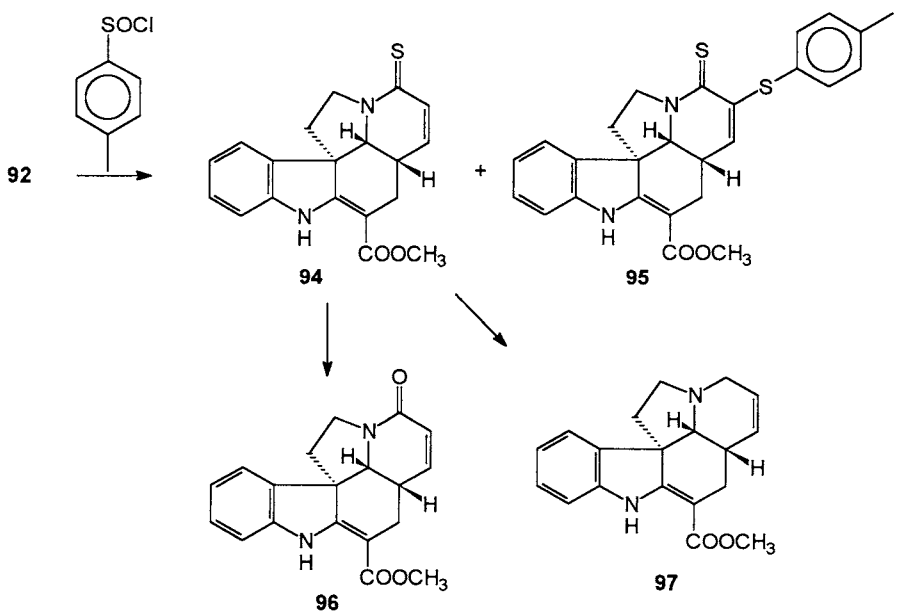
Scheme 25

The product ratios (**88:89** and **90:91**) depend on the temperature of debenzylation (Table 1). The experimental results give unequivocal evidence of the thermodynamic stability, but they do not answer the question of the sequence of debenzylation and epimerization.

Table 1. Temperature dependence of the ratio of isomers formed in the catalytic debenzylation of compounds **86** and **87** in acetic acid.

Temperature of debenzylation	Ratio **88:89**	Ratio **90:91**
20-25°C	1:1 (after refluxing in toluene)	1:10
70-75°C	10:1	1:2
85-90°C	1:0	4:5

The lactams **88** and **89** were converted into the thiolactams **92** and **93** by treatment with phosphorous pentasulfide, which could be reduced in the presence of Raney nickel to 20-deethylvincadifformine **77** and 20-deethyl-20-epivincadifformine **78**.



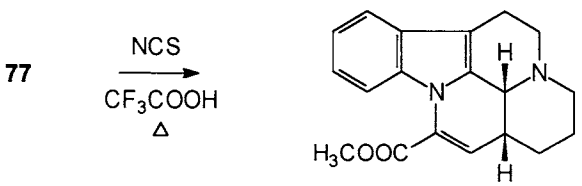
Scheme 26

In the case of derivatives **90** and **91** the intramolecular alkylation, i.e. the formation of ring D, was achieved by refluxing the starting compounds in DMF. Compound **90** was converted into 20-deethylvincadifformine **77** which has the same stereochemical structure; the reaction of **91** yielded 20-deethyl-20-epivincadifformine **78** accompanied by **77** formed as a result of epimerization.

In our earlier work a method was successfully used for the regioselective introduction of the carbon-carbon double bond into the ring D of the aspidospermane skeleton. Accordingly, **92** was allowed to react with *p*-toluenesulfinyl chloride in dichloromethane in the presence of base. In addition to the expected 20-deethyl-3-thioxotabersonine **94**, the thioether **95** was also isolated from the reaction mixture. Compound **94** was stirred with methyl iodide at room temperature, and the iminium salt - without isolation - was reduced with sodium borohydride to give 20-deethyltabersonine **97**.

In another reaction compound **94** was oxidized with *m*-chloro-peroxybenzoic acid in dichloromethane at -78° to yield 20-deethyl-3-oxo-tabersonine **96** (Scheme 26).

For the synthesis of 16-deethylapovincamine **98** 20-deethylvinca-difformine **77** was refluxed with N-chlorosuccinimide in trifluoroacetic acid (Scheme 27).

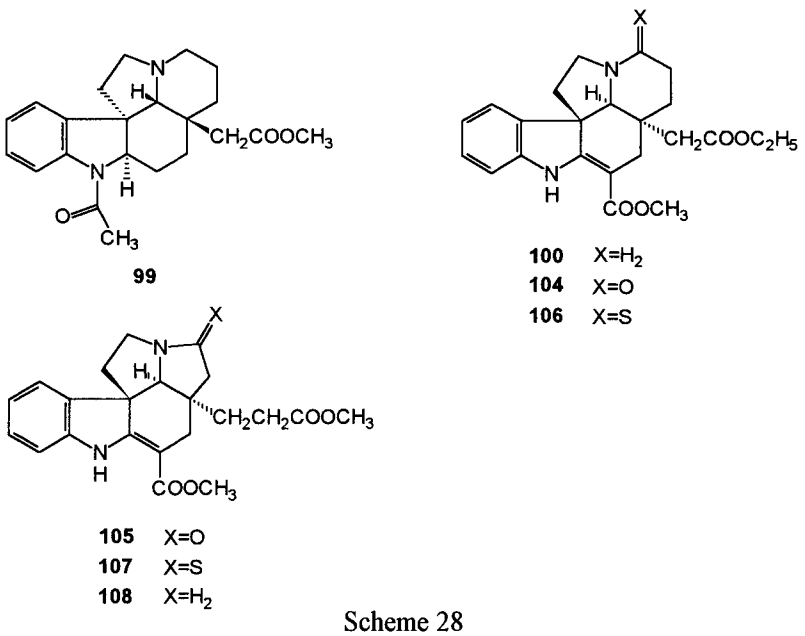


98

Scheme 27

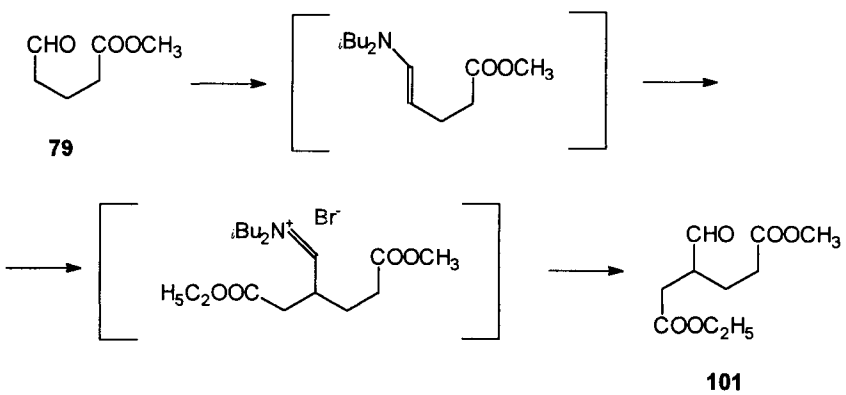
6. Cylindrocarine derivative

The above discussed synthetic strategy was further extended (13). In 1969 the isolation of twelve new alkaloids from a tree, *Aspidosperma cylindocarpon* was reported. One of the natural products was (-)-12-demethoxy-N(1)-acetylcylindrocarine **99**. The first synthesis of the racemic form of this alkaloid was achieved by Brennan and Saxton (14) in 1986; the key intermediate used was 19-ethoxycarbonyl-19-demethylvincadifformine **100** (Scheme 28).



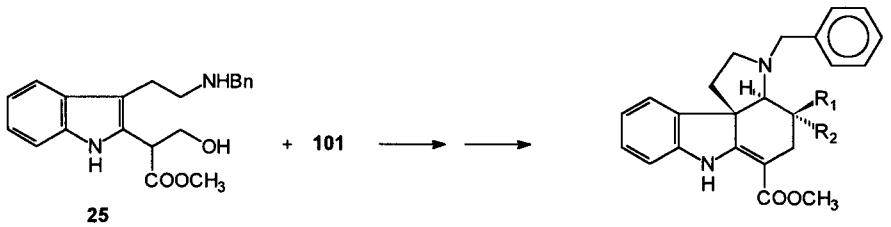
Scheme 28

Compound **100** could readily be prepared by our convergent synthesis. The reaction partners selected were the "acrylic ester" synthon **25** and the methyl 4-formyl-4-[(ethoxycarbonyl)methyl]-butanoate **101**. Compound **101,** not reported earlier in the literature, was prepared from methyl 4-formylbutanoate **79** (Scheme 29).



Scheme 29

When compound **25** and **101** were refluxed in toluene in the presence of *p*-toluenesulfonic acid, the reaction gave a 3:1 mixture of the isomers **102** and **103** (Scheme 30).

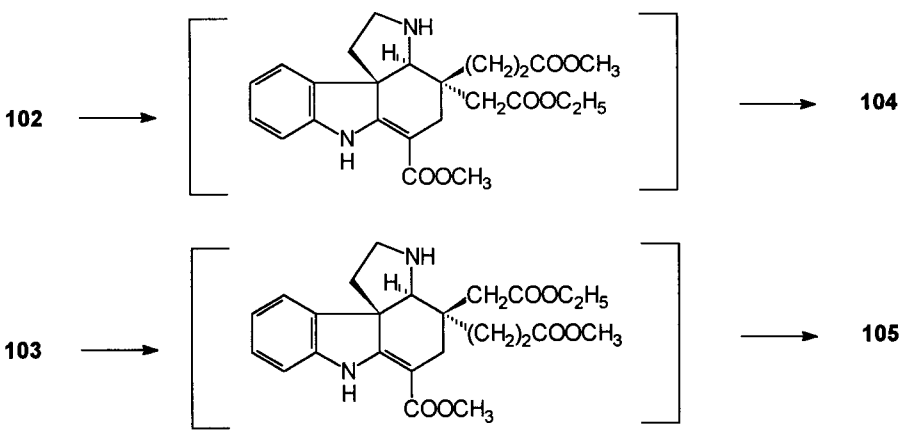


102 $R_1=(CH_2)_2COOCH_3$
$R_2=CH_2COOC_2H_5$

103 $R_1=CH_2COOC_2H_5$
$R_2=(CH_2)_2COOCH_3$

Scheme 30

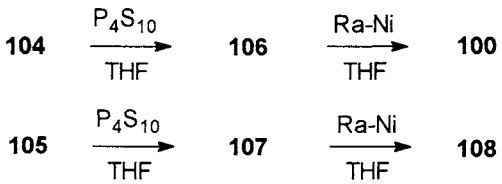
After the chromatographic separation of the C-20 epimers, they were catalytically debenzylated. The reaction was effected in glacial acetic acid at room temperature, yet the secondary amines could not be isolated; they were transformed by spontaneous intramolecular acylation into derivatives of aspidospermane and D-noraspidospermane skeleton (**103**→**105** and **104**→**106**) (Scheme 31).



Scheme 31

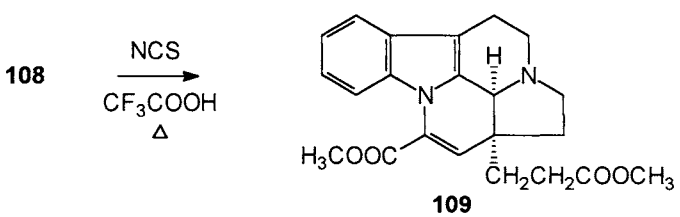
According to another observation, when the catalytic debenzylation was effected at 100° instead of room temperature, then, as a result of partial epimerisation occurring in acidic medium and at higher temperature, compound **103** gave a 3:1 mixture of the products **104** and **105**, increasing thereby the total yield of the vincadifformine derivatives.

Next **104** and **105** were treated with phosphorous pentasulfide to convert them into the thioxo analogues, **106** and **107**. The products were then reduced with Raney nickel. In the first case the reaction product was 19-ethoxycarbonyl-19-demethylvincadifformine **108**, representing the formal synthesis of the racemic form of 12-demethoxy-N(1)-acetylcylindrocarine **99.** In the second case the reduction resulted in 18-methoxycarbonyl-D(14)-norvincadifformine **109** (Scheme 32).



Scheme 32

The oxidative ring transformation of **108** was also achieved by means of N-chlorosuccinimide in trifluoroacetic acid, in analogy to the conversion of **100**; the product was 21-methoxycarbonyl-D(18)-norapovincamine **109** (Scheme 33).



Scheme 33

To sum up all the above discussed investigations we may conclude that based on earlier studies of Kuehne, Saxton and many other researchers an efficient and practical approach to synthesize compounds having aspido-spermane or pseudoaspidospermane skeleton has been created, and used extensively.

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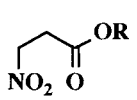
Synthesis of Natural Products *via* Aliphatic Nitroderivatives

Roberto Ballini

1. INTRODUCTION

Aliphatic nitro compounds have been proved to be valuable intermediates and the chemical literature continuously reports progress in their utilization for the synthesis of a variety of target molecules. It is now over two decades since the appearance of the bench mark review article by Seebach *et al.* (ref. 1) on the vast preparative potential of aliphatic nitro compounds and the efforts to develop efficient syntheses and utilization of functionalized aliphatic nitro derivatives have continued with increasing success.

Very often the nitro group is present in different natural products, showing biological activity, such as: *(i)* the 3-nitropropanoic acid ***1*** (R=H), first isolated in 1920 as a component of the glycoside hiptagen from the root of the Javanese tree *Hiptagamadablota* and, then, found in the roots of other plants such as *Violaodorata* (ref. 2) or molds such as *Aspergillus flavus*, *Penicilliumatrovenetum*, *A. oryzae* (refs. 2,3), and in the legume *Indigoferaendecaphylla* (ref. 4), more recently, 3-nitropropionates were found in extracts of the legumes, *Hippocrepis balearica*, *H. comosa*, *Lotus uliginosus* and *Scorpiurus muricatus* (ref. 5). Three separate nitro-propanoyl esters of glucose, 6-(3-nitropropanoyl)-α,β-*D*-glucopyranose, 1,6-di-O-(3-nitropropanoyl-β-*D*-glucopyranose (cibarian) and 1,2,6-tri-O-(3-nitropropanoyl)-β-*D*-glucopyranose (karakin) were identified in shoot extracs of *H. comosa*. Evidence for the presence of the 3-nitropropanoyl ester of 4-hydroxybutanoic acid in extracts of *H. comosa* was also reported; *(ii)* the 1-phenyl-2-nitroethane ***2***, isolated from the wood and bark of various plants such as *Aniba canellila* and *octotea pretiosa*, which is a constituent of certain



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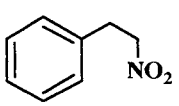

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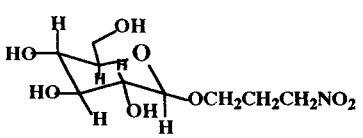

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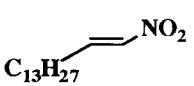
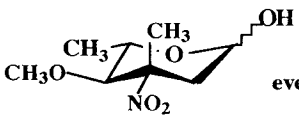


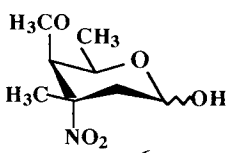
4

essential oils and possesses a quite characteristic odor (refs. 6,7); *(iii)* the miserotoxin ***3***, isolated as poisonous material from *Astragalus* (Leguminosae) *miser* (ref. 8); and *(iv)* The (*E*)-1-nitro-1-pentadecene ***4***, the defensive compound, isolated (refs. 9,10) from the frontal gland secretion of soldiers of the Cuban *Prorhinotermes simplex* (Hagen) (*Rhinotermitidae, Isoptera*). Moreover, the nitro group has been found on natural carbohydrates which occur as components of one or more antibiotics. So, *L*-Evernitrose ***5*** was discovered as a component of the everninomicins B, C and D by Ganguly et al. (ref. 12); *D*-Rubranitrose ***6*** was isolated (ref. 13) from the antitumor antibiotic rubradirin; *L*-kijanose ***7*** was obtained (ref. 14) from the antibiotic kijanimicin and the tetrocarcins A and B; while *L*-decilonitre ***8*** occurs (ref. 15) in the trisaccharide moiety of the anthracycline antibiotics



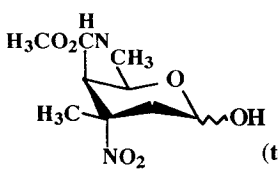
evernitrose

5



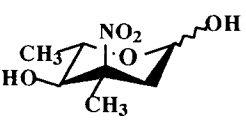
rubranitrose

6



kijanose (tetronitrose)

7



decilonitrose

8

decilorubicin and arugomycin.

The understanding of the biological role played by the nitro compounds is exceedingly limited. It may be that the natural role played by antibiotics is indeed to protect the host organisms from bacterial or fungal invasion and its consequences. Other possible roles may be those of insect attractants or repellants. For example, 1-phenyl-2-nitroethane ***2*** might serve as a repellent of parasitic insects. The 3-nitropropanoic acid ***1*** causes severe near-toxic symptoms or ingestion of the plant sources and this may serve to protect the plants.

However, the primary focus of this review is to assess the practical utility of nitroalkanes as starting material and/or key intermediates, for the preparation of important natural products in which the nitro group is no more present. Additionally, the general reactivity of nitroalkanes is recalled in order to favour a better understanding of the reported syntheses.

2. GENERAL REACTIVITY OF NITROALKANES

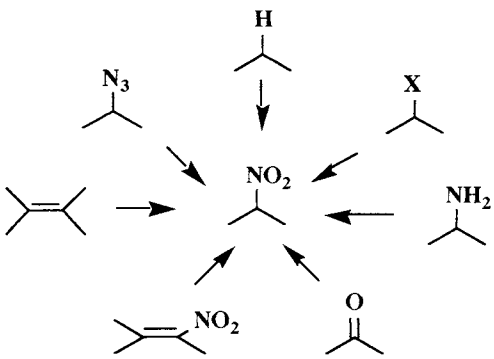
Among the many interesting developments in the field of synthetic methodology of the past years, the use of nitro compounds in organic synthesis is experiencing a burst of activity. After the first report of Seebach *et al.* (ref. 1), several excellent reviews appeared concerning the chemistry of nitroalkanes (refs. 16-28).

Aliphatic nitro compounds present a wide versatility, in fact it is possible: *(i)* to show that they are readly available, *(ii)* to have a wide range of efficient methods for its transformation into other functionalities, *(iii)* to verify the variety of carbon-carbon bond-forming processes that the nitro group

facilitates, *(iv)* to replace the nitro group by hydrogen, and *(v)* to permit the ring cleavage of cyclic compounds.

2.1 Sources of Nitroalkanes

In the Scheme 1, the most popular sources of nitroalkanes are reported (refs. 1,24). So, for istance, nitroalkanes can be obtained from alkanes by nitration or from haloalkanes by reaction with

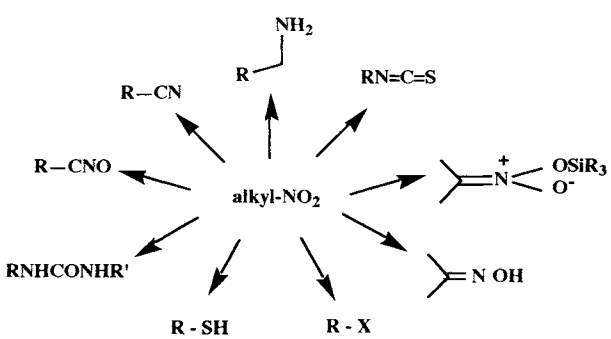


Scheme 1

sodium or silver nitrite in dipolar solvents; by nitration of alkenes followed by selective reduction; or by ozonolisis of azides.

2.2 Conversion of Nitroalkanes into Other Functionalities

In Scheme 2 many examples of typical nitro group conversion are reported; i.e., into nitrile

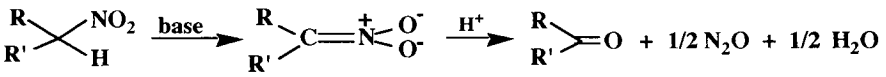


Scheme 2

oxides, nitriles, amines, oximes, aloalkanes, amides, isothiocyanathes, thiols, etc.

However the conversion of the nitro group into a carbonyl group can be considered (Scheme 3) by the far most important one, because it effectively reverses the polarity of the neighboring carbon

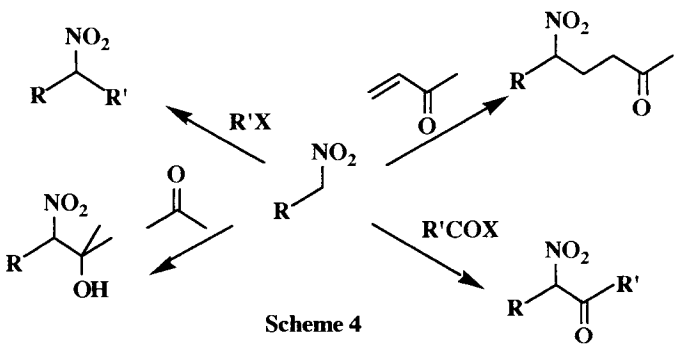
from nucleophilic to electrophilic, thus allowing a wide range of transformations to be carried out. The reaction, discovered in 1894 by Nef, is currently called "Nef reaction" (ref. 32).
The classical Nef reaction is an acidic method, however, more recent methods include modifications using excess base or acid, oxidizing agents or reducing agents, ozonolysis or neutral conditions.



Scheme 3

2.3 C-C Bond-Forming Processes *via* Nitroalkanes

In Scheme 4, it is possible to observe that nitroalkanes play an important role in organic



Scheme 4

synthesis due to the ease of carbon-carbon bond-forming reactions (refs. 1,16-35). A nitro group acting as a strong electron-withdrawing group, can activate a neighboring carbon-hydrogen bond for alkylation by reacting with (Scheme 4) alkyl bromides or iodides, with saturated (Henry reaction) or conjugated (Michael reaction) carbonyl compounds and with activated carboxylic acids.

2.4 Replacement of Aliphatic Nitro Groups by Hydrogen

Recently new reactions have been discovered that result in the displacement of the nitro group by hydrogen (ref. 36). This discovery has opened a new area in organic synthesis, because the nitro group can be removed after it has served as an activating group for the reaction in which C-C bonds

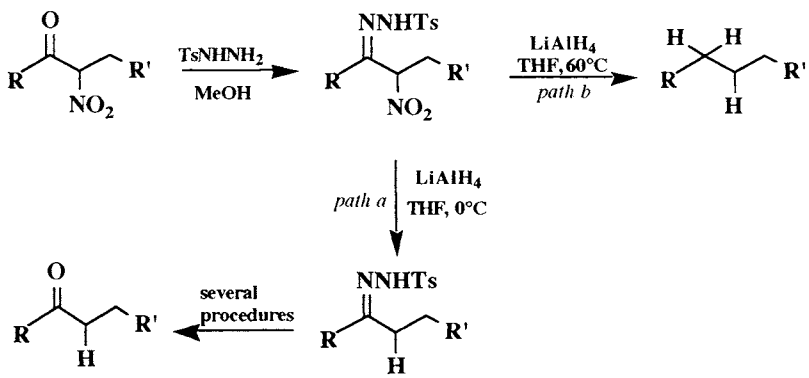
$$Bu_3SnH + In\cdot \rightarrow Bu_3Sn\cdot + InH$$
$$Bu_3Sn\cdot + R\text{-}NO_2 \rightarrow RN(O\cdot)OSnBu_3$$
$$RN(O\cdot)OSnBu_3 \rightarrow R\cdot + Bu_3SnNO$$
$$R\cdot + Bu_3SnH \rightarrow R\text{-}H + Bu_3Sn\cdot$$

Scheme 5

are formed.

After several works regarding the denitration of some types of nitroalkanes, N. Ono reported a more general method for the substitution of the nitro group with an hydrogen (refs. 36,37), using tri-*n*-butyltin hydride (Bu_3SnH) in benzene and in the presence of AIBN. The radical mechanism, as shown in the Scheme 5, has been proposed for this reaction.

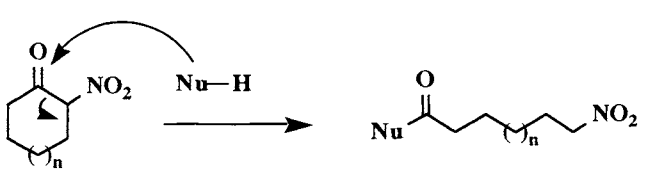
An important procedure for the denitration of 2-nitroketones has been published, using $LiAlH_4$ as the denitrating reducent. This method (refs. 38,39) consists (Scheme 6) of the conversion of 2-nitro ketones into the corresponding tosylhydrazones followed by treatment with $LiAlH_4$, in THF, at 0° C (*path a*). The tosylhydrazones thus obtained may be readily cleaved to give the corresponding ketones. However, if the reduction is performed at 60° C (*path b*) the tandem deoxygenation-denitration of 2-nitroketone is observed (refs. 40,41).



Scheme 6

2.5 Ring Cleavage of Cyclic Compounds

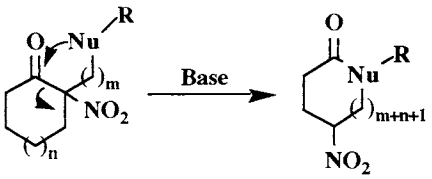
The presence of the nitro group in α-position to a carbonyl group in cycloalkanones offers a new reactivity pattern, peculiar to α-nitroketones. In fact, the C-C bond between the carbonyl group and the nitro substituted atom (Scheme 7) of cyclic α-nitroketones undergoes facile cleavage by nucleophilic agents (refs. 17,25) under mild conditions.



n = 5-15 **Nu = OH, OR, NH_2, RNH, RS.**

Scheme 7

Hesse *et al.* (ref. 42) has extensively studied the possibility of cyclic 2-nitroketones to react with internal nucleophile to give macrocyclic compounds (Scheme 8) by ring enlargement, greatly improving the synthetic utility of this class of compounds.



Scheme 8

Many internal nucleophiles, such as active methylene groups, alcohols, amines and enamines have been utilized. Thus, by this reaction ("the Zip Reaction") two, three, four or five carbon atoms, respectively, can be incorporated to the pre-existent cyclic system, and many macrocyclic molecules can be synthesized.

3. UTILIZATION OF NITROALKANES IN THE SYNTHESES OF NATURAL PRODUCTS

As reported above, aliphatic nitro compounds have demonstrated a high versatility in organic synthesis, and the many synthetic transformations which can be accomplished with them have encouraged many researchers to explore their use in the synthesis of natural products.

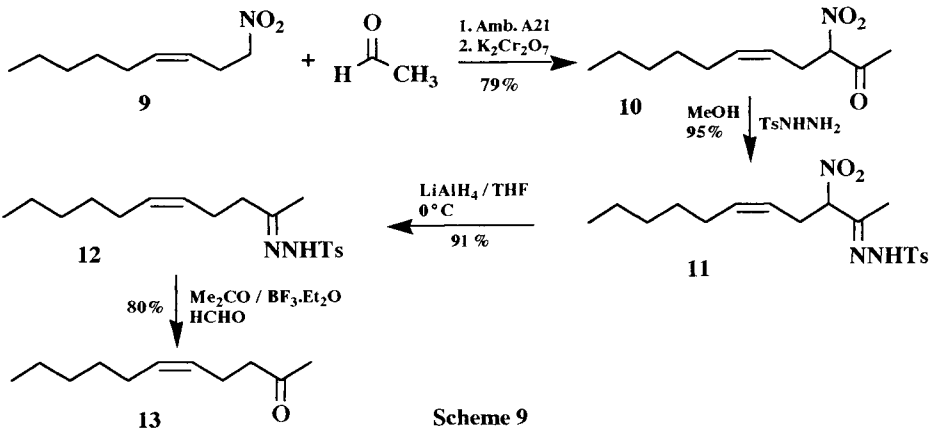
3.1 PHEROMONES

Many organisms release chemical substances that specifically affect other members of the same species some distance away from the point of release of the chemicals. Such substances therefore resemble hormones, consequently such chemicals are termed "pheromones" derived from the Greek words meaning to carry and excite. For chemical insect control the most valuable of these pheromones are the insect sex pheromones or attractants which may be regarded as chemicals which directly facilitate mating, either by attracting another insect from a considerable distance or by inducing the performance of some close-range behaviour concerned with courtship. Then, due to their important role in biological insect control, the discovery of efficient syntheses for the preparation of pheromones became very important. In this context aliphatic nitro compounds have been demonstrated to be important key building blocks for many of these preparations.

3.1.1 Enone-Pheromones

Different pheromones exibit the chemical structure of (Z)-alkenones. Functionalized nitroalkanes have proved to be very effective for the synthesis of this class of compounds, *via* Henry reaction (ref. 28) and denitration (refs. 36-39) as key steps.

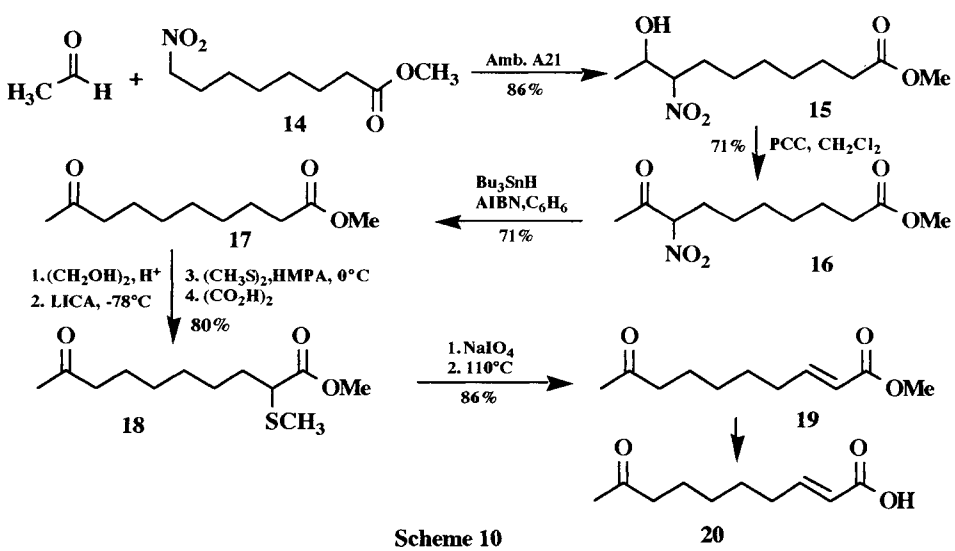
Firstly, the synthesis of (Z)-5-undecen-2-one ***13***, the principle volatile component of the pedal gland exudate of the bontebox *Damaliscus dorcas dorcas*, has been reported (ref. 43). The procedure (Scheme 9) starts from (Z)-1-nitro-3-nonene ***9*** as the (Z)-3-nonen-1-yl-anion synthon. The nitroaldol reaction of ***9*** with acetaldehydes followed by potassium dichromate oxidation, under phase-transfer catalysis, afforded the nitro ketone ***10***. Reaction of ***10*** with $TsNHNH_2$ in methanol gave the corresponding hydrazones ***11*** in high yield (95%). The purification of ***11*** was easily performed by crystallization from methanol/water.



Scheme 9

Treatment of compound *11* in THF with $LiAlH_4$ at 0°C produced the denitrated hydrazone *12*. Subsequent deprotection of *12* was carried out in acetone/water with catalytic amounts of boron trifluoride etherate and (*Z*)-5-undecen-2-one *13*, chemical purity 98% by GLC, was obtained in about 54% overall yield from *9*.

Methyl 8-nitrooctanoate *14* has been used (ref. 44) as the starting material for the synthesis of 9-oxo-(*E*)-2 decenoic acid *20* (queen substance), an important sex attractant of the queen bee also

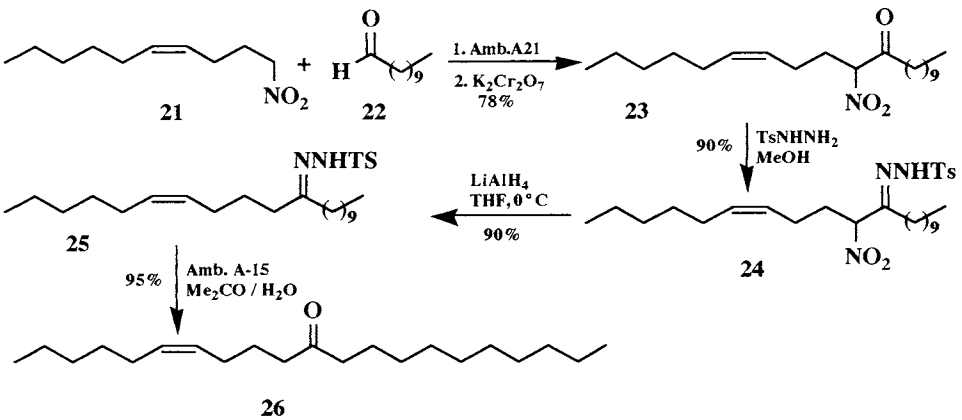


Scheme 10

implicated as a pheromone of termites (Scheme 10). The nitro ester *14*,when treated with acetaldehyde in the persence of Amberlyst A 21, gave the nitroalkanol *15*. Subsequent oxidation with pyridinium chlorochromate in dichloromethane furnished the α-nitroketone *16*, which by reaction with Bu_3SnH and catalytic amount of AIBN in refluxing benzene was denitrated to *17* (46% overal yield).

The oxo ester ***17*** can be converted into the α,β-unsaturated methyl ester of the queen substance ***19***, following the Trost's method (ref. 45), *via* sulfenylation an dehydrosulfenylation.

(Z)-Heneicos-6-en-11-one ***26***, a (*Z*)-δ,ε-alkenone isolated in 1975, is the sex pheromone of



Scheme 11

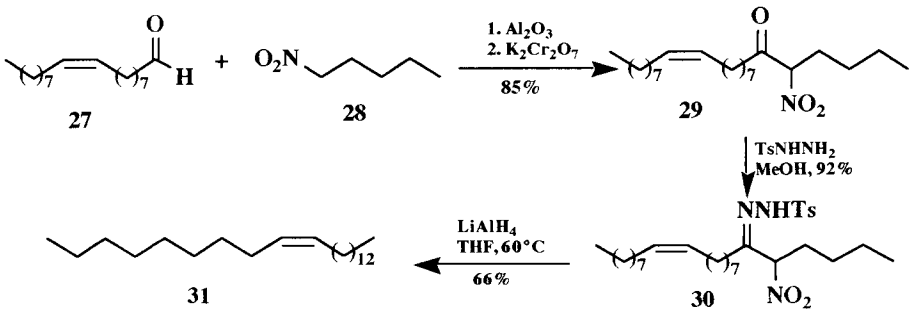
the Douglas Fir Tussock moth (*Orgyia pseudotsugata*). Douglas Fir Tussock moth is a severe defoliator of fir forests; consequently, considerable interest attends the synthesis of this pheromone. In the Scheme 11 is reported (ref. 46) a convenient preparation of ***26***, starting from (Z)-1-nitrodec-4-ene ***21***. The nitroalkene ***21*** can be easily obtained in good yield from commercial (Z)-dec-4-en-1-ol by convertion into the corresponding bromide and successive substitution (refs. 1,24) with sodium nitrite in DMF. Nitroaldol reaction of ***21*** with undecanal ***22*** on Amberlyst A-21, in the absence of solvent, followed by oxidation of the resulting nitro alcohol with potassium dichromate under phase-transfer catalyst, afforded the α-nitroketone ***23*** in 70% yield. Conversion of ***23*** into the corresponding tosylhydrazone ***24*** was performed in 90% yield. Denitration of ***24*** with $LiAlH_4$, in THF at 0°C, followed by regeneration of carbonyl group, carried out in acetone-water with the catalysis of Amberlyst A-15, gave the pheromone ***26*** in 60% overall yield and with a high isomeric purity (> 99% by ^{13}C NMR).

3.1.2 Alkane- and Alkene-Pheromones

The discovery of efficient procedures for the tandem denitration-deoxygenation of 2-nitroketones (refs. 40,41) allowed us to realize the synthesis of two important pheromones: (*Z*)-9-tricosene ***31*** and 2-methylheptadecane ***35***. The (Z)-alkene ***31*** is a sex pheromone component of the mature female housefly (*Musca domestica*) and its synthesis (Scheme 12), starts (ref. 40) by the nitroaldol reaction of oleic aldehyde ***27*** with nitropentane ***28*** on basic alumina, followed by in situ oxidation of the resulting nitro alcohol affording the α-nitro ketone ***29*** in 85% yield.

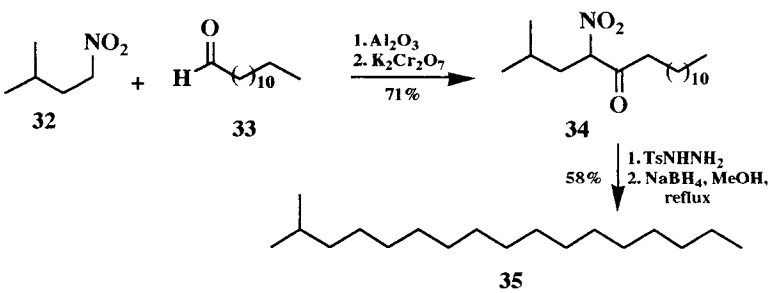
The conversion of compound ***29*** into the corresponding tosylhydrazone ***30*** was performed in 92% yield after recrystallization. Reduction of compound ***30*** with $LiAlH_4$, in THF, at 60°C produced, *via* deoxygenation-denitration of α-nitro ketone ***29***, the (Z)-9-tricosene ***31*** in 66% yield, and 98% purity.

2-Methylheptadecane *35* has been isolated and identified as an important pheromone of at least nine species of the *Artidae* family. It has also been characterized as a flavour component in four mango variety and in black soya beans, as one odorous constituent of the blue-green algae, commonly



Scheme 12

called Aoko, and as a chemical component of the essential oil from *Clematis hexapetala* Pall and *Inula nervosa* Wall. The synthesis of *35* has been achieved (ref. 47) starting from 1-nitro-3-methylbutane *32* which acts as the 3-methylbutan-1-yl anion synthon (Scheme 13). Nitroaldol reaction of tridecanal *33* with *32* on basic alumina, followed by oxidation of nitroalkanol gave the nitroketone *34* in 71% yield. The tandem denitration-deoxygenation of *34* to *35* was perfomed by converting *34* into

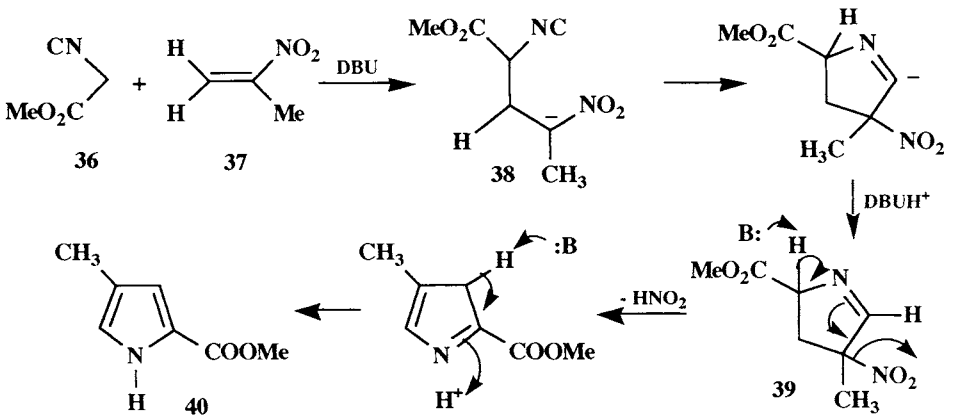


Scheme 13

the corresponding tosylhydrazone and subsequent, in situ, reduction with $NaBH_4$ at 80°C. 2-Methylheptadecane *35* was readly produced in 58% yield (41% overall yield).

3.1.3 Heterocyclic Pheromones

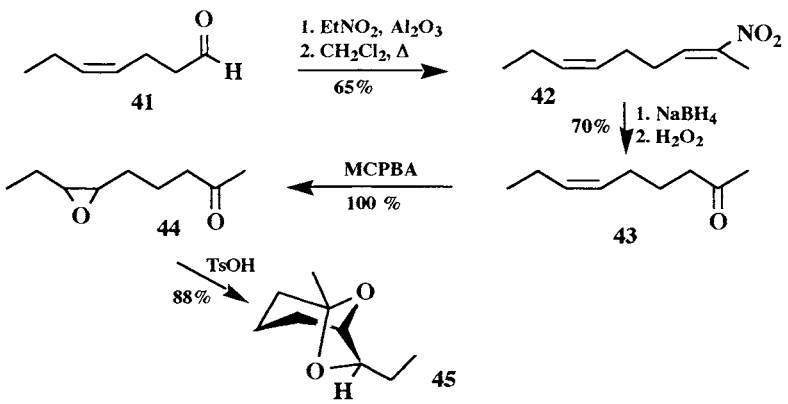
Nitrocompounds have demonstrated to be very effective also for the syntheses of pheromones showing heterocyclic structures. Barton and Zard (ref. 48) reported the synthesis of 2-carbomethoxy-4-methylpyrrole *40* which is the trail marker pheromone of the Texas leaf-cutting ant, *Atta texana* (Buckley). Thus base-catalyzed Michael addition of α-isocyanoacetate *36* (Scheme 14) to 2-nitropropene *37*, followed by cyclisation of the nitronate anion *38* onto the isocyano group led to pyrroline *39*. Finally, base-catalyzed expulsion of nitrite from the pyrroline and double bond



Scheme 14

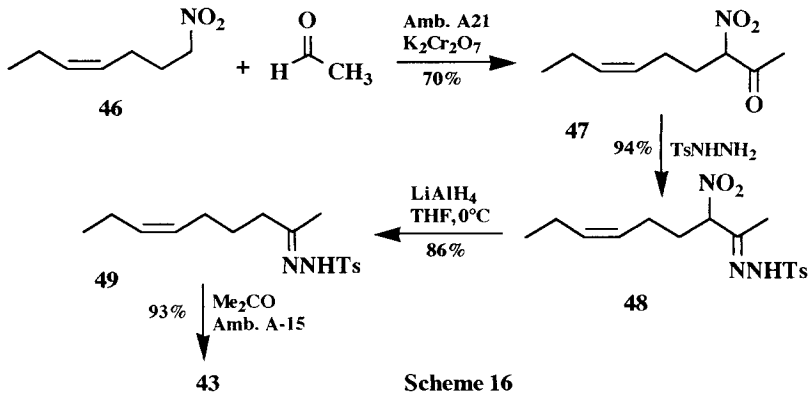
rearrangement gave the pyrrole ***40*** in 60% yield. It is important to point out that in this procedure the nitro group facilitates three different chemical reactions: C-C bond forming process, cyclisation, and C=C double bond formation.

Exo-brevicomin (ref. 45) is the principal pheromone component of the female wester pine beetle, *Dentroctonus brevicomin*, which bores into ponderosa pine, and two different approaches



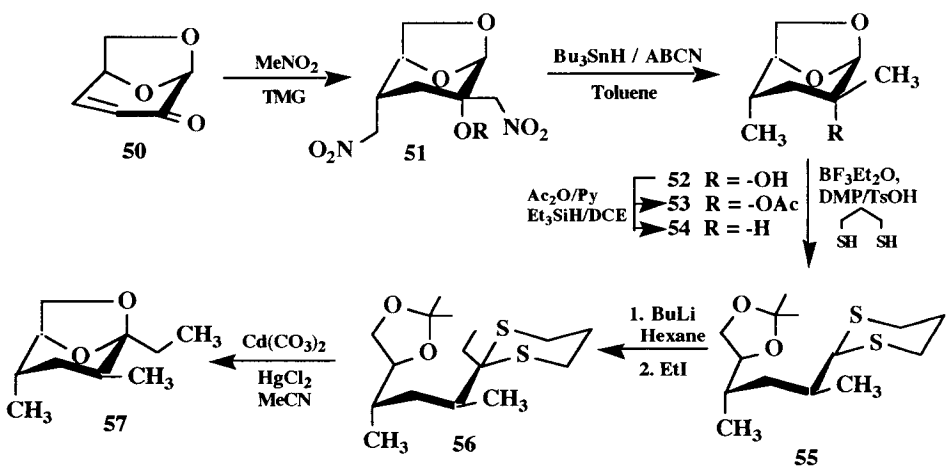
Scheme 15

have been reported to produce ***45*** using nitroalkanes. In Scheme 15 nitroethane has been (ref. 49) used in the nitro aldol condensation with (*Z*)-4-heptenal ***41***, giving the nitrodiene ***42***, in 65% yield as an (*E*),(*Z*)-2,6-isomer. Chemoselective convertion of the conjugated nitroalkene into ketone ***43*** was performed by $NaBH_4/H_2O_2$. Epoxidation of the enone ***43*** with MCPA afforded the corresponding epoxy ketone ***44*** in quantitative yield, which by *p*-TsOH provided racemic *exo*-brevicomin ***45*** in 86% yield. The enone ***43*** can be also obtained (ref. 46) from (*Z*)-1-nitrohept-4-ene ***46*** (Scheme 16) following the reaction conditions reported for the synthesis of (*Z*)-heneicos-6-en-11-one ***26*** (Scheme 11).



Scheme 16

Multistriatin, a natural component of the aggregation pheromone of the Elm bark beetle *Scolytus multistriatis* was firstly synthesized in 1976 (ref. 50). It has been reported that the (-)-α-isomer is biologically active toward the European population of the beetle and the (-)-δ-isomer ***57*** is active for the American population. Witczak and Li (ref. 51) published the synthesis of ***57*** starting by



Scheme 17

(Scheme 17) Michael 1,4-addition of nitromethane to levoglucosenone ***50***, using TMG as base. Under these reaction conditions, the simultaneous 1,4-addition of nitromethane to the enonic system and 1,2-addition to the keto function at C-2 proceeded stereoselectively, with the formation of the chiral precursor ***51*** . This efficient approach furnished an intermediate with two chiral centers at C-2 and C-4 as required. Radical removal of both nitro groups present in the adduct ***51*** was achieved by reaction with tributyltin hydride, and in the presence of a radical initiator (ABCN). Reductive removal of the -OH group at C-2 in ***52*** was accomplished by treatment with $Et_3SiH/BF_3{\cdot}Et_2O$/DCE. No

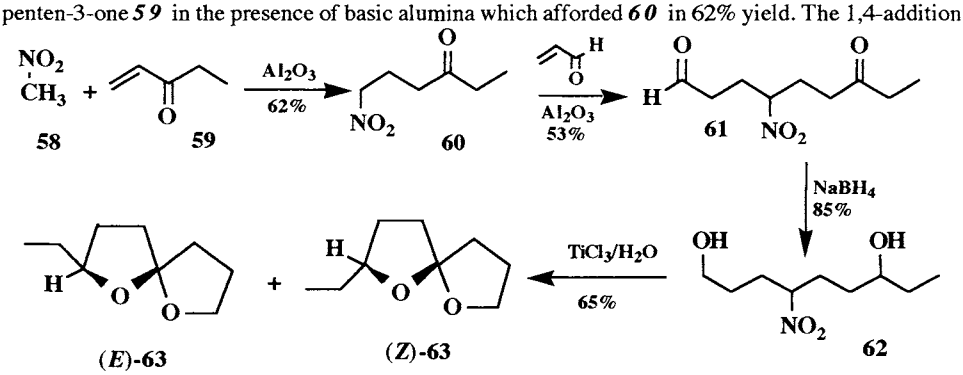
epimerization at C-2 occurred under the above reaction conditions and the formation of ***54*** as the sole product in 76% was observed. The final steps were performed according to the original Mori procedure (ref. 50). The first step was the introduction of the 1,3-dithiane group at C-1, by conventional method, giving the compound ***55*** in 72% yield. Alkylation of ***55*** with ethyl iodide, after lithiation with *t*-BuLi and in the presence of HMPA produces ***56*** . The final removal of the dithiane protecting group, by treatment of ***56*** with mercuric chloride, regenerated the carbonyl group with simultaneous transacetalation to form ***57*** in 89% yield and with an excellent optically purity, c.a. 99%.

3.1.4. Spiroketalic Pheromones

Spiroketals enjoy widespread occurrence as substructures of naturally occurring substances from many sources, including insects, microbes, plants, fungi, and marine organisms. Avermectins and milbemycins, macrocyclic lactones which have exceptional pesticidal activity; polyether antibiotics such as monesin, narasin and lonomycin; antitumoral agents including okadaic acid and phyllatocin; toxin (talaromycins) and steroidal compounds (sapogenins) are representative structures (refs. 52-55) exhibiting a spiroketal moiety. However, the discovery that simple spiroketals are components of insect pheromones is an achievement of W. Francke and his coworkers (ref. 56) that confirms the great importance of the acetal and ketal groups in well-known semiochemicals.

Nitroalkanes have been demonstrated to be very efficient for the preparations of several spiroketalic pheromones. The first synthesis is dated 1986 (ref. 57) and describes the production of 1,6-dioxaspiro[4,4]nonane (Chalcogran) ***63***, the main component in the aggregation pheromone of the bark beetle *pityogenes chalcografus*, also named Kupferstecker.

The starting point of this synthesis (Scheme 18) is the 1,4-addition of nitromethane ***58*** to 1-penten-3-one ***59*** in the presence of basic alumina which afforded ***60*** in 62% yield. The 1,4-addition

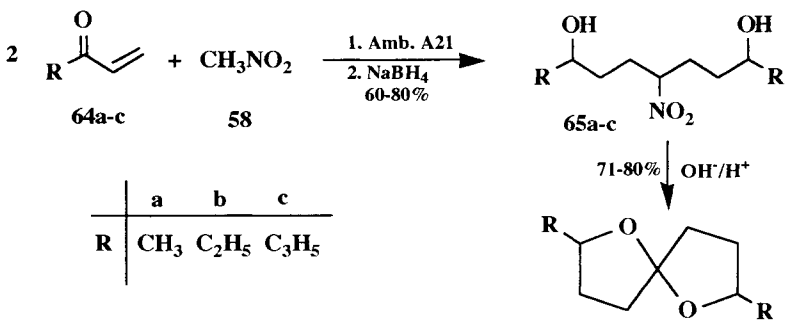


Scheme 18

of ***60*** to acrolein under identical conditions gave ***61*** (53%). On reduction with $NaBH_4$/EtOH ***61*** furnished 1,7-dihydroxy-4-nitrononane ***62***, the open-chain chalcogran precursor. Finally, the transformation of the nitro group into a carbonyl group by reaction with aqueous solution of $TiCl_3$ offered, *via* spontaneous cyclization, the desidered chalcogran [(*E*)-***63***/(Z)-***63*** = 39/61]. Thus, the nitromethane, used as d,d' multiple coupling reagent, can be considered as a carbonyl dianion synthon.

Moreover, this double Michael additon of nitromethane represents a novel strategy for the synthesis of 1,6-dioxaspiro[4,4]nonane derivatives.

Recently we hAVE published (ref. 58) a simple, two step, inexpensive, large scale preparation of 2,7-dialkyl-1,6-dioxaspiro[4.4]nonanes ***66***, important pheromone components (ref. 56) of *Andrena Wilkella*, *A. Ocreata*, *A. Ovatula*, and *A. Haemorroa*, in which the first step (Scheme 19) is the double 1,4-addition of nitromethane ***58*** with two moles of enone ***64***, on Amberlyst A-21, without solvent, followed by in situ reduction with $NaBH_4$. Thus the nitrodiols ***65*** were obtained in one pot,

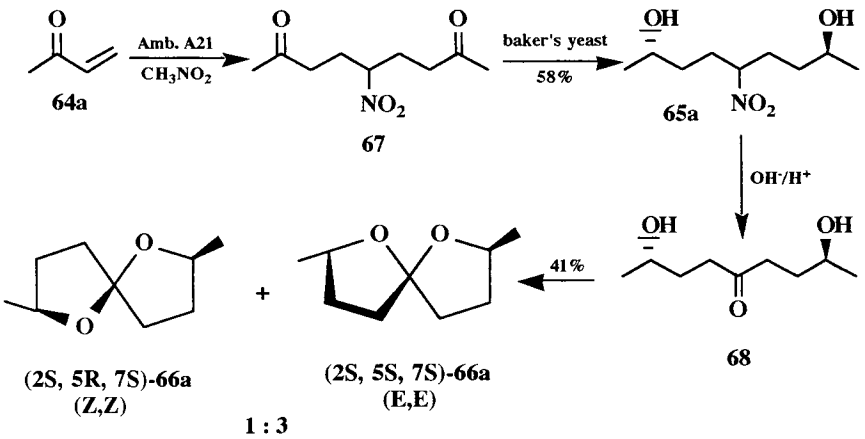


Scheme 19

66a-c

and in good yields (60-80%). The transformation of the nitro group into a carbonyl group, under basic conditions (sodium hydroxide), to the corresponding nitronate, followed by acidification in a two-layer (sulfuric acid/pentane) solution, afforded, by spontaneous spiroketalization, the compounds ***66*** as a mixture of diastereoisomers. Following this procedure Guarna *et al.* (ref. 59) proposed an enantioselective synthesis of 2,7-dimethyl-1,6-dioxaspiro[4.4]nonanes, **66a** (Scheme 20). The symmetrical diketone **67** was prepared, as above reported, by addition of nitromethane to the vinylketone **64a** in the presence of Amberlyst A 21. The successive baker's yeast reduction of **67** was carried out in aqueous solution at 30°C with glucose as nutrient and in aerobic conditions. It is known that the baker's yeast reduction of symmetrical diketones having the two carbonyl groups in 1,4 or more distant positions occurs independently on the two oxo groups. In such compounds the bioreduction afforded (*S*,*S*) diols. The result of the bioreduction of **67** was consistent with these general observations, and yielded (58%) the diol **65a** with (2*S*, 8*S*) absolute configuration. The bioreduction of **67** is highly diastereoselective because no *meso* forms of **65a** were detected in the products.

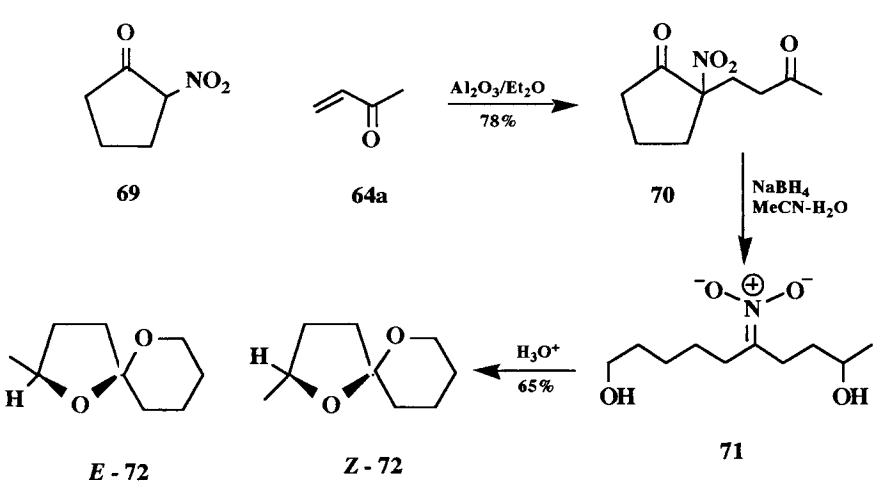
Treatment of (2*S*, 8*S*)-**65a** with NaOH in ethanol and then with the two-layers system, dil. H_2SO_4/hexane at 0°C for 1h, afforded **66a** through spontaneous cyclization of the intermediate **68**, in 41% yield. The analysis of ^1H- and ^{13}C-NMR spectra of **66a** showed the absence of the (*E*,*Z*) diastereoisomer derived from the spiroketalization of the two diol *meso* forms (2*S*, 5*R*, 8*R*)-**66a** and (2*S*, 5*S*, 8*R*)-**66a**.



Scheme 20

In these three last Schemes it has been stated that nitromethane, used as a d d' multiple coupling reagent and acting as a carbonyl dianion synthon, it is an excellent starting molecule for the preparation of spiro[4.4]nonanes.

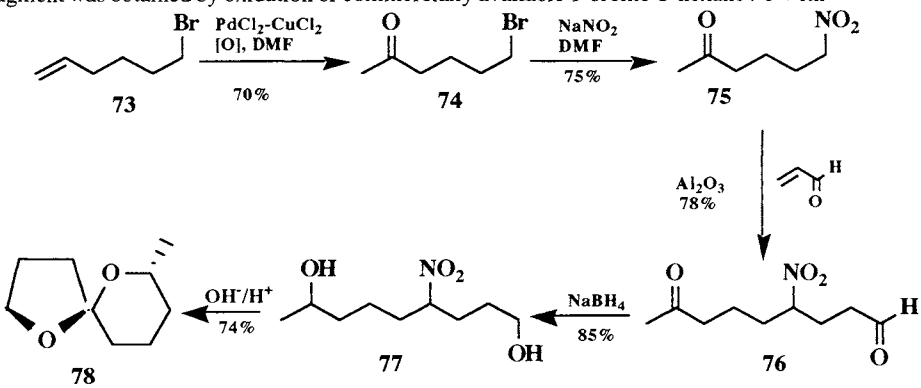
However, continuing our efforts to verify the utility of functionalized nitroalkanes in the synthesis of natural products, we have worked out (refs. 60,61) a solution to the preparation (Scheme 21 and 22) of 1,6-dioxaspiro[4,5]decanes ***72*** and (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane ***78***, starting from nitro ketones. The spiroketals ***72*** and ***78*** were identified as important components of odours of common wasp *Pararespula vulgaris* which might serve as repellants or aggregation inhibitors. 1,6-Dioxaspiro[4,5]decanes ***72*** have been prepared by 1,4-addition of 2-nitrocyclopentanone ***69*** to the vinylketone ***64a***. The obtained adduct ***70*** was directly converted into



Scheme 21

the spiroketals ***70*** by regiospecific reductive cleavage (refs. 17,25) with sodium borohydride in acetonitrile/water (3:2) at room temperature. The tandem reductive ring cleavage and spiroketalization proceeded, very likely, *via* the dihydroxynitronate ***71*** that, by acidification, converted into a carbonyl derivative which spontaneously cyclized to the spiroketals ***72*** as a mixture of *E*/*Z*-isomers (1:2).

In Scheme 22 1-nitrohexan-5-one ***75*** has been chosen as the reagent for the 5-oxohexyl anion synthon, needed for the synthesis of (*E*)-7-methyl-1,6-dioxaspiro[4.5]decane ***78***. This specific C-6 fragment was obtained by oxidation of commercially available 1-bromo-5-hexane ***73*** with



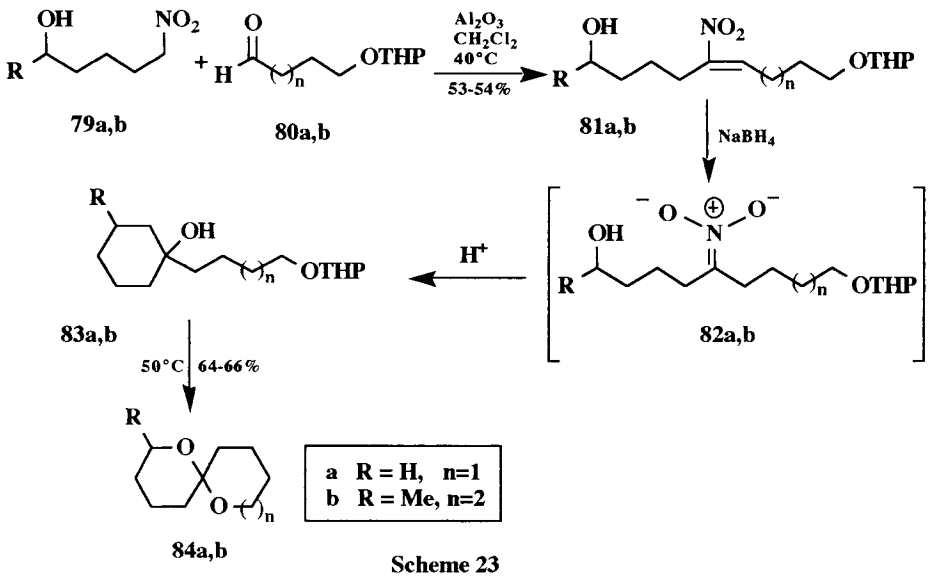
Scheme 22

$PdCl_2/CuCl_2$/benzoquinone and subsequent displacement of bromine with sodium nitrite (refs. 1,24) in DMF. The conjugate addition of ***75*** to an equimolar amount of acrolein was performed on Amberlyst A-21. Under these conditions the synthesis of compound ***76*** was achieved in 78% yield. This was reduced to the corresponding dihydroxy derivative ***77*** by conventional treatment with $NaBH_4$/EtOH. The conversion of ***77*** to a carbonyl derivative and its concomitant spiroketalization was easily performed *via* solvolysis of its *aci*-nitronate in a two-layer solution of aqueous sulfuric acid and pentane. In this case the cyclization occurred, in good yield, giving the single (*E*)-isomer ***78***. This high stereoselectivity in spiroketalization of ***77*** was not unexpected in view of the strong conformational stabilization since the methyl group of a pyranose ring prefers an equatorial position whereas the oxygen atom of the neighboring ring occupies an axial site. This fact depends (ref. 62) on stereoelectronic effects and steric interactions between the oxygen lone pair and the anti bonding orbitals of adjacent bonds (anomeric effect).

As reported above, nitroalkanes have been demonstrated to be very effective precursors for the preparation of spiro[4,4] and spiro[4,5]ketal systems. However, hydroxy-functionalized conjugated nitroolefins can act as immediate precursors for the preparation of spiro[5,5] and spiro[5,6]ketal systems (Scheme 23), by a key step performed by their reduction with sodium borohydride.

In this way 1,7-dioxaspiro[5,5]undecane ***84a***, the major component of the olive fruit fly (*Dacus oleae*) sex pheromone and (*E*)-2-methyl-1,7-dioxaspiro[5,6]dodecane ***84b***, a component of the pheromone of *Andrena haemorrhoa* have been (ref. 63) constructed.

The starting point of these syntheses was the solvent free nitroaldol reaction between protected hydroxy aldheides ***80*** and 5-nitroalcohols ***79*** on alumina at room temperature. In situ dehydration,



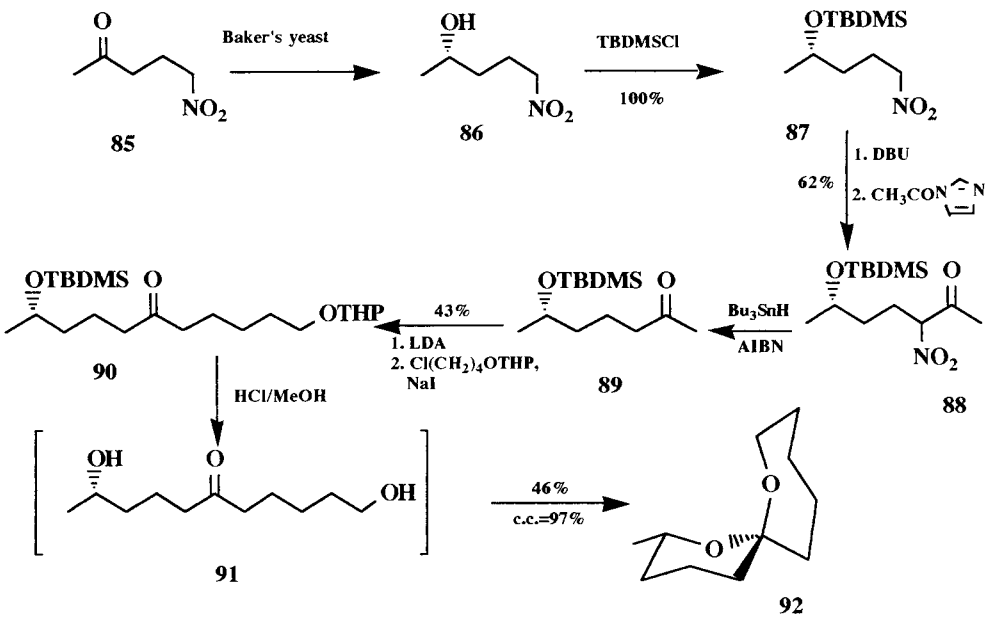
Scheme 23

with addition of CH_2Cl_2 and warming at 40°C, provided, in a one-pot reaction, the nitroalkenes ***81***, which were converted directly into the spiroketals ***84*** by reduction with sodium borohydride in methanol. The tandem reduction-spiroketalization of the nitroalkene ***81*** probably proceeds *via* the nitronate ***82***, that by acidification was converted into a carbonyl which spontaneously cyclized to the hemiketals ***83***. Removal of the tetrahydropyranyl group by heating the acidic mixture at 50°C, afforded, in a one-pot reaction from ***81***, the desired spiroketals ***84*** in 64-66% yields.

Chiral nitro alcohols with primary nitro group are potentially useful chiral building blocks in organic syntheses. Nakamura *et al.* (ref. 64) reported that the reduction of 5-nitro-2-pentanone ***85*** with baker's yeast gave the corresponding (*S*)-alcohol ***86***, enantioselectively. The nitroalkanol ***86*** has been than used as the chiral precursor for the asymmetric synthesis of (2*S*,6*R*)-(-)-2-methy-1,7-dioxaspiro[5.6]dodecane ***92***, a pheromone for *Andrena haemorrhoa* F.

The whole sequence for the total synthesis of ***92*** is shown in the Scheme 24. Protection of the hydroxy group in (*S*)-***86*** with TBDMS was achieved by reacting it with *t*-butyldimethylsilyl chloride at 0°C in the presence of imidazole in DMF. The TBDMS-protected nitro alcohol [(*S*)-***87***], obtained quantitatively, was acetylated by acetylimidazole under the catalyst of DBU in THF. A 1:1 diastereomeric mixture of the acetylated compound, (*S*)-***88***, was obtained in 62% yield. The next step was to eliminate the nitro group from ***88***. In fact, the reaction of (*S*)-***88*** with Bu_3SnH/AIBN (refs. 36,37) gave the TBDMS-protected (*S*)-6-hydroxy-2-heptanone ***89*** in 92% yield. The ω-hydroxybutylation of the methyl group of the compound ***89*** was accomplished by generating the enolate with LDA and reaction with 4-chloro-1-(tetrahydropyranyloxy)butane, in the presence of NaI. The product ***90*** was obtained in 43% yield. Deprotection of ***90*** with 6M HCl in methanol produced the spiroketal ***92*** in 46% yield. The optical purity of the product has been measured to be 97%.

In conclusion it has been demonstrated that nitroalkanes can be considered as useful building blocks for the synthesis of spiro[4.4], spiro[4.5], spiro[5.5], and spiro[5.6]ketal-pheromones.

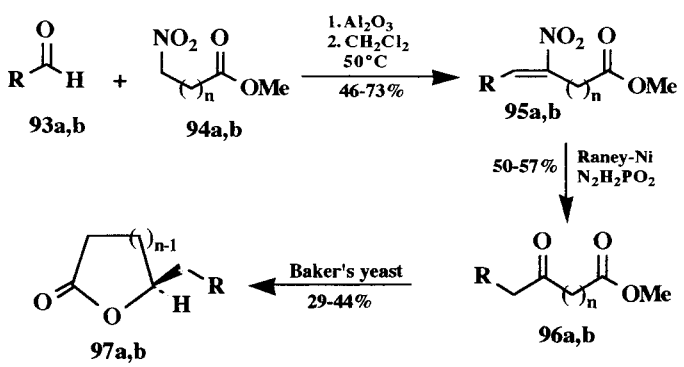


Scheme 24

3.1.5 Lactonic Pheromones

Nitro compounds have also been largely applied for the preparation of important lactones. In one of the next paragraph the preparation of natural medium- and macro-lactones will be reported, while now the syntheses of some lactone-pheromones are describes.

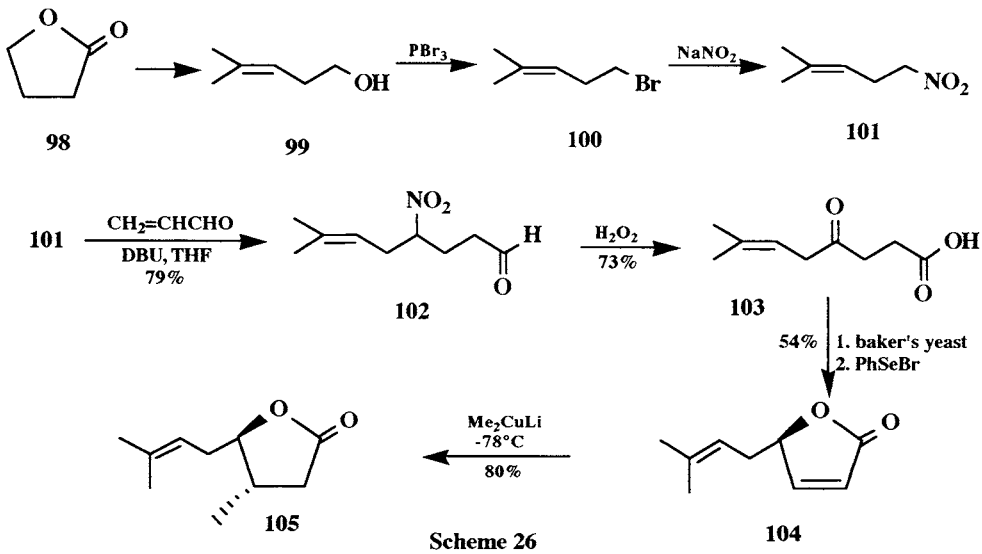
ω-Nitroesters have been used (ref. 65) to obtain (*R*)-(+)-γ-caprolactone ***97a***, the pheromone of *Trogoderma glabrum*, and the (*R*)-(+)-δ-hexadecanolide ***97b***, the queen substance of the oriental hornet *Vespa orientalis*. Nitroaldol condensation of ω-nitroesters ***94*** (Scheme 25) with suitable



Scheme 25

aldehydes ***93***, on the surface of alumina, followed by in situ dehydratation, with CH_2Cl_2 at 50°C, gave the nitroalkenes ***95***. The nitroolefins ***95*** were then converted into the carbonyl derivatives ***96***, by a direct method, with sodium hypophosphite. The lactonization of the keto esters ***96*** was performed with baker's yeast, affording the, enantiomerically pure, lactone ***97***.

Another important lactone pheromone is the natural eldanolide (+)-***105***, (3*S*,4*R*)-3,7-dimethyl-6-octen-4-olide, the pheromone of the male African sugarcane stem borer, *Eldana saccharina* (W.K.). The lactone ***105*** has been obtained (ref. 66) from (Scheme 26) the nitroalkane ***101***, prepared from the butyrolactone ***98*** following the standard method (refs. 1,24). Thus ***101***, after Michael reaction with acrolein, gave 4-nitroalkanal ***102***, which was directly converted to the ketoacid ***103*** with hydrogen peroxide. As illustrated in Scheme 26, the fermenting baker's yeast reduction

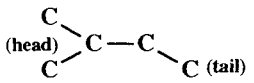


Scheme 26

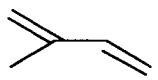
of ***103***, followed by α,β-elimination (PhSeBr), gave the lactone ***104*** in 54% yield. Methylation of ***104*** with Me_2CuLi at -78°C furnished the *R*-(+)-eldanolide ***105*** in 80% yield.

3.2 ISOPRENOIDS

Natural products often possess a carbon framework comprised of units of the five carbon arrangement ***106***. Such compounds are called terpenes. The monomeric unit ***106*** is called isoprenic, because of its relationship to the diene isoprene ***107***, and it is commonly indicated by the symbol C_5. Most terpenes possess a carbon content in multiples of this five carbon arrangement: hemiterpenes (1 x C_5), monoterpenes (C_{10} = 2 x C_5), sesquiterpenes (C_{15} = 3 x C_5), diterpenes (C_{20} = 4 x C_5), sesterpenes (C_{25} = 5 x C_5) and triterpenes (C_{30} = 6 x C_5).

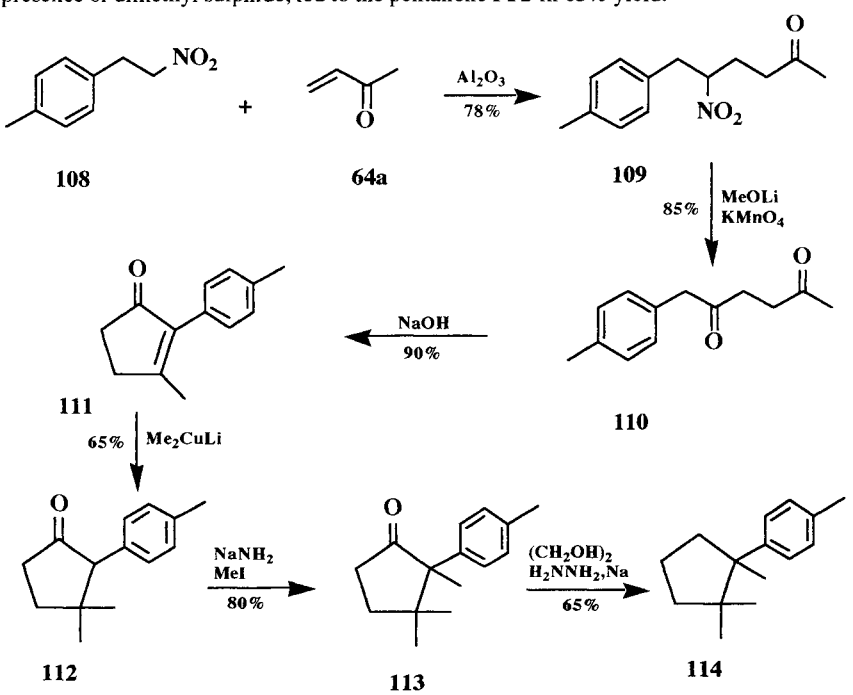


106



107

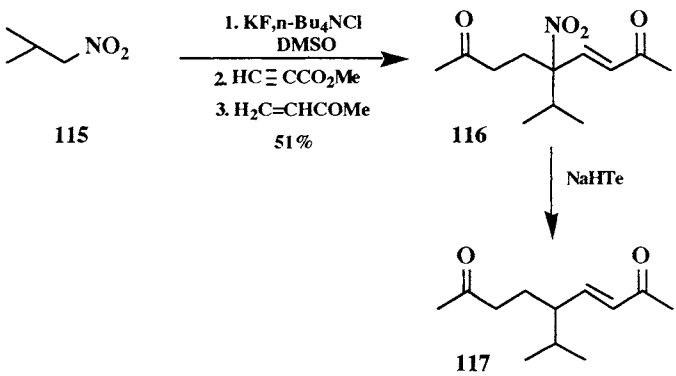
Several isoprenoids have been prepared through nitroderivatives. The sesquiterpene cuparene ***114***, firstly isolated in 1958, can be obtained (refs. 67,68) from phenyl-1-methyl-4-(2-nitroethyl) ***108*** *via* its Michael reaction with the enone ***64a*** (Scheme 27) on alumina surface. The nitro ketone ***109*** furnished by Nef reaction, performed with potassium permanganate, the diketone ***110*** which over base-cyclization, afforded the cyclopentenone ***111*** in 60% overall yield. Thus, 1,4-addition of methyl group to the cyclopentenone system of compound ***111***, effected with lithium dimethylcuprate in the presence of dimethyl sulphide, led to the pentanone ***112*** in 65% yield.



Scheme 27

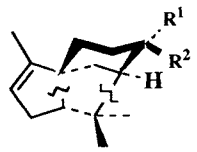
Methylation at the benzylic position of ***112*** yielded ***113*** which could be converted into the cuparene ***114*** by Barton's modification (ref. 69) of the Wolf-Kishner reduction.

Double Michael reaction of 2-methyl-1-nitropropane is the key step (ref. 70) for the synthesis of (±)-norsalanadione ***117***. (-)-Norsalanadione is a terpenoid isolated from aged Buley, Turkish, and Greek tobacco leaves, and it is an attractant for *tabakoshibanmushi*, a pest insect that infests stored foods and tobacco. In addition, norsalanadione is a flavourant and flavour enhacer for tobacco. In Scheme 28, treatment of ***115*** with 3-butyn-2-one and then with methyl vinylketone, in the presence of KF, *n*-Bu_4NCl and DMSO, gave the double Michael adduct ***116***, in 51% yield. Successive reduction of the nitro compound ***116*** with sodium hydrogen telluride (NaHTe) produced (±)-norsolanadione ***117*** in 64% yield.



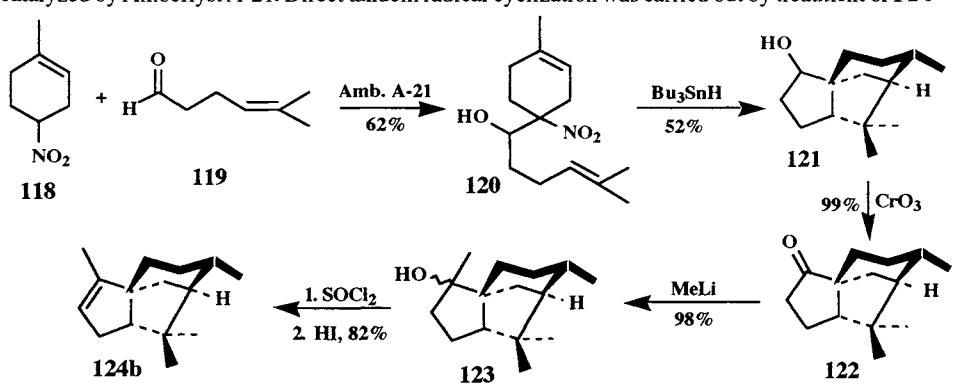
Scheme 28

The tricyclic sesquiterpene Δ^2–cedrene ***124a,*** an isomer of α-cedrene, has been found in vetiver oil. Chen *et al.* (ref. 71) reported a new synthesis of (±)-Δ^2–8–epicedrene ***124b*** *via* tandem



124a R_1=CH_3,R_2=H
124b R_1=H,R_2=CH_3

radical cyclization. As it is well known, the nitro group can active α-carbon to make C-C bonds (refs. 1,16-35) and can be removed by tin hydride (ref. 36) by a radical process. The 1-methyl-4-nitrocyclohexene ***118***, was chosen (Scheme 29) as a starting material. The cyclization precursor ***120*** was readily available in 62% yield from the Henry reaction of ***118*** with 5-methyl-4-hexenal ***119***, catalyzed by Amberlyst A-21. Direct tandem radical cyclization was carried out by treatment of ***120***

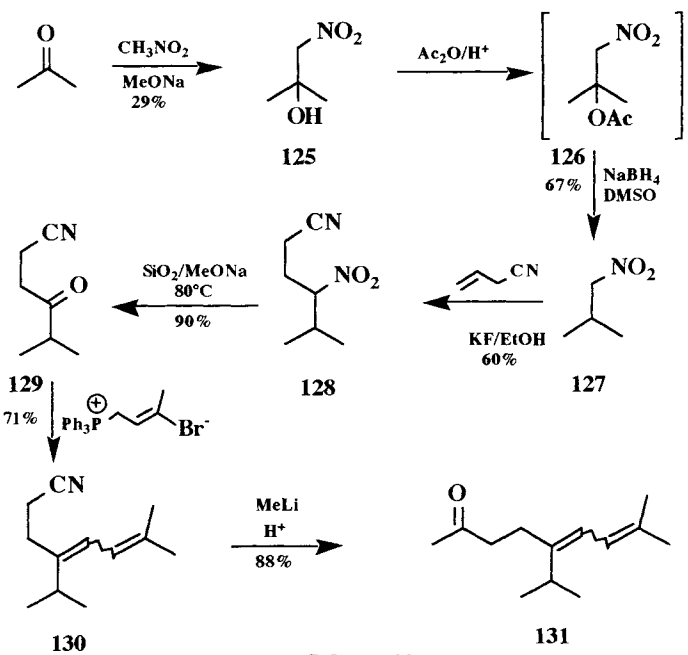


Scheme 29

with tributyltin hydride in the presence of AIBN, in benzene at 80°C. The secondary alcohol ***121*** was isolated in 52% yield. Subsequent oxidation of ***121*** with Jones reagent gave ***122***, as a colorless crystal, in nearly quantitative yields. Treatment of ***122*** with an excess of methyllithium in THF at -78°C, gave the tertiary alcohol ***123*** in 98% yield. Finally, dehydration of ***123*** with thionyl cloride in

pyridine at 0°C, afforded in 84% yield a mixture of hydrocarbons (4:1). Without further separation, the mixture was directly treated with a catalytic amount of 57% HI in benzene at room temperature, to give 88% yield of (±)-Δ^2-8-epicedrene ***124b***.

Lee *et al.* (ref. 72) have described a total synthesis of isosolanone ***131***, starting from acetone and nitromethane (Scheme 30). The compound ***131*** is a member of terpenoids and represents an unique structure in that it apparently violates the isoprene rule. It is a clear, colorless, mobile oil with a faint aroma reminescent of carrots. Moreover, it has been detected in the Burley, Greek tobacco and marijuana. Thus, nitroaldol reaction of acetone with nitromethane, in the presence of MeONa, gave

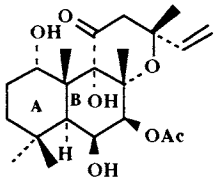


Scheme 30

the nitroalcohol ***125***, which by acetylation, and subsequent sodium borohydride reduction of the resultant acetate ***126***, yielded 2-methyl-1-nitropropane ***127***. Michael reaction of ***127***, under basic conditions (KF/EtOH), with acrylonitrile afforded the nitronitrile ***128***. Nef conversion of the nitro group resulted in the corresponding ketonic nitrile ***129***. Wittig olefination of the carbonyl group with 3-methyl-2-butenyl triphenylphosphonium bromide gave the dienenitrile ***130***, which upon treatment with MeLi, followed by acidic work up, provided isosolanone ***131*** in 88% yield.

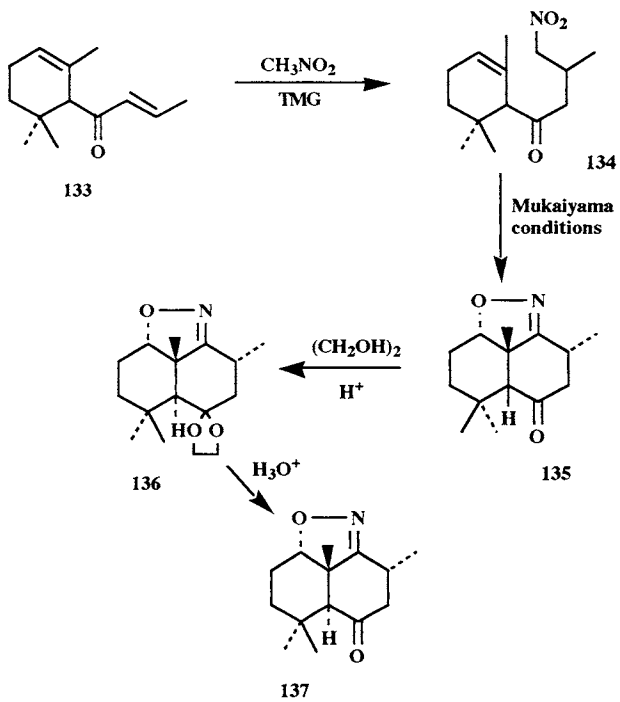
Forskolin ***132***, the major diterpenoid isolated from the Indian plant *Coleus forskohlii*, is a challenging synthetic target owing to its unusual structure with the presence of eight asymmetric centers, the high degree of functionalization and, last but not least, the important biological properties. Pollini *et al.* (refs. 73,74) reported a new strategy for the construction of the AB ring system of ***132*** starting from a nitroalkane ***134***. Thus, commercially available α-demascone ***133*** underwent

tetramethylguanidine-catalysed nitromethane addition to afford a 95% yield of the Michael adduct ***134***. Treatment of ***134***, under Mukaiyama conditions for generating a nitrile oxide (Scheme 31) furnished a 20% yield of ***135***, in which some of the required stereochemistry has been introduced.



132

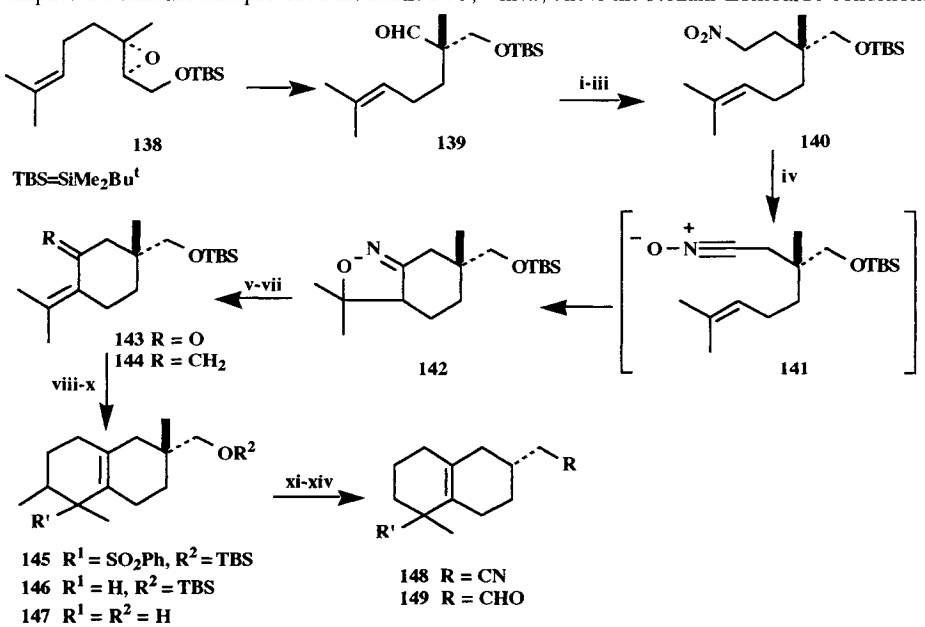
Acetalization of ***135*** under standard conditions proceeded with concomitant epimerization giving ***136***



Scheme 31

in 55% yield. Removal of the acetal moiety, by the usual aqueous acid treatment, led quantitatively to the *trans*-fused ketone ***137***. So a highly functionalized AB ring system along the route to forskolin ***132*** has been synthesized.

Recently the total enantioselective synthesis of the marine sesquiterpene nanamoal ***149*** has been published (ref. 75). The terpene ***149*** is a fragrant sesquiterpene aldehyde, isolated from the dorid nudibranch *Acanthodoris nanaimoensis*, and in Scheme 32 is depicted its preparation using nitro compounds. In fact, reaction of the epoxy silyl ether ***138*** (prepared by Sharpless asymmetric epoxidation of geraniol, using *L*-(+)-diethyl tartrate, followed by silylation) with 2 equiv. of methylaluminium bis(4-bromo-2,6-di-*tert*-butylphenoxide) proceeded *via* configuration inversion to provide the aldehyde ***139*** with (*S*)-configuration in 97% yield (95% ee). The quaternary stereogenic center present in ***149*** was thus constructed. On sequential addition of nitromethane, acetylation and sodium borohydride reduction, ***139*** was converted into the nitroalkene ***140*** in 55% overall yield. Treatment of ***140*** with *p*-chlorophenylisocyanate and triethylamine gave quantitatively the isoxazoline ***142***, *via* the [3+2] dipolar cycloaddition of alkenyl nitrite oxide ***141***. Subsequent reductive hydrolysis of ***142*** followed by immediate exposure of the resulting β-hydroxy ketone to a catalytic *D*-camphor-10-sulfonic acid provided the enone ***143***, which, under the Nozaki-Lombardo conditions,



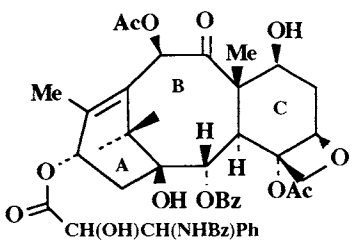
Scheme 32

Reagent and Conditions: i, $MeNO_2$, KF; ii, Ac_2O, DMAP; iii, $NaBH_4$, 55%; iv, *p*-ClC_6H_4NCO, Et_3N, benzene, 100%; v, H_2, Raney Ni; vi, CSA; vii, CH_2Br_2, Zn, $TiCl_4$, 48%; viii, $PhSO_2CH{=}CH_2$, 43%;ix, 5% Na-Hg, MeOH; x, Bu^n_4NF, THF, 49%; xi, *p*-TsCl, Py; xii, NaCN, DMSO, 86% for 2 steps; xiii, Bu^i_2AlH, (1:1),70%; xiv, $NaBH_4$, 73%.

was methylated affording the exocyclic diene ***144*** in 48% overall yield from ***142***. On heating a solution of ***144*** and vinylsulfone in benzene at 160°C, the Diels-Alder adduct ***145*** was obtained in

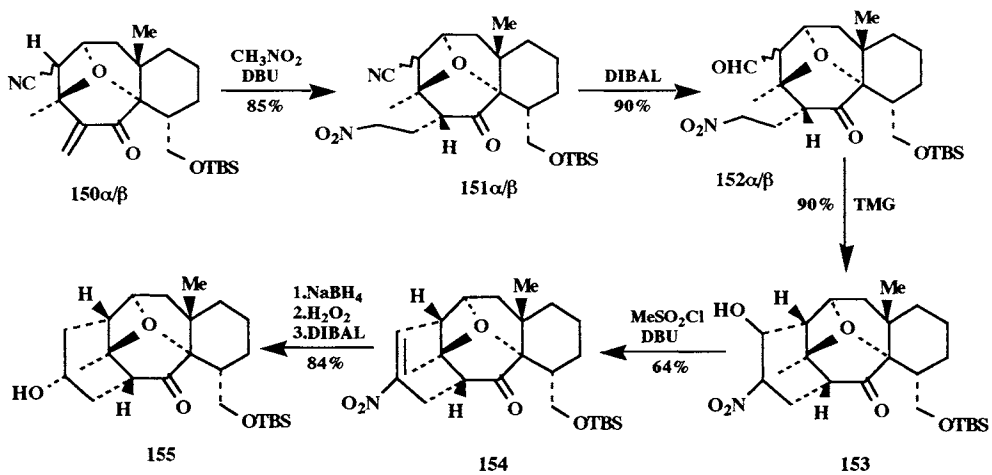
43% yield as an inseparable diastereomeric 1:1 mixture. Desulfonylation with sodium amalgam followed by desilylation of the resulting ***146*** yielded the alcohol ***147***, which was converted into the cyanide ***148*** by a standard procedure. Finally, reduction of***148*** with diisobutylaluminium hydride, followed by acidic work-up, afforded nanamoal ***149*** in 70% yield.

The nitroaldol (Henry) reaction (ref. 28) has been recently used for the formation of the A-ring of taxol. The story of taxol, a molecule that received much attention in the early 1990s, goes back to ancient times. Julius Caesar mentions in his *Gallic Wars* that Catuvolcus, a chieftain of the Eburones, committed suicide by taking extracts from the yew tree. The modern history of this natural product began in 1962, when A. Barclay collected the bark from *Taxus brevifolia*. Taxol's unique biological action stimulated a renewed bark interest in taxol as a potential drug candidate. The problem of procuring adequate supplies of taxol became more acute when environmentalists raised objections to the destruction of the ancient forest. A promising solution is the discovery of new synthesis of this compound and its derivatives. In this context Magnus and Pye (ref. 76) have reported (Scheme 33)



(Taxol)

the formation of the A-ring of the taxol *via* the Henry reaction. Thus, the exomethylene ketones ***150α/β*** were treated with nitromethane and DBU/CH_2Cl_2, to give the conjugate addition adducts ***151α/β*** (85%) as a 2:1 mixture at C-11, and a single stereoisomer at C-1. The epimeric nitrile



Scheme 33

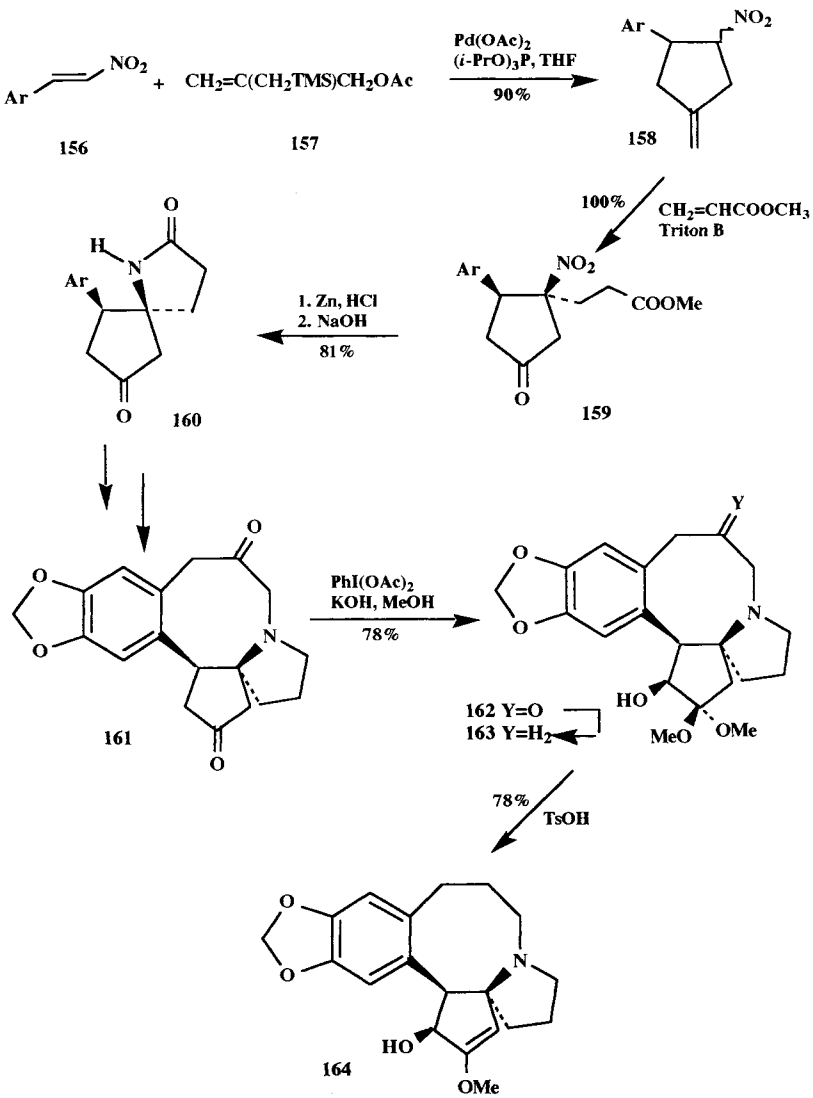
151α/β were readily separated, and each reduced with DIBAL/CH_2Cl_2 at -78°C, affording the nitroaldehydes ***152α/β*** (90%). Merely stirring a solution of ***152α/*** in CH_2Cl_2 with Et_3N produced the nitroalcohol ***153*** (100%) as a single stereoisomer. The compound ***152β*** did not cyclize under these conditions (no epimerization at C-11). However, treatment of ***152α/β*** with TMG(cat.)-CH_2Cl_2 resulted in C-11 epimerization and cyclization leading to ***153*** in 90% yield. However, the nitro alcohol ***153*** need not be isolated, but can be directly dehydrated ($MeSO_2Cl$-DBU) affording ***154***. Treatment of the α,β-unsaturated nitro compound ***154*** with $NaBH_4$ followed by a Nef-type reaction (H_2O_2), and DIBAL-H reduction gave the required C-13 alcohol ***155*** (84%) with the correct (α)-stereochemistry. These results show how the A-ring of the taxanes can be well constructed by nitroaliphatic derivatives.

3.3 ALKALOIDS

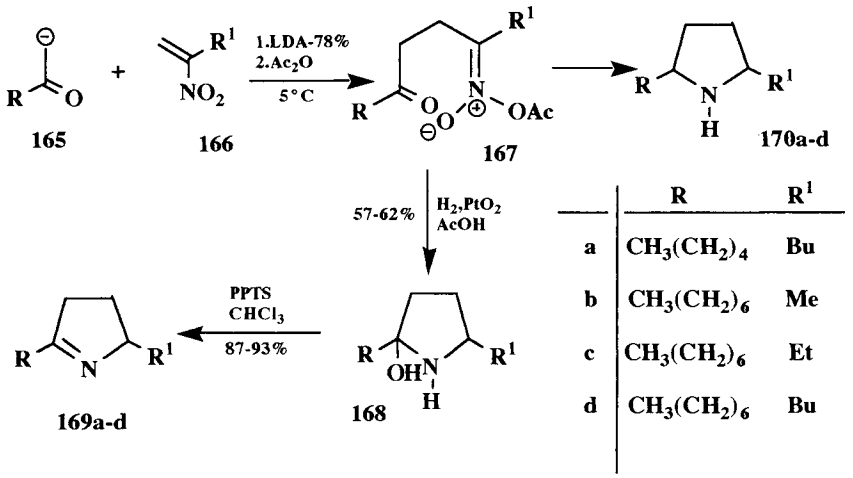
Akaloids (ref. 77) are cyclic compounds, containing nitrogen in a negative oxidation state, of great structural complexity which occur in plants. In spite of the diverse nature of alkaloid structures, two structural units, i.e. fused pyrrolidine and piperidine rings in different oxidation states, appear as rather common denominators.

Different total syntheses of alkaloids have been reported, in which a nitrogroup occupies a central role. In this context, an important example is the total synthesis of(±)-cephalotaxine ***164***, which is the predominant alkaloid of the *cephalotaxus* species. The compound ***164*** has attracted much attention from synthetic chemists due to its unique structural features and the antileukaemic activity of its ester derivatives. An important synthesis of ***164*** starts (ref. 78) from the (Scheme 34) commercial nitrostyrene ***156***, which, by palladium-mediated methylenecyclopentane annelation, gave the methylenecyclopentane derivative ***158*** as a diastereoisomeric mixture (8:2), in 90% yield. Michael addition of the isomeric mixture of ***158*** to methyl acrylate produced the nitroester ***159*** as a single stereoisomer. Reduction of the nitro group with zinc, in ethanolic HCl, followed by treatment with alkali and oxidative cleavage of the resulting lactam (OsO_4-$NaIO_4$) afforded the ketolactam ***159***. The compound ***159***, after several steps, was converted into the ketolactam ***161***. The conversion of ***161*** with $PhI(OAc)_2$, followed by Red-Al [Aldrich] reduction and then treatment of the hydroxy acetal ***163*** with TsOH in THF afforded (±)-***164*** in 21% overall yield (18 steps) from nitrostyrene.

In the same year Yoshikoshi *et al.* (ref. 79) reported the synthesis of some ant venom alkaloids ***169a-c*** and ***170a,b,d***, isolated from the genera *Solenopsis* and *Monomorium*, *via* nitroalkanes (Scheme 35). Ketone enolates ***165***, generated with LDA in THF, react with a variety of conjugated nitroalkenes ***166*** producing the acetyl nitronates ***167***. Hydrogenation of ***167***, over 5% Rh on Al_2O_3 in MeOH, directly produced 2,5-dialkyl pyrrolidines ***170***, while hydrogenation over PtO_2 in acetic acid yielded 2,5-dialkyl-2-hydroxypyrrolidines ***168***. The conversion of ***168*** to ***169*** was easily performed by treatment of the former with PPTS in $CHCl_3$ at 0°C with excellent yields of the latter.

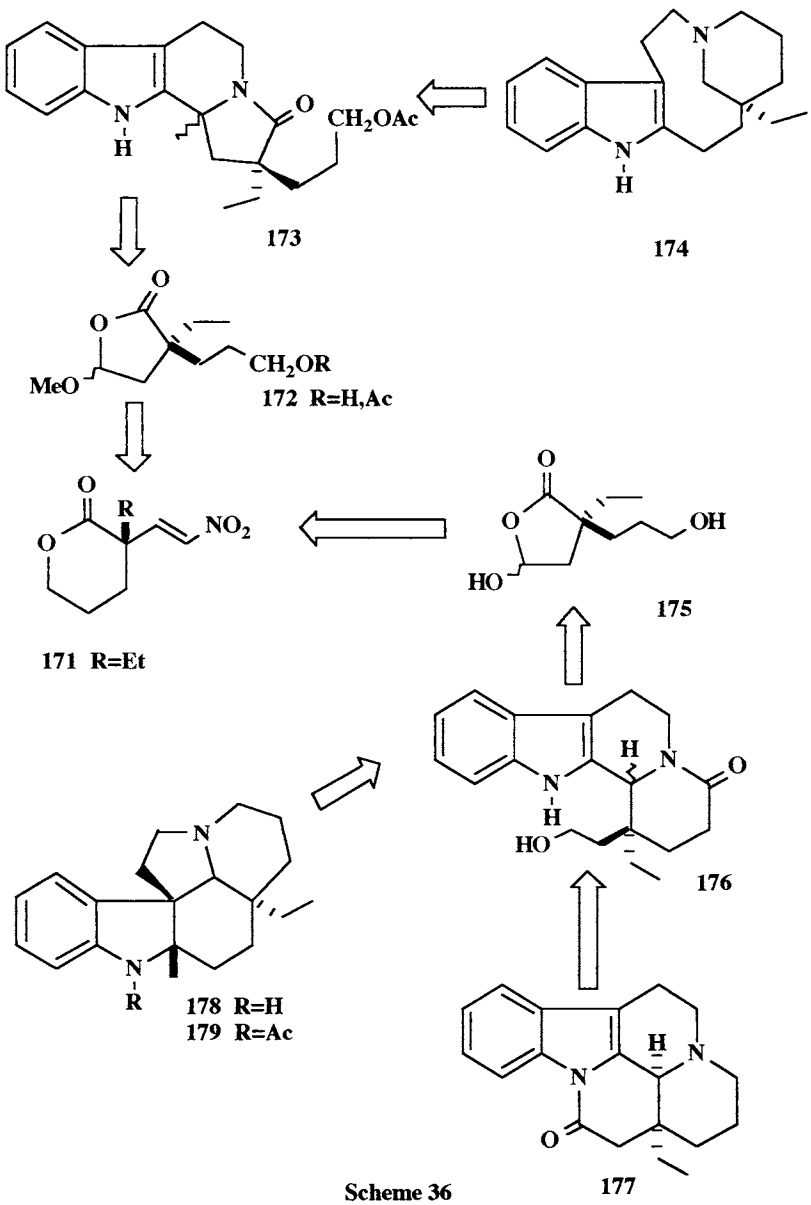

Ar
NO2
+ CH2=C(CH2TMS)CH2OAc
156
157
Pd(OAc)2
(i-PrO)3P, THF
90%
158
100%
CH2=CHCOOCH3
Triton B
159
COOMe
1. Zn, HCl
2. NaOH
81%
160
PhI(OAc)2
KOH, MeOH
78%
161
162 Y=O
163 Y=H2
HO
MeO
OMe
78%
TsOH
164

Scheme 34



Scheme 35

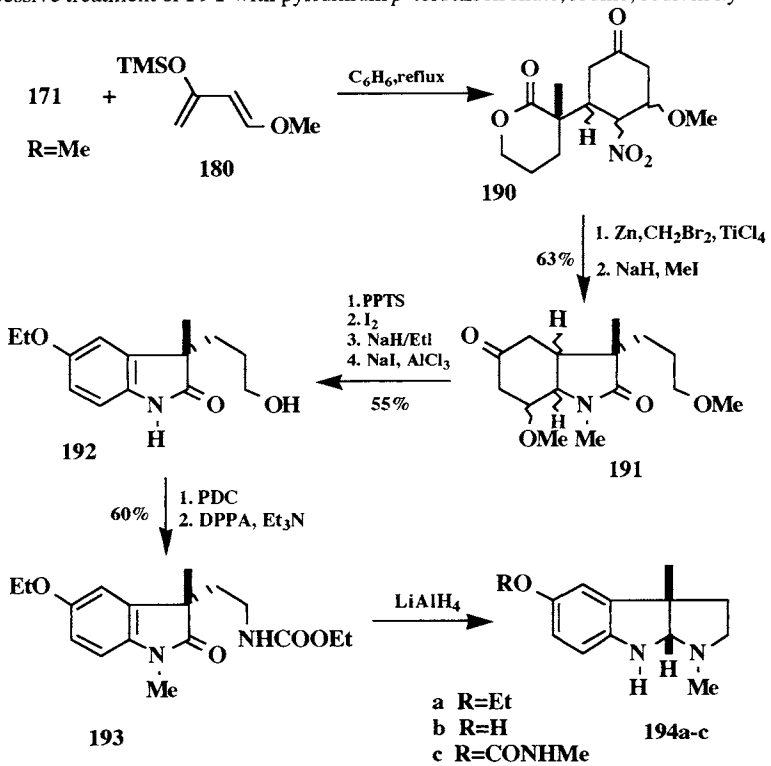
The nitroalkene ***171*** has been demonstrated to be a very important chiral building block for different natural products. Firstly ***171*** was employed (ref. 80) to prepare optically active diterpenoids of C-20β series. Soon after, this nitroalkene has been used (refs. 81-83) in the synthesis of important alkaloids. In Scheme 36 the syntheses of (+)-quebrachamine ***174***, (-)-aspidospermidine ***178***, (+)-demethoxyaspidospermine ***179***, (three *Aspidosperma* alkaloids), and (-)-eburnamonine (an *Hunteria* alkaloid), starting from (*S*)-(-)-**171** are depicted. The alkaloid ***174*** was prepared *via* the acetal ***172*** obtained by Nef rection ($TiCl_3$) of ***171*** (R=Et). Condensation of ***172*** with tryptamine yielded ***173*** in epimeric mixture. Reduction of ***173*** ($LiAlH_4$/THF), mesylation, and reduction with Na-EtOH in liquid ammonia furnished the (+)-quebrachamine ***174***. The reductive denitration of ***171*** in DMF afforded the hemiacetal ***175***. Reduction of ***175***, followed by acetalyzation, condensation with tryptamine produced an α/β mixture (1:1) of ***176***. (-)-Eburnamonine ***177*** has then been obtained by treatment of ***176*** with boron trifluoride etherate (40°C). Finally, boron trifluoride etherate treatment, at 100-110°C, followed by reduction with $LiAlH_4$, yielded the alkaloids ***178*** . Acetylation of ***178*** gave (+)-demethoxyaspidospermine ***179***.



Scheme 36

The same nitroalkene ***171*** (R=Me) has been described (ref. 83) to be very useful to prepare the (-)-physostigmine ***194c***, a principal alkaloid of the calabar bean, clinically used for glaucoma, myastenia gravis, as a antidote in organophosphate poisoning and for treating Alzheimer's disease. The Diels-Alder reaction of optically active nitroolefin ***171*** with the Danishefsky diene ***180*** afforded a diastereomeric mixture of ***190*** in good yield. Though a stereochemical control at the newly created asymmetric carbons was poor, a complete regioselectivity was observed in this cycloaddition.

Reaction of ***190*** with the Nozak reagent and methylation of the obtained hydroxy-free group gave ***191***. Successive treatment of ***191*** with pyridinium *p*-toluensolfonate, iodine, sodium hydride-ethyl

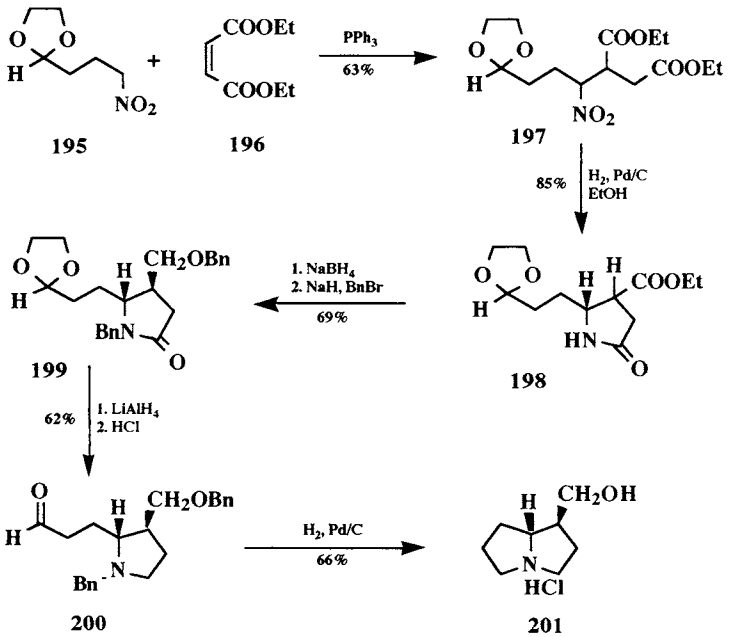


Scheme 37

iodide, and aluminium chloride-sodium iodide, yielded the alcohol ***192***. Oxidation of ***192*** with pyridinium dichromate (PDC) converted the hydroxy group to a carboxylic acid which, by Curtius degradation, afforded the (-)-eserethole ***194a***. Since ***194a*** can be converted to (-)-physostigmine ***194c*** *via* (-)-eseroline ***194b*** (ref. 84), the total synthesis of optically pure (-)-eserethole ***194a*** constitues a formal synthesis of naturally occuring (-)-physostigmine ***194c***.

The nitroacetal ***195*** has been employed (ref. 85) to synthesize (±)-trachelanthamidin ***201***, an alkaloid isolated from *Eupatorium maculatum*, *Heliotropium strygosum*, and *Heliotropium*-Arten. Michael addition (Scheme 38) of ***195*** to diethylfumarate ***196*** afforded ***197*** (63% yield), which, by hydrogenation (Pd-C), gave the pyrrolidine ***198***. Reduction ($NaBH_4$) of the ester, followed by the double protection of the hydroxy and NH groups as benzyl derivatives, afforded ***199*** in 69%. Reduction of the amide ($LiAlH_4$) and removal of the acetal gave the trachelanthamidin precursor ***200***, which, by H_2-Pd-C, yielded the desired ***201***.

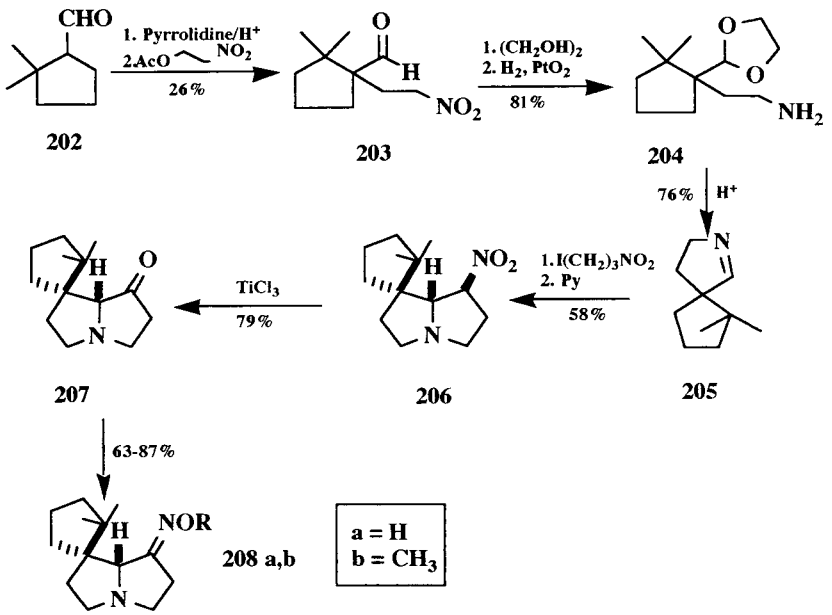
Hutchinson *et al.* (ref. 86) elaborated the synthesis of spiropentanopyrrolizine oxime ***208a*** (alkaloid **222**) and the corresponding *O*-methyl oxime ***208b*** (alkaloid **236**), two alkaloids isolated in the eighties from skin extracts of the Panamanian poison frog.



Scheme 38

The route (Scheme 39) involved the synthesis of nitropolyzonamine ***206***, a known millipede alkaloid, from ***202*** in five steps. After conversion of ***206*** to the ketone ***207*** (Nef reaction), the oxime ***208a*** and the *O*-methyl oxime ***208b*** were obtained.

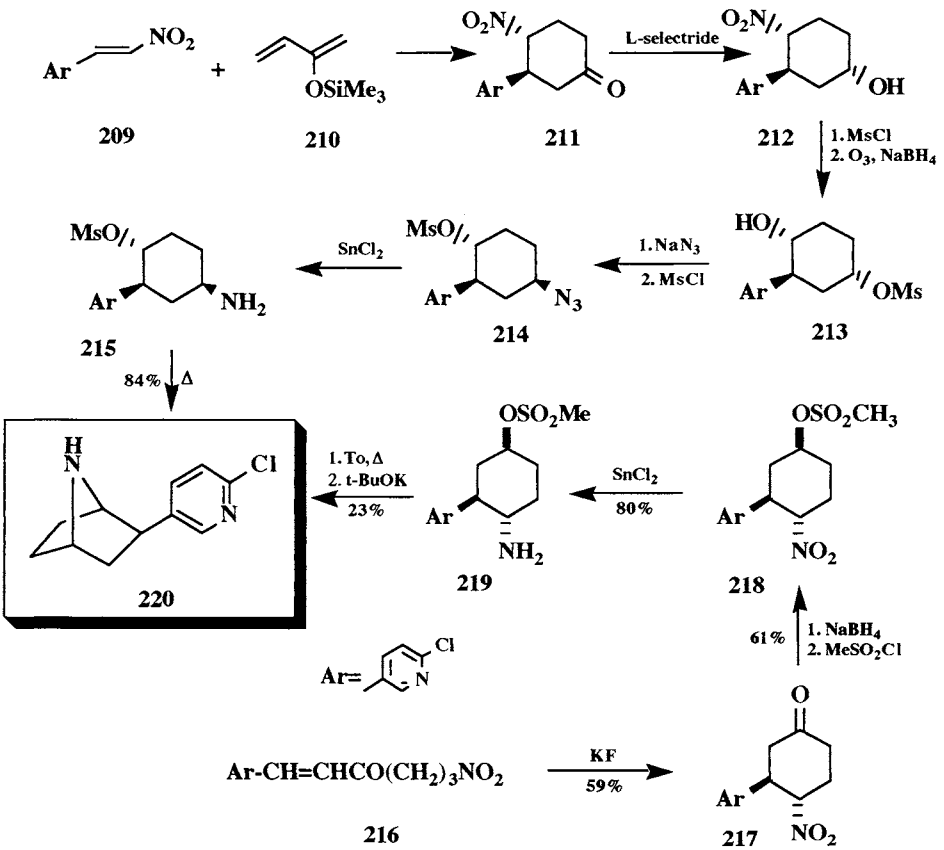
In the same year (1994) Pollini's group (ref. 87) and Szantay's group (ref. 88) published the total synthesis (Scheme 40) of epibatine ***220***, an alkaloid isolated from Equadorian poison frog, *Epipedobates tricolor*.



Scheme 39

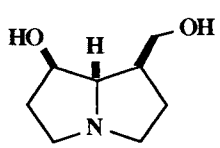
The approach of the first group is centered on the tandem Michael reaction occurring by mixing the nitroolefin ***209*** with ***210*** yielding the nitrocyclohexanone ***211***, a key intermediate for a stereocontrolled synthesis of the alkaloid ***220***. Transformation of ***211*** to the epibatine requires a five steps sequence, including: a) *L*-Selectride reduction; b) mesylation; c) direct conversion of the nitro group into an hydroxyl group with the complete retention of configuration at the involved center; d) azide formation; e) reduction of the azide to the corresponding amine with $SnCl_2$; f) production of epibatine ***220*** by heating the compound ***215*** in chloroform.

The route of Szantay's group utilizes the nitrocompound ***216***, easily available in a few steps from nitromethane, which by an internal Michael addition furnished the cyclohexanone derivative ***217***. Reduction of the keto group, followed by mesylation ***218*** and subsequent reduction of the nitro group gave the amine ***219***, which on heating resulted in the epimer of epibatidine. On boiling the latter compound in *t*-butanol and in the presence of potassium *t*-butoxide, epimerization occurred and the racemic epibatidine ***220*** was obtained.



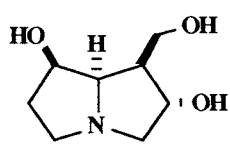
Scheme 40

The hetero Diels-Alder cycloaddition constitutes an important class of reactions for the synthesis of alkaloids due to its ability to construct funcionalized, heterocyclic rings with up to four



221

(-)-hastenecine

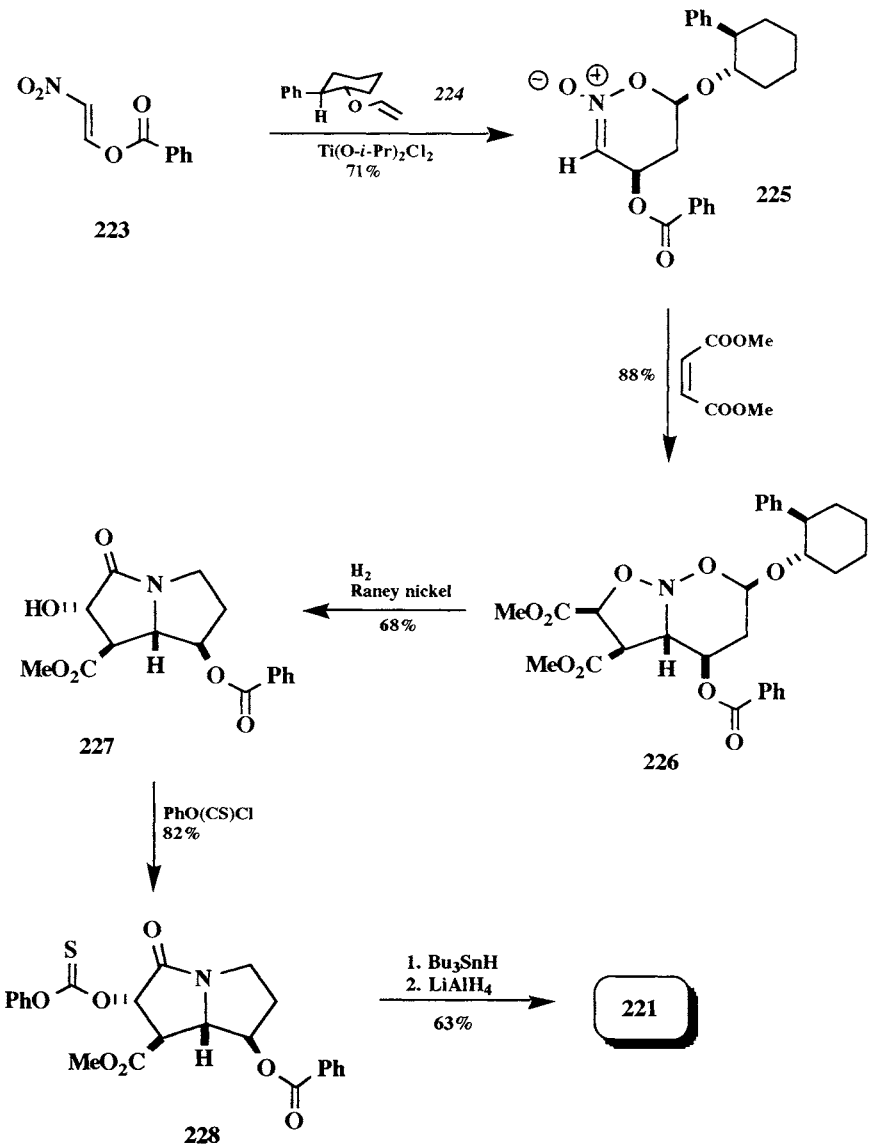


222

(-)-rosmaricenine

contiguous stereogenic centers (ref. 89). Over the past few years Denmark *et al.* (ref. 90) have extensively developed the use of nitroalkenes as heterodienes in [4+2] cycloaddition reactions. These cycloadditions have been shown to proceed with high diastereoselectivity utilizing chiral vinyl ethers.

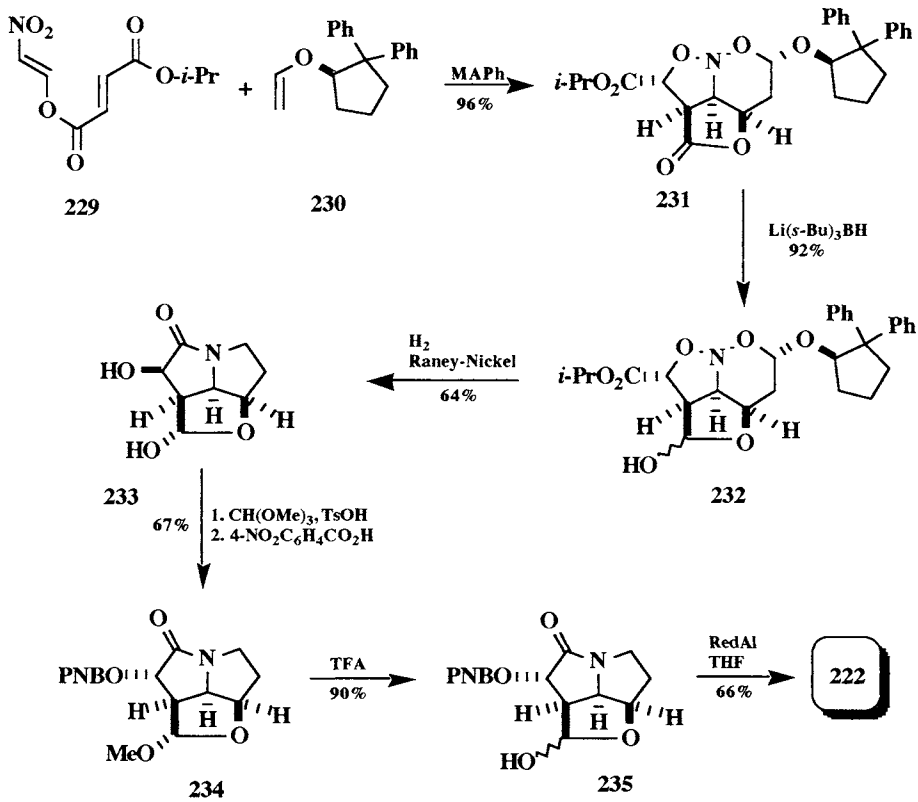
This behavior provides access to important natural alkaloids such as (-)-hastanecine ***221*** and (-)-rosmarinecine ***222***. (-)-Hastanecine is the necine base of hastacine which was isolated from *Cacaliahastata*, family Compositae (tribe Seniocioneae). The synthesis of (-)-hastanecine proposed by Denmark *et al.* (ref. 91), proceeds through a sequential inter [4+2]/inter [3+2] cycloaddition as the key reaction (Scheme 41).



Scheme 41

The benzoyloxy nitroalkene ***223*** was employed as a diene in Lewis acid-promoted cycloaddition with the chiral vinyl ether ***224***.

The use of titanium diisopropoxide dichloride as the Lewis acid afforded the nitronate (+)-***225*** with high selectivity. The nitronate ***225*** was found to be a reactive dipole which combined with dimethyl maleate at room temperature to afford nitroso acetal ***226*** in 88% yield as a single diastereomer. The cleavage of nitrosoacetal ***226***, with Raney nickel (W-2) at 260 psi of hydrogen, afforded the α-hydroxy lactam (-)-***227*** in 72% yield.



Scheme 42

Lactam (-)-***227*** contains all the stereocenters and functionality needed for (-)-hastanecine. In fact, it is over-functionalized for this particular target and the second half of the synthesis involves deoxygenation at C(1), *via* activation of the alcohol, with Bu_3SnH, and reduction of the amide and the esther with lithium aluminium hydride.

In the Scheme 42 is detailed the synthesis of (-)-rosmarinecine ***222*** in which the tandem [4+2]/[3+2] nitroalkene cycloaddition reaction, to generate substituted pyrrolizidine ring, with high degree of stereocontrol, is employed.

(-)-Rosmarinecine was isolated from *S. rosmarinifolius* Lin. Its synthesis reported (ref. 92) in Scheme 42 starts from the nitroalkene ***229*** which was subjected to the tandem [4+2]/[3+2] cycloaddition sequence using methylaluminum bis(2,6-diphenylphenoxide) (MAPh) as the promoter. The reaction of ***229*** with 3 eq. of the chiral vinyl ether (-)-***230***, to afford the nitrosoacetal ***231*** in 96% yield and an overall 25/1, *exo/endo* ratio of diastereomers. The acetal ***231*** was then reduced very selectively with lithium tri(*sec*-butyl)borohydride to afford the lactol (-)-***232***. The lactol (-)-***232*** was then subjected to hydrogenolysis conditions leading to the tricyclic α-hydroxy lactam-lactol (+)-***233***. After protection of both hydroxy groups in ***233*** as the methyl ether and as 4-nitrobenzoate, respectively, the compound ***234*** was obtained with 67% yield and with inversion of configuration at C(6).

The completion of the synthesis of (-)-rosmarinecine required the deprotection of the methyl acetal (+)-***234*** and the reduction of the resulting lactol (+)-***235***.

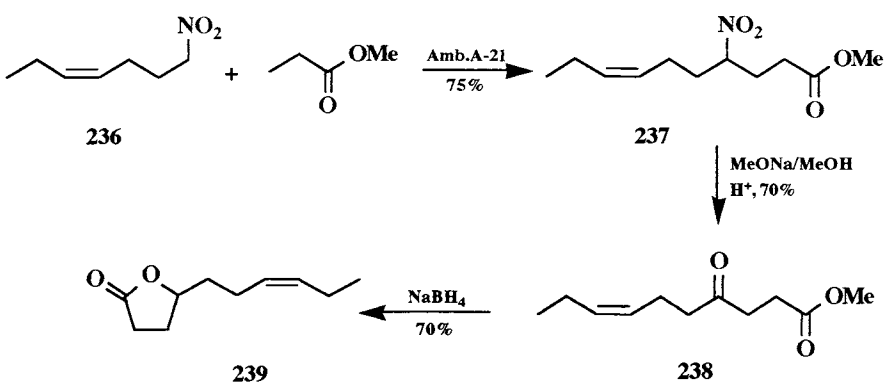
Thus, the synthesis of (-)-rosmarinecine has been accomplished in eight steps and in 14.8% overall yield. Moreover, all the stereocenters were installed in a single transformation with high selectivity clearly demonstrating the utility of the tandem [4+2]/[3+2] cycloaddition strategy, involving nitroalkenes.

3.4 LACTONES

This section deals with the use of the nitrocompounds as precursors of different lactone-skeletons which are often found as natural products. The synthesis of some lactonic pheromones is reported, while the preparation of other lactones (non pheromonic) will be described next.

3.4.1 γ-Lactones

The lactone skeleton has been often found in natural products. Nitroalkanes have been demonstrated

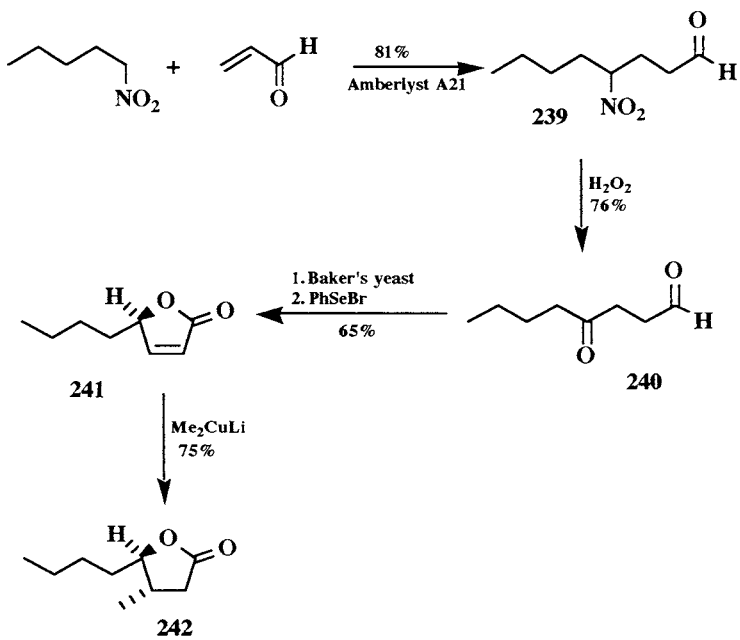


Scheme 43

to be very useful for the synthesis of these kind of compounds, through two key reaction steps: *(i)* C-C bond-forming reaction; *(ii)* conversion of the nitro group into a carbonyl (Nef reaction).

An example coming from our laboratory (ref. 93) is the synthesis of γ-jasmolactone ***239***. The lactone ***239*** is one of the most important constituents of the essential oil of jasmin flowers (*Jasminium grandiflorum*). Conjugate addition (Scheme 43) of *(Z)*-7-nitro-3-heptene ***236*** to methyl acrylate, followed by Nef reaction to the nitro esther ***237***, afforded the keto esther ***238***, reduction of which ($NaBH_4$) yielded the lactone ***236*** (37% overal yield).

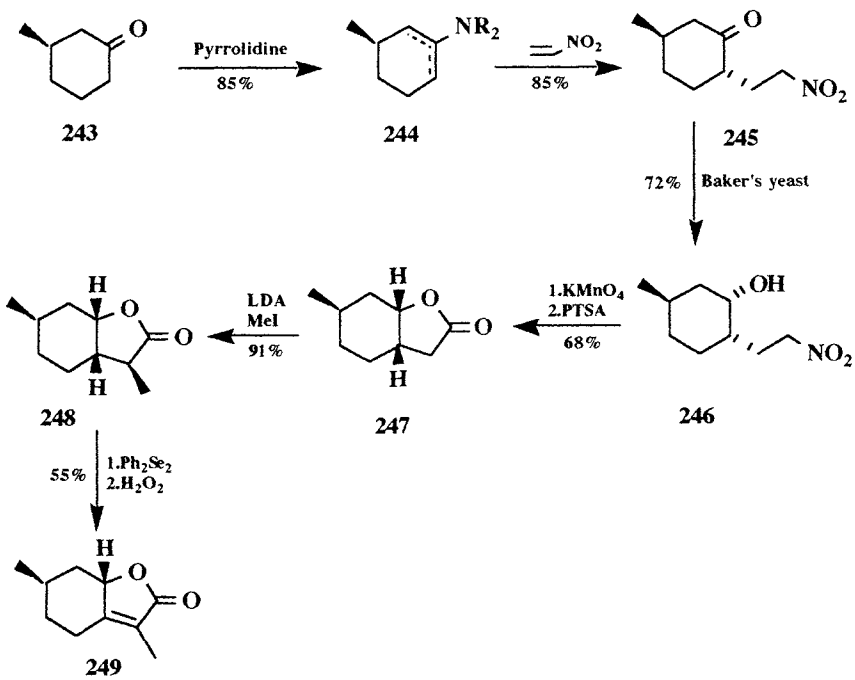
Another representative example is the synthesis of (+)-*trans* whisky lactone ***242*** following (Scheme 44) the synthetic sequence of the natural eldanolide ***105*** (see Scheme 26) (ref. 66).



Scheme 44

During the synthesis of optically active condensed γ-lactones, a new method for the preparation of (+)-isomintlactone ***249***, isolated from a sample of american peppermint oil in 1980, has been discovered (ref. 94). *(R)*-3-methylcyclohexanone ***243*** was chosen as the starting material. The pyrrolidino enamine of the 3-methylcyclohexanone ***244*** was reacted with nitroethylene, affording the nitro ketone ***245***. This latter compound was prepared by the well-known Michael-type addition of nitro-olefins to enamines, followed by hydrolysis of the resulting nitroalkylated enamines. Baker's yeast reduction of the carbonyl yielded the alkanol ***246***. Nef reaction of the nitro group, and

successive acidic work-up converted the nitroalcohol ***246*** to the condensed lactone ***247***. Methylation of ***247***, then, α-phenylselenylation and oxidation of the obtained ***248***, yielded (+)-isomintlactone.



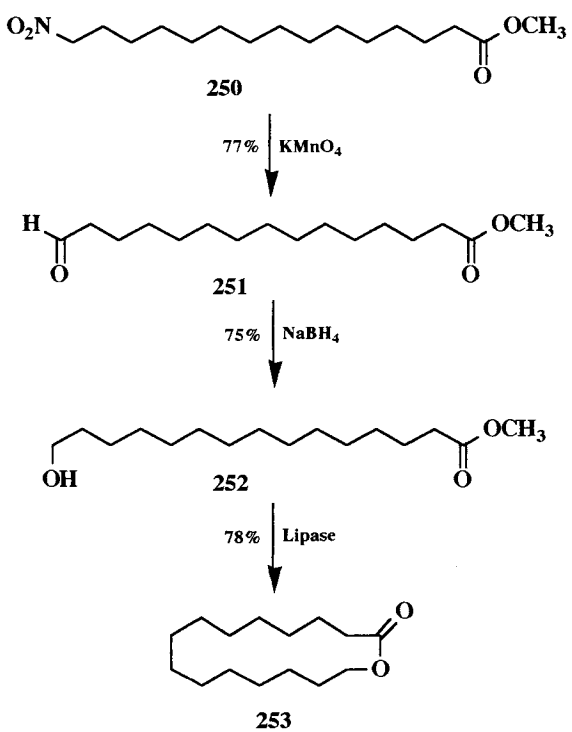
Scheme 45

3.4.2 Medium and Macro Ring Lactones

Medium (those having a ring size in the range 8 to 11) (ref. 95) and macro ring compounds are becoming increasingly important in organic chemistry, as they are contained in an ever-growing number of natural products. Hydrocarbons, as well as heterocyclic compounds (ethers, lactones, amines, amides) have been isolated and a number of their syntheses, *via* nitrocompounds have been published.

Thus, exaltolide ***253***, an important perfume isolated from angelica root oil, has been prepared (ref. 96) starting from the easly available ω-nitroester ***250*** (Scheme 46). Conversion of the nitro group to an aldehyde (Nef reaction) yielded the methyl-15-oxopentadecanoate ***251*** which, by selective reduction ($NaBH_4$), gave the hydroxy ester ***252***. Cyclization of ***252***, performed by Lipase P in benzene, afforded, in 45% overall yield, exaltolide ***253***.

(±)-Phoracantholide ***257a***, a 10-membered lactone isolated from metasternal secretion of the eucalypt *Phoracantha synonyma*, and (±)-recifeiolide ***257b***, a 12-membered lactone isolated from *Cryptolestes ferrugineus*, have been prepared (ref. 97) from α-nitrocycloalkanones (Scheme 47). Thus, $C_{\alpha'}$ methylation of the nitro ketones ***254*** with methyl iodide/LDA, furnished the methylated

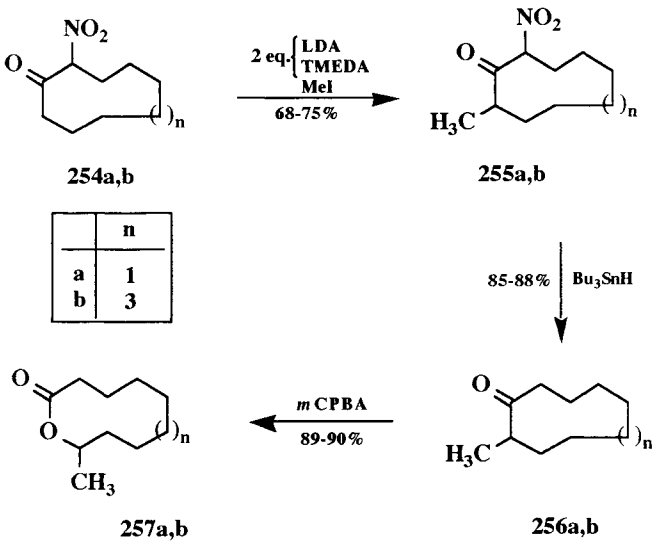


Scheme 46

products ***255***. Subsequent denitration, with tributyltin hydride, afforded compounds ***256*** in 85-88% yield. Bayer-Villiger oxidation of ***256*** provided the racemic lactones ***257a,b*** in 89% and 90% yields, respectively (57% and 54% overall yield).

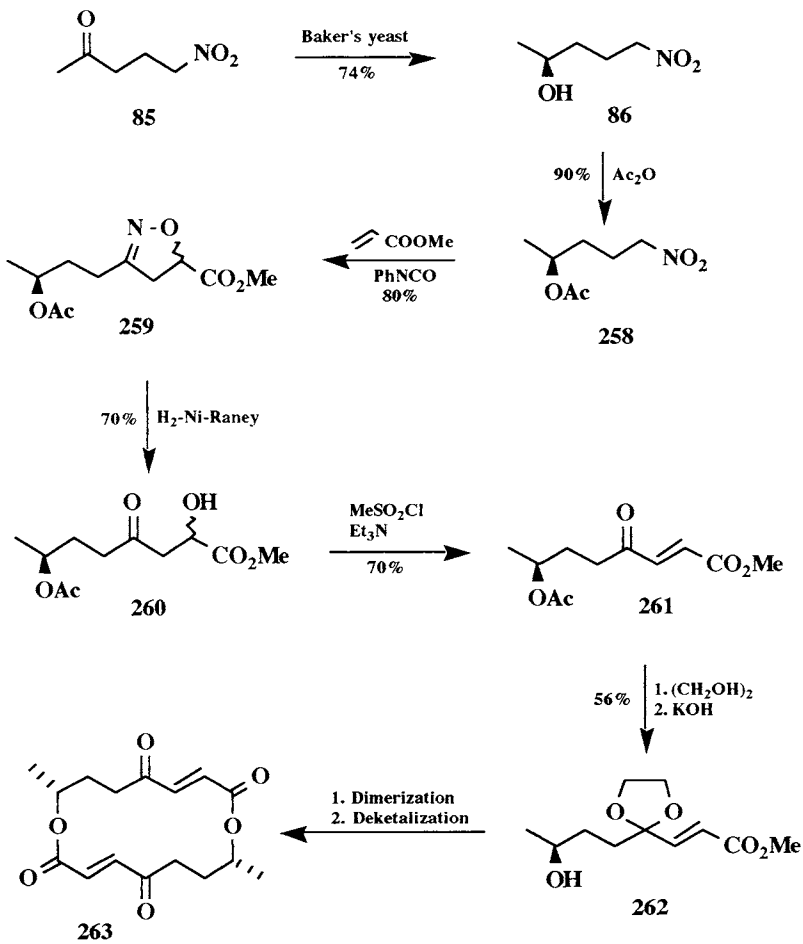
An interesting synthesis of (-)-pyrenophorin ***263***, starting from the known (See Scheme 20) nitro ketone ***85***, has recently been published (ref. 98). The dilactone ***263*** is an antifungal antibiotic produced by the plant pathogenic fungi *Pyrenophora avenae* and *Stemphylium radicinum*. Thus, as summarized in Scheme 48, the required eight carbon atom skeleton ***262*** (precursor of the natural target ***263***) has been constructed by coupling of two fragments of five and three carbon atoms

(through a dipolar [3+2] cycloaddition): 1) the starting fragments ***85***, which incorporates both the primary nitro group as nitrile oxide precursor, and 2) a suitably placed prochiral carbonyl group



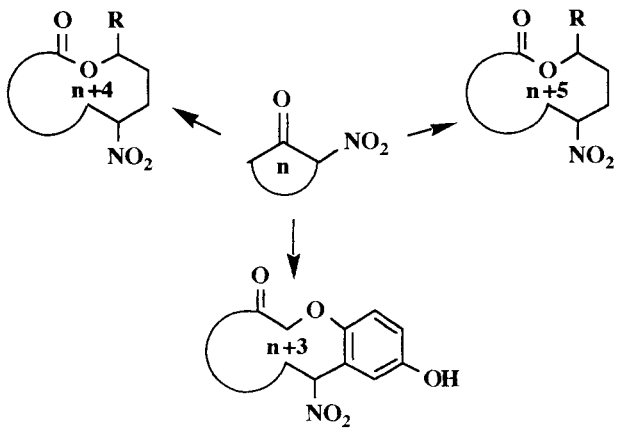
Scheme 47

required for introducing the correct chirality, and methyl acrylate respectively. Submitting ***85*** to the action of Baker's yeast following the conditions described by Guarna *et al.* (ref. 59), yielded the (*S*)-5-nitro-2-pentanol ***86*** in 99% e.e., which was subsequently transformed into the corresponding acetal derivative ***258***. The stage was set for performing the key cycloaddition step, which was accomplished generating the nitrile oxide from the primary nitro group under classical Mukaiyama conditions. The compound ***259*** was obtained in 80% yield as an inseparable mixture of diastereomers at the newly created chiral center. Reductive hydrolysis of the isoxazoline ring system with Raney-Ni under hydrogen atmosphere gave rise to ***260*** which was immediately dehydrated to give ***261*** which contains the complete γ-keto acrylate function. After protection of the carbonyl function of ***261*** as cyclic ethylene ketal ***262***, the latter underwent saponification of the ester groups by alkaline hydrolysis to provide the hydroxy acid ***262*** upon acidification. Cyclodimerization of ***262*** followed by removal of the protecting groups provided (-)-pyrenophorin.



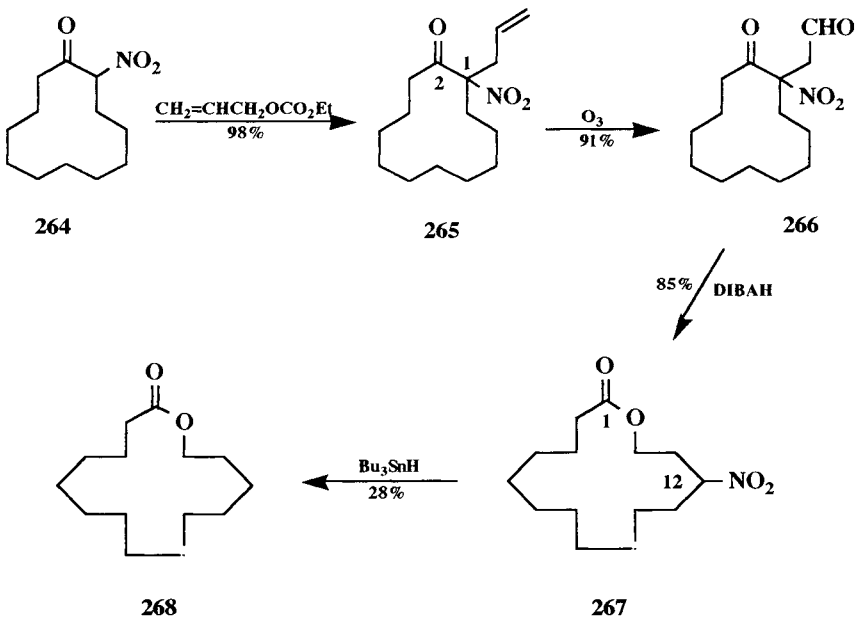
Scheme 48

The possibility of cyclic 2-nitroketones to react with internal nucleophiles giving macrocyclic compounds by ring enlargement (see above, sec. ***2.5***), has been extensively studied by Hesse *et al.* Many internal nucleophiles have been utilized to prepare important macrocyclic derivatives. By this reaction two, three, four or five carbon atoms, respectively, can be incorporated to the pre-existent cyclic system and, if a hydroxy group is used as the internal nucleophile, different macrolactones can be prepared (Scheme 49). The results obtained up to 1988 were reported in a review (ref. 42) by Hesse and Stach who have devised an incisive name: "The Zip Reactions" for these processes. As representative examples of the the zip reaction, the synthesies the lactones ***268***, and ***272*** were detailed in the following Schemes 50 and 52.



Scheme 49

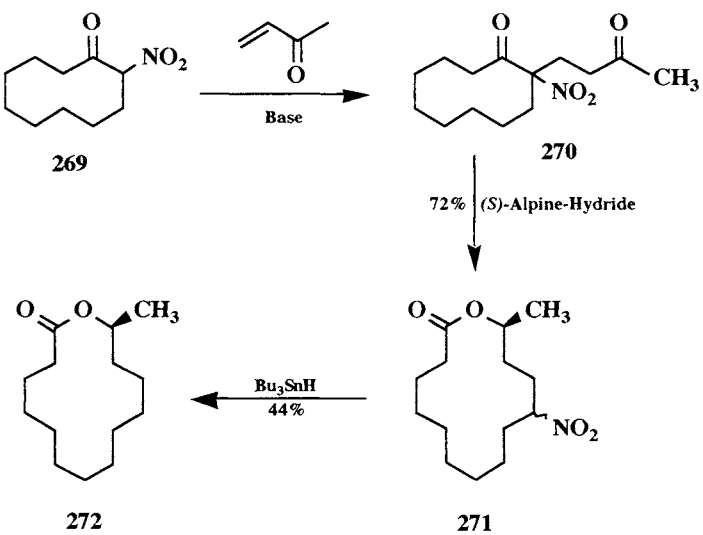
Thus, the preparation of tetradecano-14-lactone *268*, isolated from the*Ferula galbaniflua* and *F.*



Scheme 50

rubicaulis , (ref. 99) is depicted in Scheme 50. The aldehyde ***266***, prepared by ozonolysis of 2-allyl-2-nitrocyclododecanone ***265***, gave the ring-enlargement product ***267*** by treatment with DIBAL-H. Its transformation to the tetradecano-14-lactone ***268*** was performed by reduction with Bu_3SnH/AIBN/toluene.

Another representative utilization of the zip reaction is the synthesis (ref. 100) of (+)-*(S)*-tetradecan-13-olide ***272***, a commercial product which is obtained from *Ferula galbaniflua* and *F. rubicaulis*, outlined in the Scheme 51. Michael addition of 2-nitrocyclodecanone ***269*** to methyl acrylate, under standard conditions yielded ***270***. Reduction of (±)-***270***, with *(S)-alpine-hydride* at -78°C in THF, gave the nitrolactone (+)-***271*** (72%). Denitration of ***271*** with Bu_3SnH/AIBN afforded the lactone (+)-***272*** in 44% yield.



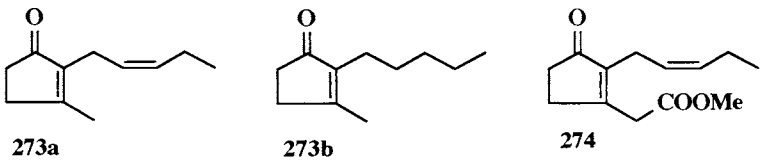
Scheme 51

3.5 CYCLOPENTANE DERIVATIVES

Many natural products present a five-membered ring as the main structural feature. Because of the high versatility of nitro compounds, the latter have often been used to prepare natural compounds with a cyclopentane-skeleton.

3.5.1 Perfumes

Analytical efforts have revealed that a considerable number of interesting constituents of the essential oil of jasmin flowers (*Jasminum grandiflorum*) contain a cyclopentanone-ring. Most prominent among them are *(Z)*-jasmone ***273a***, dihydrojasmone ***273b*** and *(Z)*-methyl jasmonate ***274*** .

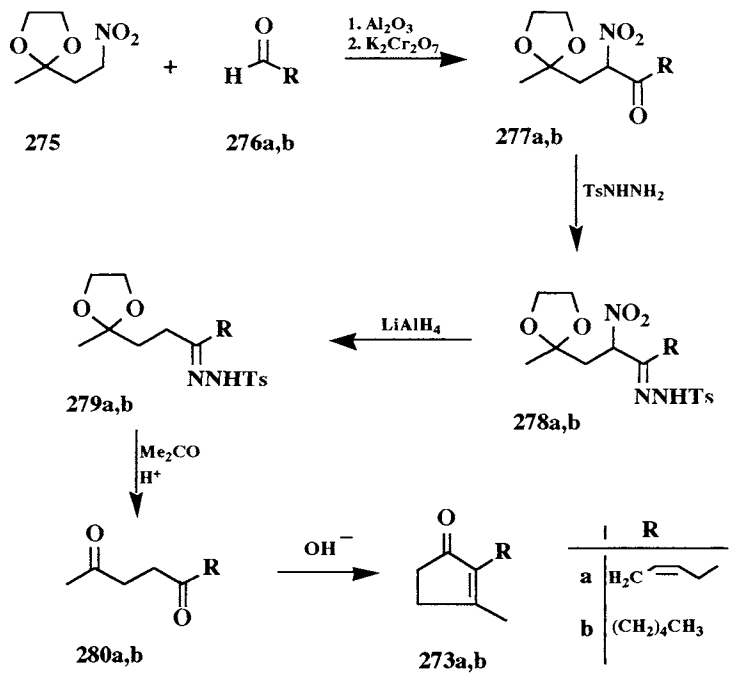


273a **273b** **274**

Nitro compounds have been demonstrated to be very useful in the syntheses of these perfume components.

Since the first report of Mc Murry and Melton (ref. 101), on the synthesis of *(Z)*-jasmone ***273a*** from nitroalkanes, many authors have described the use of these perfumes *via* nitro derivatives. Here some representative synthetic routes to the componds ***273*** and ***274*** have presented.

In Scheme 52 *(Z)*-jasmone ***273a*** and dihydrojasmone ***273b*** have been prepared (ref. 102) starting from the nitro ketal ***275***. This nitroaldol reaction of ***275*** with the right aldehydes ***276*** is the



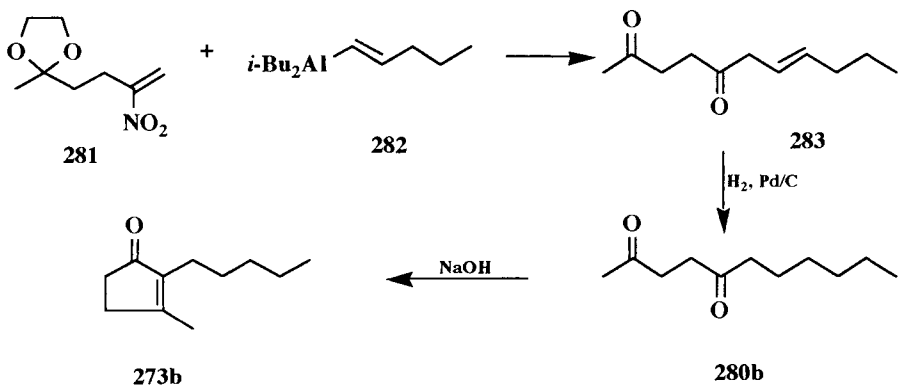
275 **276a,b** **277a,b** **278a,b** **279a,b** **280a,b** **273a,b**

Scheme 52

chainlengthening reaction followed by oxidation and denitration, *via* the *p*-toluenesulfonylhydrazones of the corresponding α-nitro ketones ***277***. Removal of the protecting groups yielded the 1,4-diketones ***281*** which were then cyclized with alkali. As depicted in Scheme 52, 1,4-diketones

represent the most widely employed intermediates and efforts to find new efficient synthesis of these compounds.

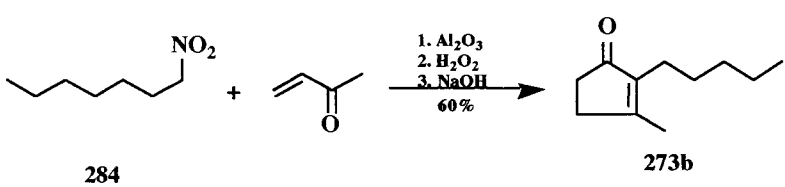
Since triorganoalanes (AlR_3) react with α-nitro olefins, Pecunioso and Menicagli (ref. 103) published the synthesis of dihydrojasmone ***273b*** (Scheme 53) starting from nitroalkenes. Thus, the nitroolefins ***281*** and diisobutyl-1-pentenylaluminium, gave the crude β,γ-unsatured diketone ***283***, which after hydrogenation afforded the undecane-2,5-dione ***280b*** (85% overall yield); cyclization of this latter



Scheme 53

compound yielded (90%) the dihydrojasmone ***273b***.

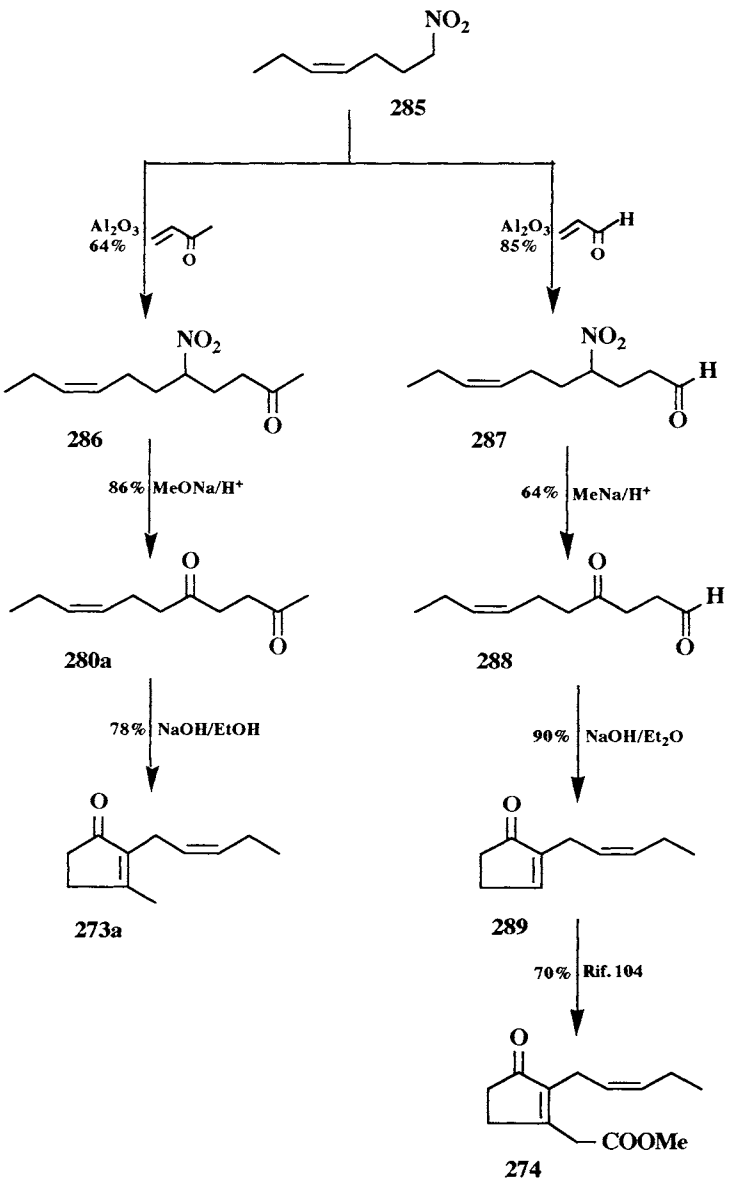
An improved strategy for the one-pot synthesis of ***273b***, based on the utilization of alumina in the Michael addition of a nitroalkane to MVK, is depicted in Scheme 54. Conjugate addition of 1-nitroheptane ***284*** to 3-buten-2-one (MVK) on alumina, followed by *in situ* oxidation with hydrogen peroxide in methanol, and then *in situ* basic cyclization with 0.5 N sodium hydroxide, affords, in one-pot and in 60% overall yield, the dihydrojasmone ***273b***.



Scheme 54

(Z)-7-Nitro-3-heptene ***285*** has been proposed (ref. 93) as the central intermediate for the synthesis of *(Z)*-jasmone ***273a*** and methyl jasmonate ***274***. Addition (Scheme 55) of ***285***, by heterogeneous catalysis (Al_2O_3), to methyl vinyl ketone or acrolein, followed by Nef reaction

afforded *(Z)*-undec-8-ene-2,5-done ***280a*** and *(Z)*-1,4-dioxodec-7-ene ***288***, respectively. Basic cyclization of the latter compounds furnished jasmone ***273a*** and the jasmonate percursor ***289***.

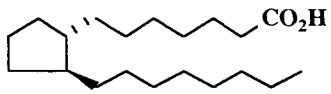


Scheme 55

Following the Buchi and Egger method (ref. 104) compound ***289*** was converted to *(Z)*-methyljasmonate ***274***.

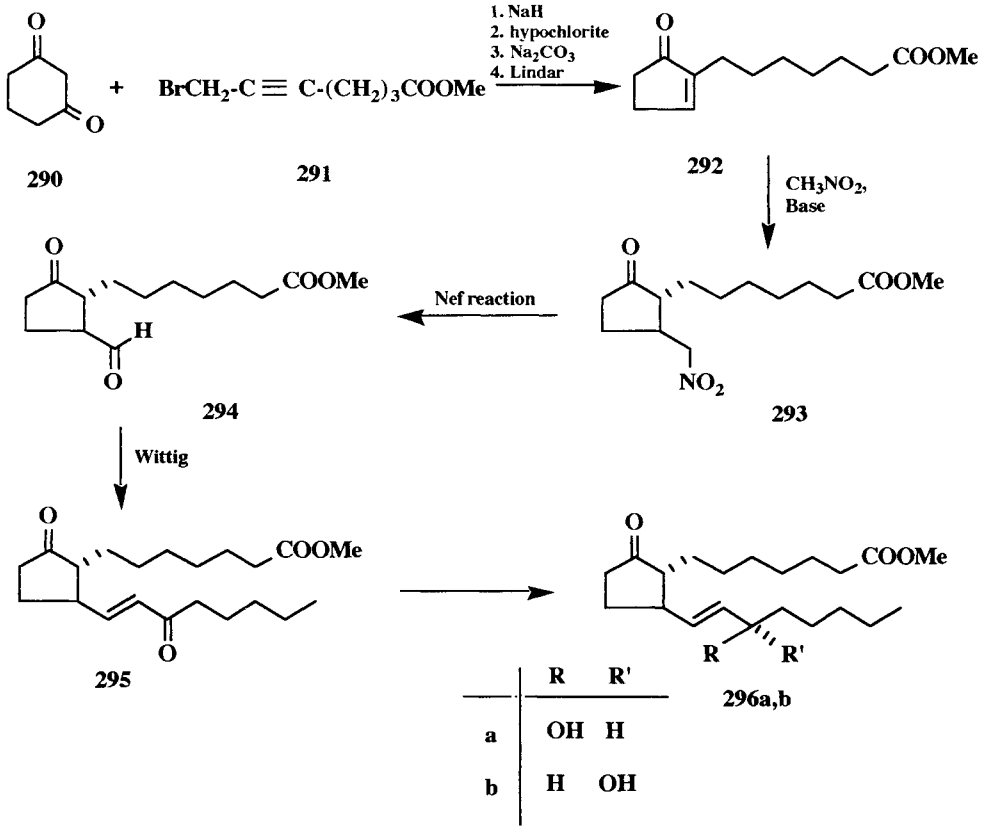
3.5.2 Prostaglandins

The name prostaglandin was given originally to a lipid fraction of human seminal fluid which was found to stimulate isolated strip of uretine muscle. It is known (ref. 105) that there are more than a dozen naturally occurring prostaglandins with similar structures and that one or more has been found in almost every mammalian tissue examined.



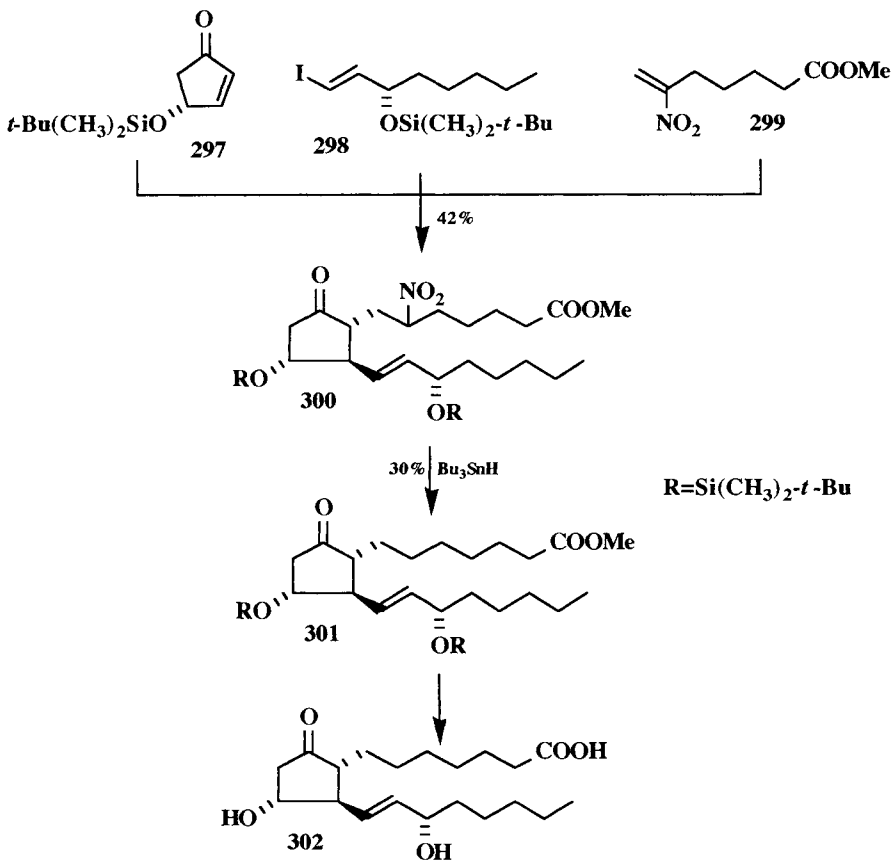
289 (prostanoic acid)

Prostaglandins (PGs), which are functionalized derivatives of prostanoic acid ***289*** show a five-membered ring with different substitutions. Nitro compounds have been occasionally employed for the synthesis of prostaglandins. Bagli and Bogri (ref. 106) employed nitromethane to obtain some PGE_1 and PGE_2 derivatives (Scheme 56).



Scheme 56

(-)-Prostaglandin E_1 ***302*** has been prepared (Scheme 57) from (*R*)-protected hydrogen cyclopentenone ***297*** using, as a key operation, the tandem organocopper conjugate addition/nitrogen Michael trapping of the resulting enolate intermediate (ref. 107). First, a vinylcopper reagent was



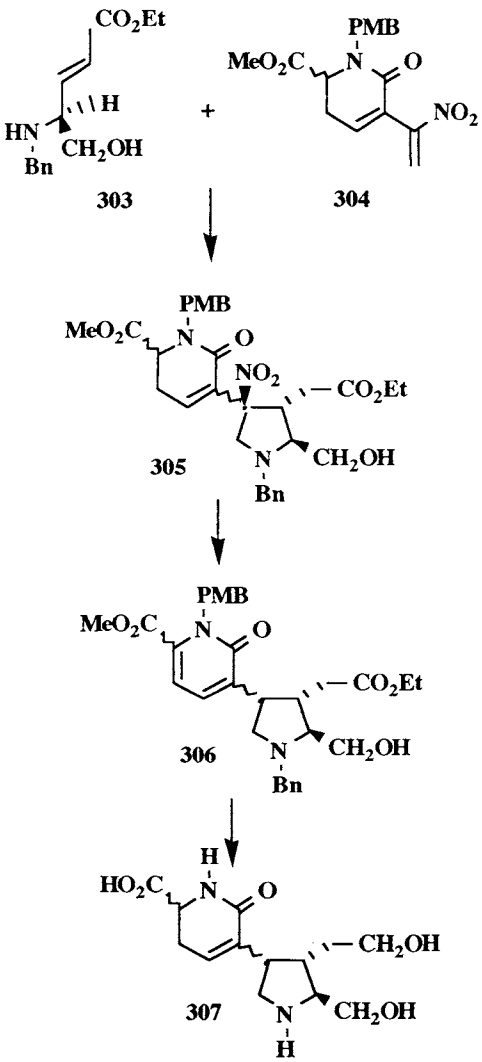
Scheme 57

prepared from optically active iodide ***298*** followed by Michael additon to the enone ***297***. Treatment of the obtained adduct to the nitroolefin ***299*** gave the condensation product ***300***. Reduction of the nitro group with Bu_3SnH/AIBN yielded the denitrated compound ***301***. Desilylation and hydrolysis of the ester with pig liver esterase completed the synthesis of (-)-PGE_1 ***302***.

3.6 AMINO ACIDS

As continuation of synthetic effords in the area of kainoids (ref. 108), a family of non-proteinogenic amino acids having a pyrrolidine dicarboxylic acid ring as common structural feature, Pollini's group published (ref. 109) an enantioselective formal synthesis of acromelic acid A ***307***, a potent neurotoxin isolated from the poisonous mushroom *Clitocybe acromelalga.*

The formal enantioselective synthesis of ***306*** is depicted in Scheme 58, starting with the cyclocondensation of the subunit ***303*** and ***304*** to afford the key intermediate ***305***. Subsequent

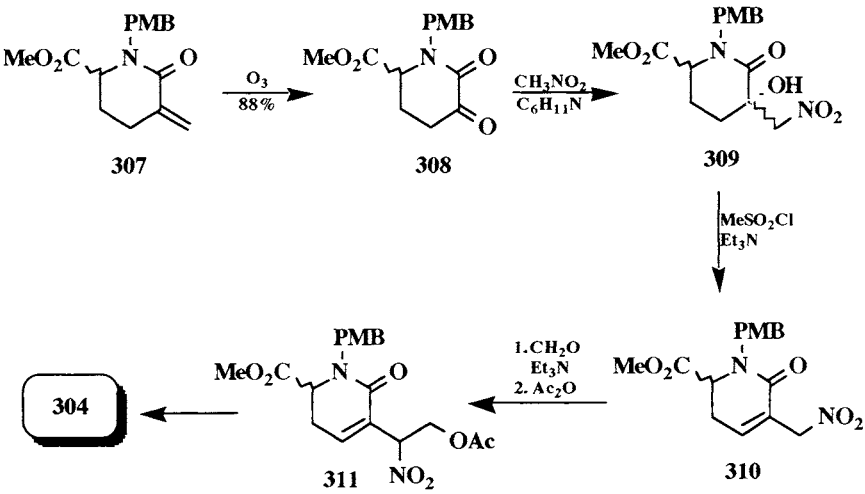


Scheme 58

removal of the allylic nitro group following Ono's methodology proceeded regio- and stereoselectively to give rise to ***306***. The synthesis of ***307*** was completed in five steps by appropriate use of different protecting groups.

However the first stage of the Pollini's approach was concerned with the preparation of 2-nitro-1,3-diene ***304*** or an equivalent thereof incorporated into a dihydropyridone moiety. With this in

mind, the piperidone ***307*** was selected as starting material for the preparation of the precursor ***311*** of the nitrodiene ***304***. The overall sequence, summarized in the Scheme 59, began from the keto lactam

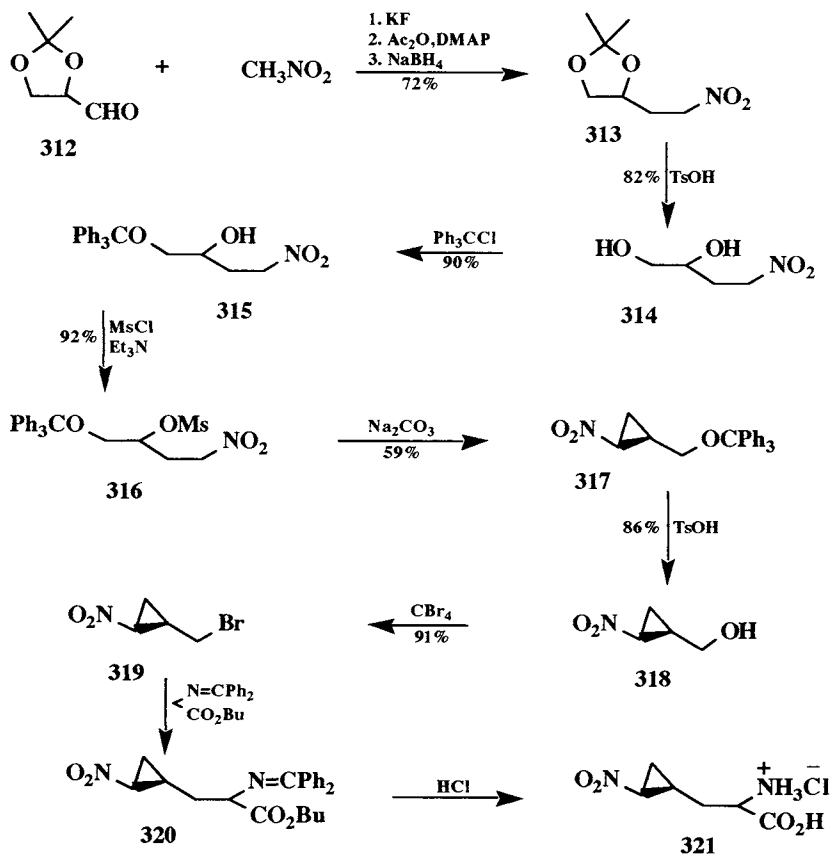


Scheme 59

308 obtained by ozonolysis of ***307***. Its subsequent pyperidine-catalyzed Henry reaction with nitromethane produced the adduct ***309***, then easly dehydrates to the nitroethylene derivative ***310***. Base catalyzed hydroxymethylation with formaldehyde, followed by acetylation of the obtained alcohol, yielded ***311***, the requisite precursor of the nitrodiene ***304*** as a mixture of diastereoisomers.

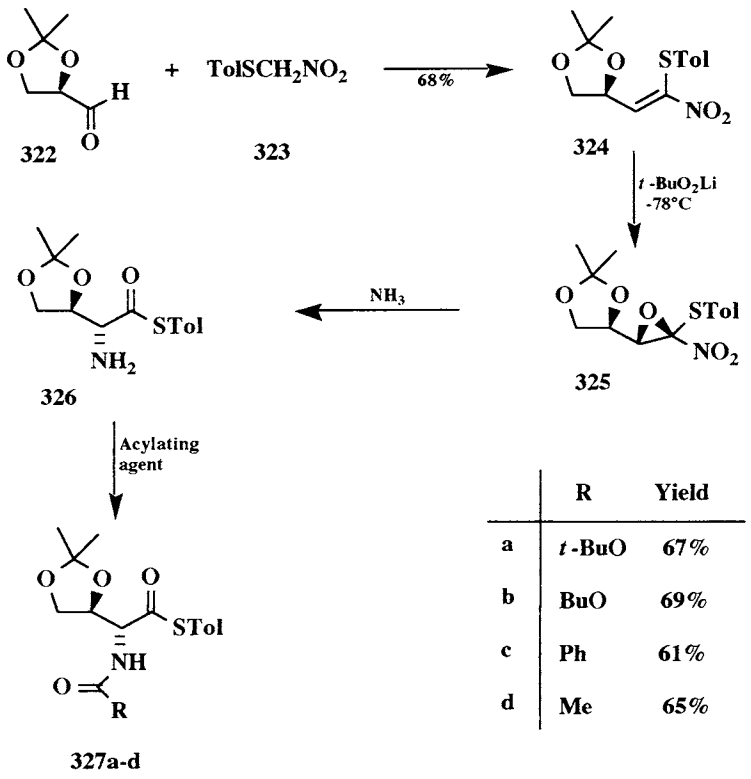
The peptide-lactone hormaomycin produced by *Streptomyces griseoflavus* shows a selective antibiotic effect against some *gram*-positive bacteria. This intercellular signal substance has an influence on the formation of air mycel as well as the production of secondary metabolites in streptomyces. The structure includes two molecules of 3-(*trans*-2-nitrocyclopropyl)-alanine ***321*** with a *trans* oriented nitro group and both possible configurations at C-2. Zeeck *et al.* (ref. 110) proposed (Scheme 60) a synthetic strategy towards ***321*** which called for an enantioselective construction of 4-nitrobutane-1,2-diol ***314***, its transformation into a derivative with a protected primary hydroxy group (***315***) as well as a reasonable secondary leaving group (***316***) and ring closure by γ-elimination under S_N2 conditions with inversion of configuration to give the (*trans*-2-nitrocyclopropyl)methyl derivative ***317***. After cleavage of the ether, the primary alcohol ***318*** can be converted to the bromide ***319*** which can be combined with a suitable glycine equivalent. The protected amino acid ***320*** is deprotected with HCl giving the hydrochloride of 3-(*trans*-2-nitrocyclopropyl)alanine ***321***.

Over the past 50 years, many nonproteinogenic amino acids have been isolated from natural sources. β-Hydroxy-α-amino acids represent an important class of these compounds, and, in this context, Jackson *et al.* (ref. 111) reported the syntheses of γ-hydroxy threonine derivatives ***327***,



Scheme 60

and polyoxamic acid ***341*** *via* polyfunctionalized nitroalkenes. Thus, the first sequence, depicted in the Scheme 61, starts from the nitroalkene ***324*** prepared from *D*-isopropylideneglyceraldehyde ***322***,

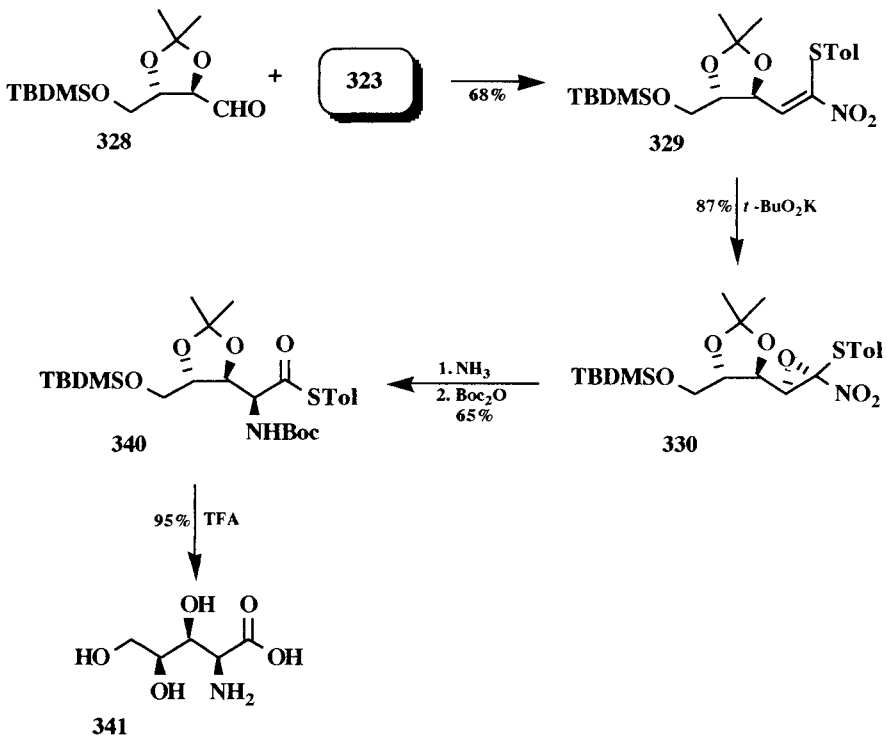


	R	Yield
a	*t*-BuO	67%
b	BuO	69%
c	Ph	61%
d	Me	65%

Scheme 61

by condensation with [(4-methylphenyl)thio]nitromethane ***323***. Epoxidation of ***324*** with *t*-BuO_2Li, at -78°C, gave the oxirane ***325***. Reaction of ***325*** in dichloromethane with aqueous ammonia yielded the α-amino thioester ***326***, which was treated with a number of different acylating agents providing the corresponding α-amino-*S*-tolyl thioesters ***327*** in good yield. Each of these products was diastereoisomerically pure.

In Scheme 62, following the same starting sequence as in Scheme 61, the alkene ***329*** was prepared by condensation of ***323*** with the aldehyde ***328***, itself prepared from commercially available 2,3-isopropylidene-*L*-threitol. Nucleophilic epoxidation of the nitro alkene ***329*** gave the oxirane ***330***. Reaction of the *anti* oxirane ***330*** with ammonia, followed by treatment with *t*-butyl pyrocarbonate, gave the *syn* Boc-protected ***340***. Subsequent treatment of ***340*** with trifluoroacetic acid afforded polyoxamic acid ***341*** (95%).



Scheme 62

3.7 OTHERS

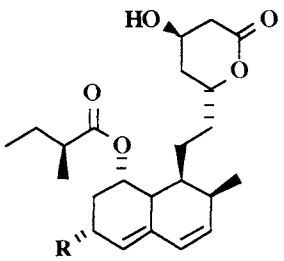
As reported in the above sections, aliphatic nitro compounds have been extensively employed in the syntheses of many classes of natural products. In this section will be presented the preparation of some single natural compound *via* nitroalkanes.

3.7.1 Mevinic Acid Derivatives

In 1975 three active compounds from the culture broth of the fungus *Penicillium citrinum*, were isolated. The main compound, ***342*** ML-236B, was also isolated as an antifungal agent from *P. brevicompactum* and was named compactin. A second, more active compound, mevinolin ***343***, was later isolated from *Monascus ruber* and from *Aspergillus terreus*.

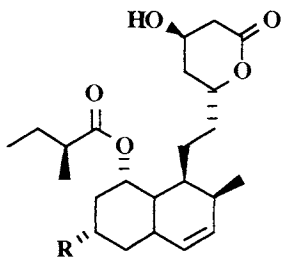
Two related compounds, dihydrocompactin ***344*** and dihydromevinolin ***345***, were subsequently isolated as minor metabolites from the cultures of these fungi.

Since their discovery, compactin ***342*** and mevinolin ***343*** have attracted considerable attention due to their biological activity as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterogenesis in man. Dihydromevinolin ***345***, which exhibits biological similar to mevinolin, is produced in small quantities during the fermentation.



342 R = H (+)-Compactin

343 R = Me (+)-Mevilonin

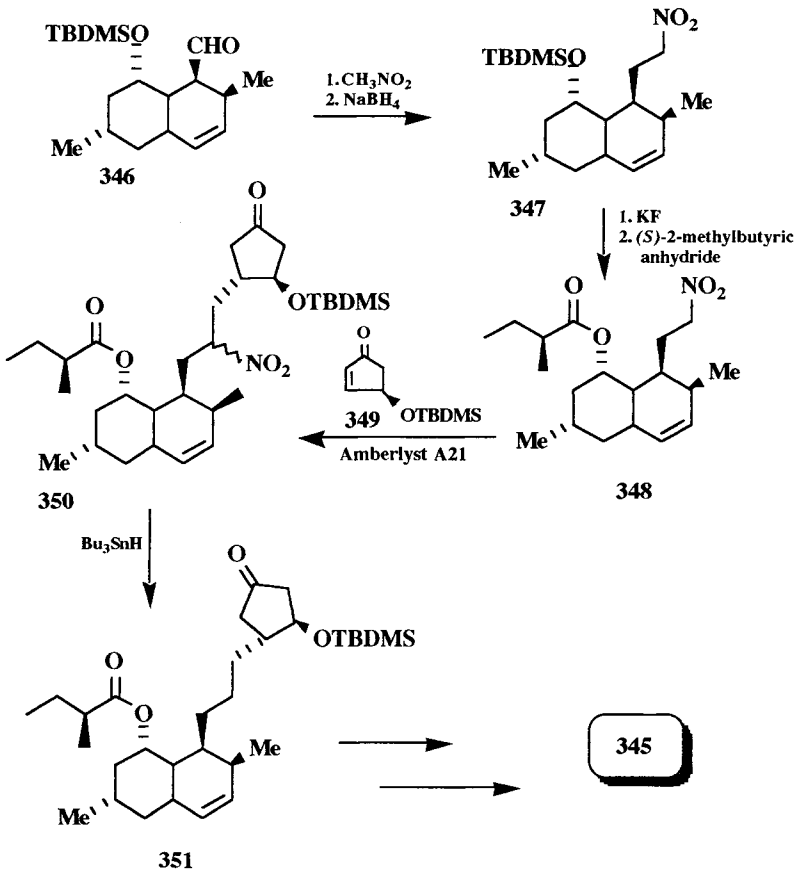


344 R = H (+)-Dihydrocompactin

345 R = Me (+)-Dihydromevilonin

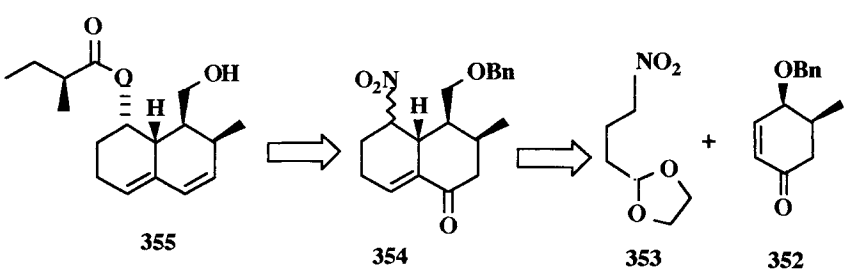
Due to the considerable value of these molecules, different researchers have been attracted to their syntheses. In this context Hanessian *et al.* (ref. 112) reported the total synthesis of (+)-dihydromevinolin ***345*** through nitroalkenes (Scheme 63).

Treatment of the aldehyde ***346*** with nitromethane anion followed by mesylation of the resulting mixture of nitroalcohols gave a nitroolefin, which by reduction ($NaBH_4$) afforded the nitroalkane***347***. It is at this stage in the sequence that it was decided to remove the TBDMS group and to introduce the required ester function leading to the compound ***348***. The next step was a critical Michael addition which was done by allowing the nitroalkane ***348*** to come into contact with *(R)*-4-[(*t*-butyldimethylsilyl)oxy]cyclopent-2-enone ***349*** in the presence of Amberlyst A-21, or basic alumina. The epimeric mixture of nitroalkane ***350***, thus formed in 70% yield, was treated with tributyltin hydride in the presence of a catalytic amount of AIBN to give the corresponding des-nitro derivative ***351*** in 55% yield. The target ***345*** was then obtained by a sequence of eight steps.



Scheme 63

Later, Pollini *et al.* (ref. 113) published an interesting procedure, starting from a nitroalkane, for the enantioselective synthesis of the hexahydronaphtalene nucleous ***355*** of (-)-compactin ***342*** (Scheme 64).

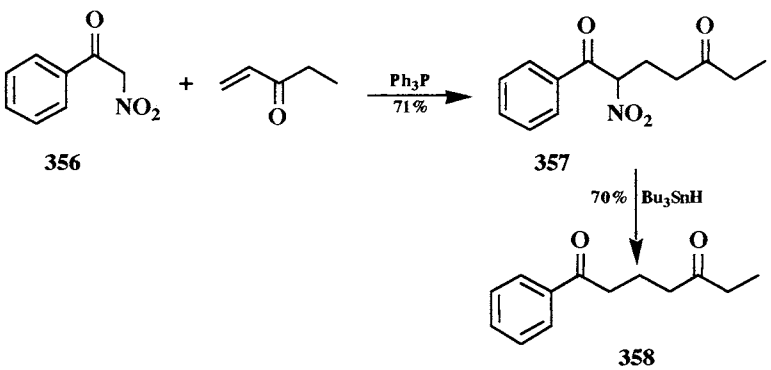


Scheme 64

Thus, the hexahydronaphthalene nucleus of the known precursor ***355***, with its full complement of stereogenic centers, was envisaged to arise from the intermediate ***354*** containing strategically situated functions for the elaboration of the conjugated diene and a functional group at the C-1 position that could be manipulated to the required oxygenated function. Ring A could in turn be built onto a preformed ring B portion ***352*** with the help of the nitroacetal ***353***.

3.7.2 1-Phenylheptane-1,5-dione

Different phenylheptanes have been isolated from the decayed heart wood of aspens infected with the fungus *Phellinus tremulae* (Bond.) Bond & Borisov (Hymenochaetaceae). The isolation of 1-phenylheptane-1,5-dione ***358*** directly from the fungus, was reported in 1993. This was the first documentation of compound ***356*** as a natural product. A two step synthesis of ***358*** has been



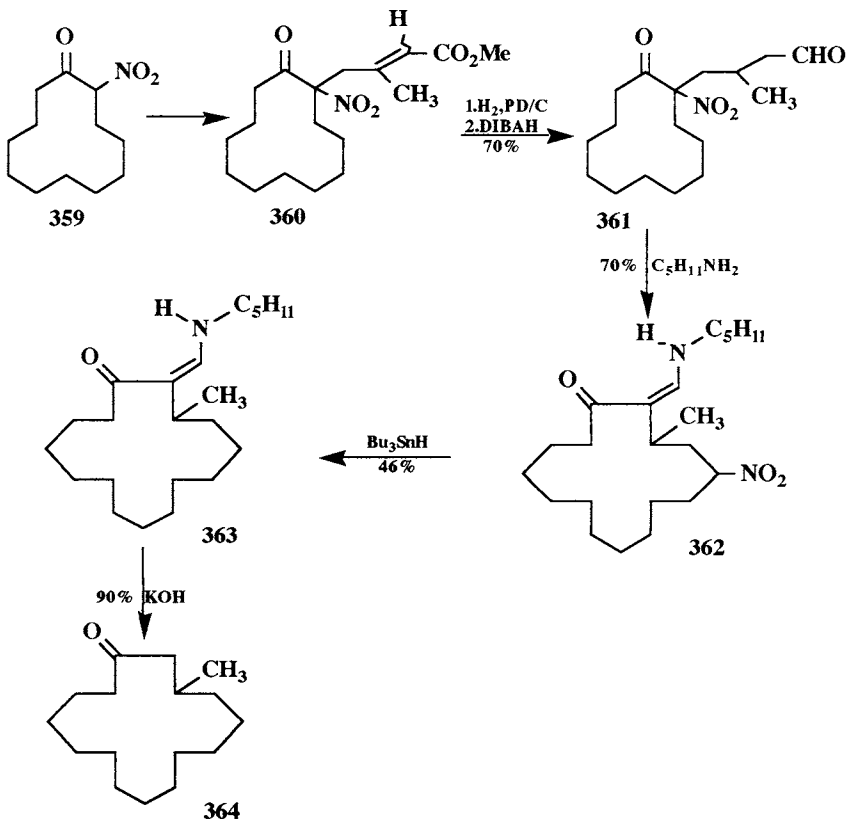
Scheme 65

published (ref. 114) starting from the, commercially available, benzoylnitromethane ***356***, Michael addition of the latter to ethyl vinyl ketone in THF in the presence of a catalytic amount of triphenylphosphine gave ***357*** in 71% yield. Denitration of ***357*** was carried out by heating a stirred mixture of ***357***, tributyltin hydride and AIBN, in benzene. The 1-phenylheptane-1,5-dione ***358*** was obtained in 70% yield (49.7% overall yield).

3.7.3 Muscone

Muscone ***363*** is a rare and valuable perfumery ingredient isolated from the male musk deer *Moschus moschiferus*. α-Nitrocycloalkanones have proved to be very useful for the synthesis of muscone. Hesse and Bienz (ref. 115) started from α-nitrocyclododecanone ***359*** (Scheme 66) which,

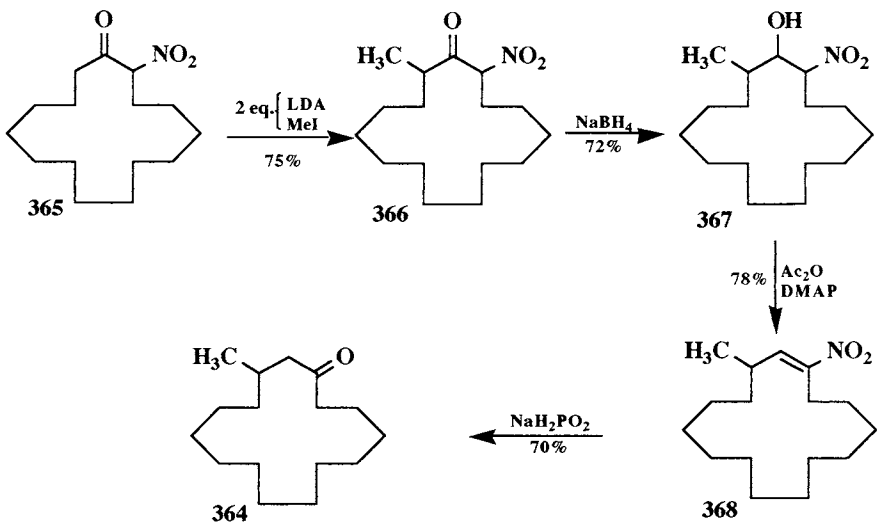
after an appropriate α-alkylation, gave the α,β-unsaturated ester ***360***. Reduction of the C=C double bond (H_2,Pd/C) and conversion of the ester functionality to an aldehyde (DIBAH) afforded the nitroaldehyde ***361***. Treatment of the aldehyde with pentylamine, in EtOH at room temperature, yielded the enlarged (by three members) ring cycloalkanone ***362***. Denitration of the latter (Bu_3SnH) and hydrolysis (KOH) of the enamine ***363*** produced the muscone ***364***.



Scheme 66

Another source (ref. 95) of the perfume component ***364*** is 2-nitrocyclopentadecanone ***365*** which was converted (Scheme 67) to the muscone ***364*** through two key steps: *(i)* regioselective

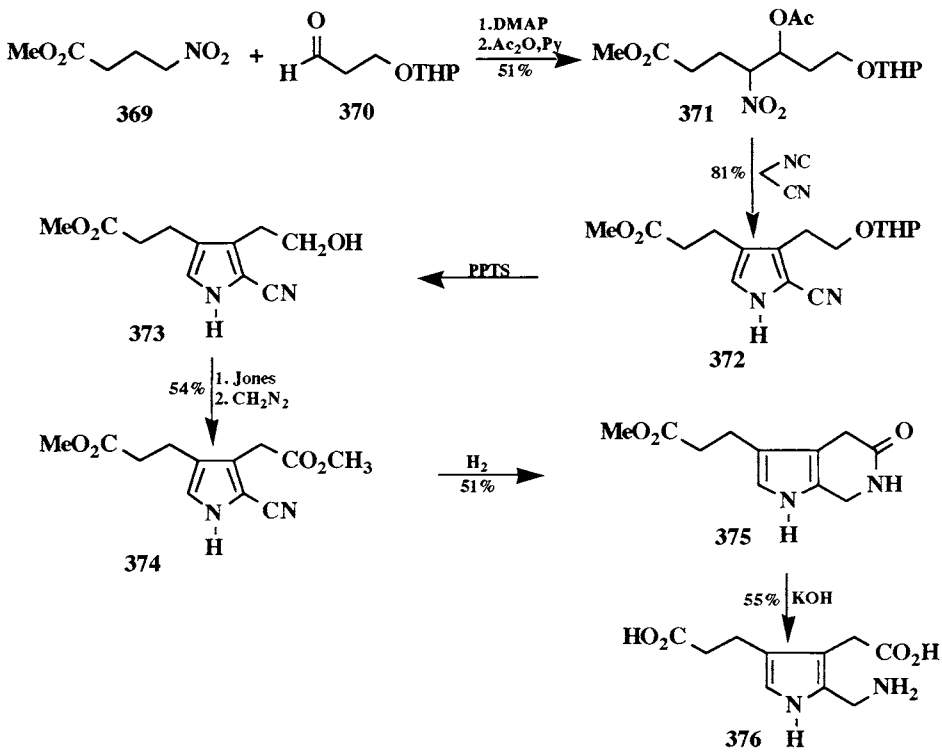
alkylation of the nitro ketone ***365***, *(ii)* shift of the carbonyl group to an adjacent position. Thus, $C_{\alpha'}$ methylation of ***365*** with methyl iodide, using LDA as base, furnished the methylated product ***366***, which, by $NaBH_4$ reduction, afforded the nitro alkanol ***367***. Dehydration of ***367*** by dehydroacetylation with alumina/DMAP in refluxing dichloromethane completed the formation of the nitroalkene ***368***. Treatment of ***368*** with sodium hypophosphite and Raney nickel (Nef reaction) gave (±)-muscone ***364*** in 40% overall yield, based on the starting nitro cycloalkanone ***365***.



Scheme 67

3.7.4 Porphobilinogen (PBG)

Porphobilinogen (PBG) ***376*** was isolated in 1952 from the urine of patients with acute porphyria. PBG ***376*** is the key building block in the biosynthesis of tetrapyrrolic natural products such as porphyrins, chlorophylls, corrins and vitamins B_{12}. A convenient and versatile method for the synthesis of PBG ***376*** involves (ref. 116) the addition (Scheme 68) of methyl 4-nitrobutyrate ***369*** with aldehyde ***370*** (Henry reaction). Acetylation of the obtained nitro alcohol yielded the polyfunctionalized nitro derivative ***371***, which, after reaction with isocyanoacetonitrile in THF and in the presence of DBU afforded the 2-cyano-3,4-substituted pyrrole ***372***. The tetrahydropyranyl ether ***372*** was cleaved by PPTS giving the corresponding alcohol ***373***. The hydroxy functionality in ***373*** was then converted to the methyl ester ***374***, first by oxidation with Jones reagent followed by



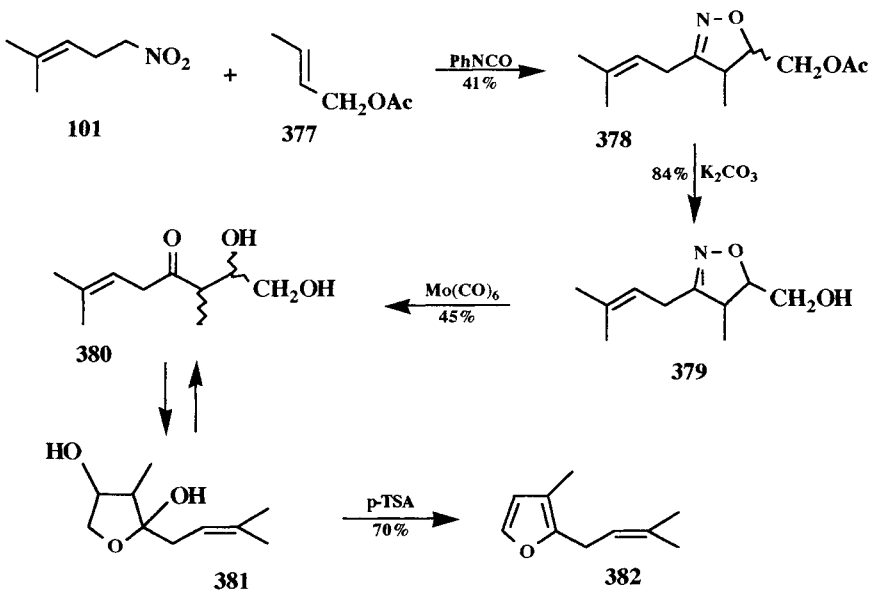
Scheme 68

esterification of the resulting crude acid with diazomethane. Now the only remaining transformation in the synthesis of PBG was the conversion of cyanogroup to the aminomethyl functionality. Thus hydrogenation of ***374*** and successive hydrolysis of the obtained ***375*** yielded the PBG-***376***.

3.7.5 Rosefuran

Rosefuran ***382*** is a trace component of the high prized oil of rose, so it has become the target of considerable synthetic attention. In a recent publication (ref. 98) an approach to rosefuran (Scheme 69) entailed on the [3+2] cycloaddition of the nitrile oxide generated under classical Mukayama conditions from the known (see Scheme 26) nitro compounds ***101***, containing in addition the feliciously placed required unsaturation, into the suitable protected crotyl alcohol ***377*** to afford the key 3,4,5-substituted isoxazoline ***378***. Saponification of the acetate easily occurred at room temperature (Na_2CO_3) to provide the free alcohol ***379*** in 84% yield. The stage was set for demasking the required α,β-dihydroxyketone moiety contained in ***379***, which was conveniently accomplished by means of molybdenum hexacarbonyl, leading to the formation of ***380*** in 45% yield. Its exposure

to catalytic *p*-toluenesulfonic acid in methylene chloride proceeded with the formation of ***382*** in 70% yield.

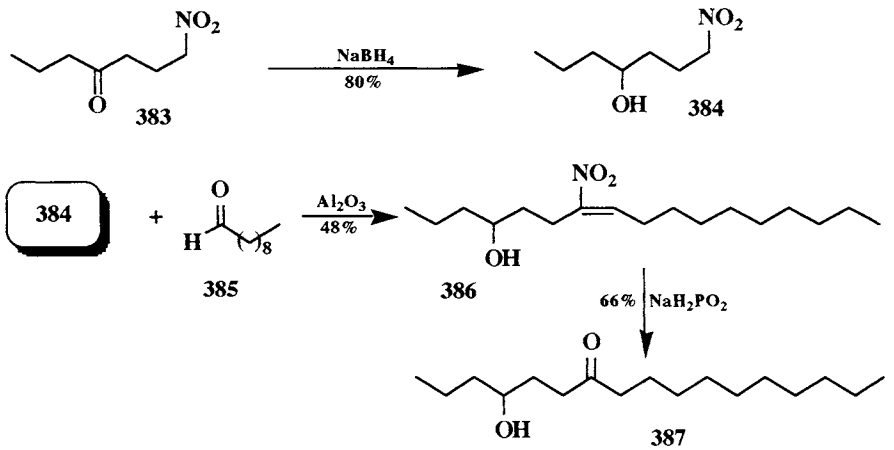


Scheme 69

3.7.6 Hydroxy Ketones

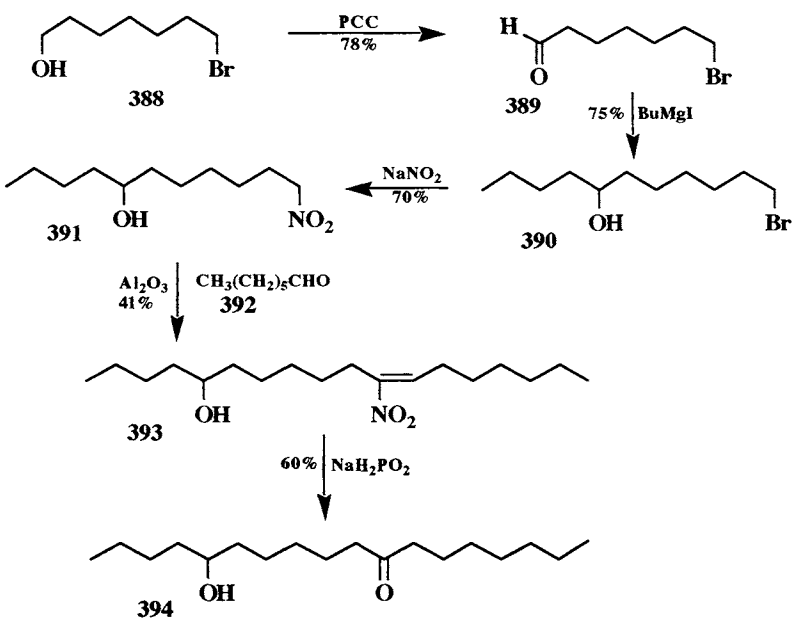
The roots of *Chiococca alba* (*Anguifuga, Brachiata, Racemosa,* and *Trisperma*), of Rubiaceae family, are reported to be used in folk medicine as a tonic for ganglion inflammation, a diuretic, an antivirus, an antioedema, and as an aphrodisiac. Neither chemical nor pharmacological studies of the constituents of this genus were reported up to 1991 when, after an examination of a leaf-exstract, two new hydroxy ketones (4-hydroxyheptadecan-7-one ***387***, and 14-hydroxyoctadecan-8-one ***394***) were found as the main components.

Due to the high value of these compounds, two important syntheses (ref. 117) of both the hydroxy ketones ***387*** and ***394***, starting from hydroxy-functionalized nitro alkanes ***384*** and ***391***, have been recently reported. The key steps of these syntheses are two chemoselective reactions in which the hydroxy group, present in the nitro derivatives, must be preserved: *(i)* nitroaldol condensation with basic alumina, and *(ii)* direct Nef conversion of nitroalkenes to carbonyl derivatives with sodium hypophosphite.



Scheme 70

The synthesis of ***387*** was achieved (Scheme 70) by $NaBH_4$ reduction of nitroketone ***383*** to ***384***, followed by nitroaldol condensation of ***384*** with decanal ***385*** under basic conditions (Al_2O_3), and successive Nef conversion of the obtained (*E*)-nitroalkene ***386*** using sodium hypophosphite.

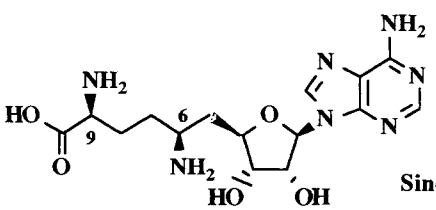


Scheme 71

The preparation of ***394*** (Scheme 71) started with PCC oxidation of commercially available alcohol ***388*** to the aldehyde ***389***. Grignard addition of butylmagnesium iodide to ***389*** gave ***390*** which by subsequent substitution with $NaNO_2$ in DMF was converted to the nitroalkanol ***391***. Following the above procedure for the conversion ***384*** to ***387***, nitroaldol condensation of ***391*** with heptanal ***392*** produced ***393*** which, on sodium hypophosphite treatment, furnished the 14-hydroxyoctadecan-8-one ***394***.

3.7.7 Sinefungin

Sinefungin is an antifungal antibiotic (**A-9145**) isolated from cultures of *Streptomyces griseolus* at Lilly Research Laboratories in 1971. Sinefungin, later assigned structure ***395***, is also produced by *Streptomyces incarnatus*. Considering its structural similarity to *S*-adenosylhomocysteine



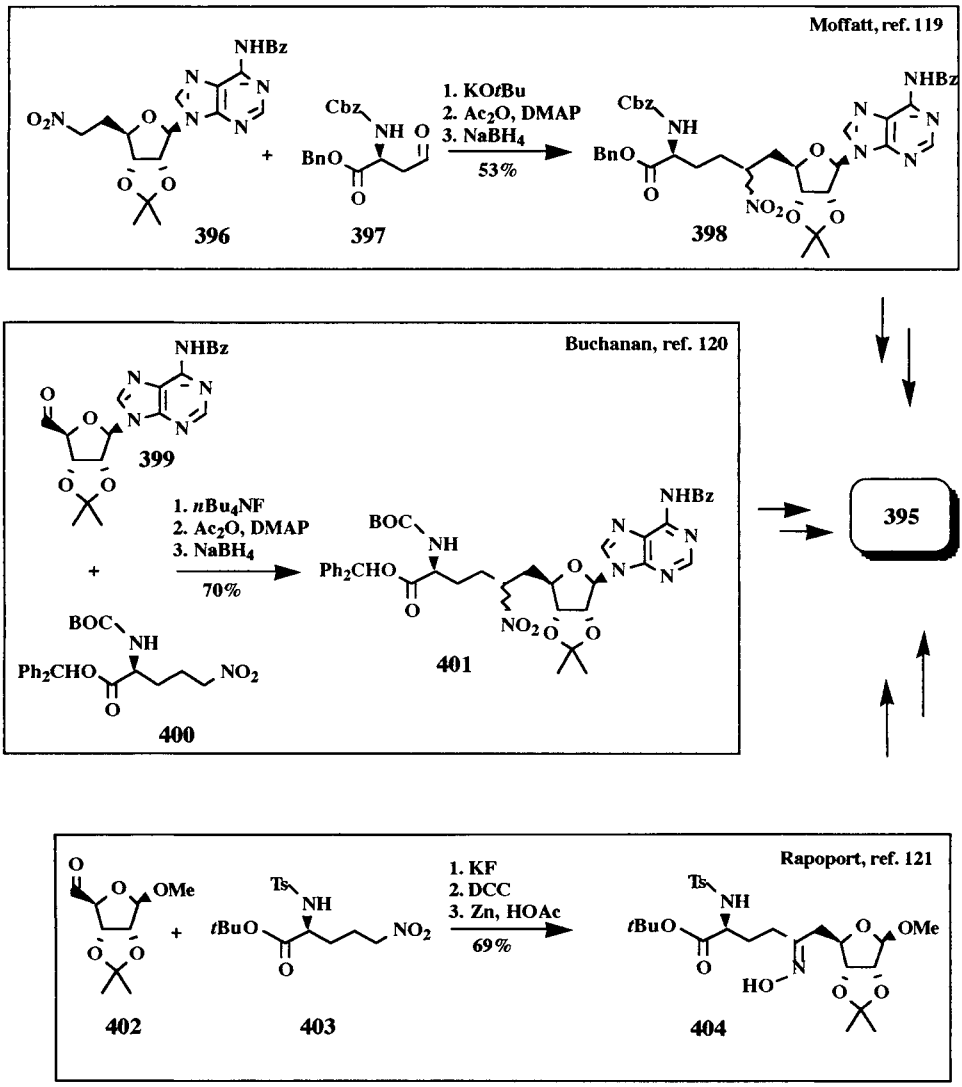
Sinefungin

395

(SAH) and *S*-adenosylmethionine (SAM), the inhibitor effect of sinefungin was studied on several *S*-adenosylmethionine-mediated transmethylation reactions in cell culture and in vivo. The compound was shown to inhibit the growth of various fungi, virus replication, cell transformation, and has interesting antiparasistic affects in vivo and in vitro. Despite these significant biological activities, sinefungin cannot be considered for clinical use because it is nephrotoxic and causes marrow depression in laboratory animals.

Three total syntheses of sinefungin, recently reviewed by Knapp (ref. 118), utilize the nitroaldol (Henry) condensation as the key step for carbon-carbon bond formation. As depicted in Scheme 72, the Moffatt synthesis (ref. 119) illustrates the application of the nitroaldol condensation of the nitrocompound ***396*** with the aldehyde ***397***, while the nitro derivative ***400*** and the aldehyde ***399*** were proposed by Buchanan *et al.* (ref. 120). Finally, Rapoport (ref. 121) introduced the aldehyde ***402*** and the functionalized nitro alkane ***403*** for this kind of reaction.

These condensations produced nitroalkenes, which, after appropriate reduction, gave the polyfuntionalized nitro derivatives ***398***, ***401***, and ***404***, respectively. The latter compounds possess the basic structure of sinefungin ***395***, which can be easily obtained by their further elaboration. The above syntheses show how the nitro group facilitates the formation of the C-C bond and serves as precursor of the amino group at C-6 of the sinefungin ***395***.

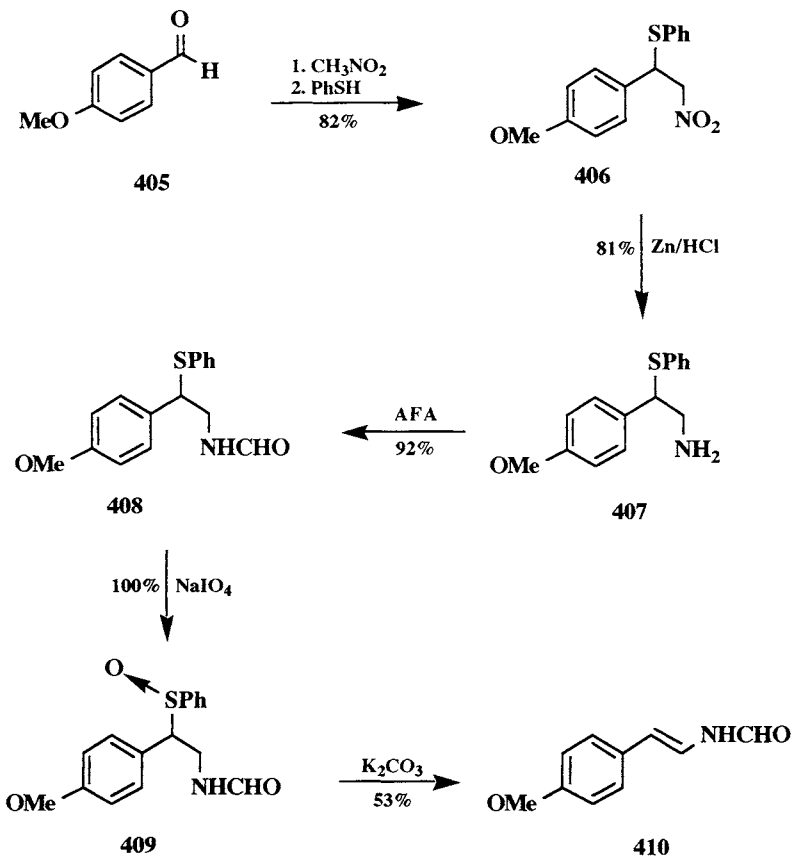


Scheme 72

3.7.8 Tuberine

Tuberine ***410*** is a simple enamide isolated from *Streptomyces amakusaensis* and has some structural resemblance to Erbastatin, an enamide which has received much attention in recent years as an inhibitor of Tyrosine-specific kinases.

An important synthesis of ***410*** started (ref. 122) by nitroaldol reaction (Scheme 73) of *p*-anisaldehyde ***405*** and nitromethane, in the presence of thiophenol, yielding the compound ***406***.



Scheme 73

Reduction of the nitro group, followed by formylation of the obtained amino group, ***406*** gave the compound ***408***. Successive oxidation and elimination of the sulfoxide, produced tuberine ***410*** in 32% overall yield.

4. CONCLUSIONS

Many naturally occurring compounds have been synthesized efficiently through pathways based on the intermediacy of nitroalkane derivatives. Although many other works have been published in this area, the aim of this review is to demonstrate how the versatility of nitroalkanes can be successfully applied to the synthesis of several classes of important natural products. I believe that the easy

accessibility of nitroalkanes and their different reactivities make these compounds modern and useful precursors and intermediates in the syntheses of natural compounds. I hope that this account will provide an incentive for further studies.

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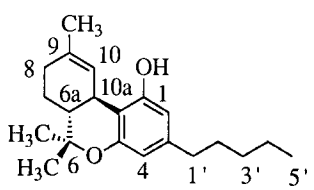
Stereospecific Cannabinoid Synthesis: the Application of New Techniques to a Classical Problem

Marcus A. Tius

Introduction

In the years since the appearance of Razdan's ground-breaking review on the synthesis of cannabinoids, research activity has continued apace.[1,2] The interest which this area engenders is due in part to the challenge which the structures pose to the synthetic organic chemist, and also because of the diverse and useful pharmacological activities which many of these materials express. The chemical structures of the naturally occuring tricyclic cannabinoids, typified by Δ^9-tetrahydrocannabinol (Δ^9-THC), are very simple: there are only two stereogenic carbon atoms, two carbocycles and the dihydrobenzopyran ring.[3] The functionality is in most cases limited to the phenolic C1 hydroxyl and to one or two oxygen-bearing functional groups. One would be justified in questioning whether this class of compounds is of sufficient complexity to continue to interest the organic chemist. The difficulties of the synthesis belie the simplicity of the structure, and are due, at least in part, to the following: (a) The materials are typically non-crystalline, and are often quite difficult or impossible to separate and purify without recourse to HPLC. (b) The aromatic portion of the molecule is very sensitive to oxidation, particularly in the presence of base or transition metals.[4] (c) The Δ^9 unsaturation is thermodynamically disfavored relative to Δ^8 unsaturation. There is also no general method by which to favor Δ^9 unsaturation kinetically. These issues will become clearer in the course of the discussion which follows.



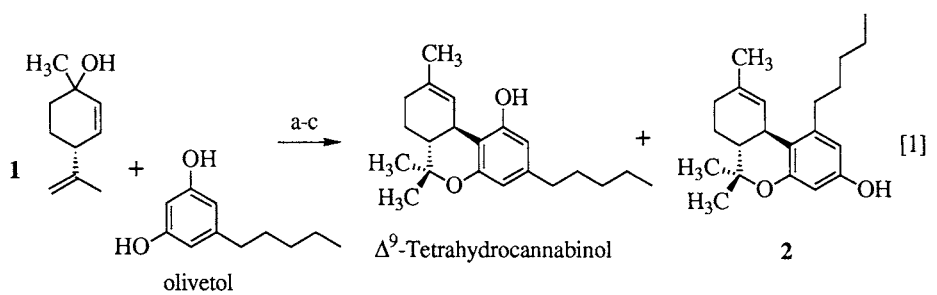
Δ^9-Tetrahydrocannabinol

Interest in the pharmacology of these materials goes back many thousands of years.[5] Herodotus' account of the Scythians' use of *Cannabis sativa* as an intoxicant makes it clear that the psychotropic properties of the producing plant were recognized since antiquity.[6] In addition to uses as anaesthetics, spasmolytics and hypnotics, cannabinoids have been used to combat emesis and nausea induced by cancer chemotherapy, and also in the treatment of glaucoma. In recent times, cannabinoids have achieved a certain notoriety due to their abuse potential. A significant portion of the synthetic effort has been directed toward the preparation of some of the oxygenated human urinary metabolites of Δ^9-THC for use in forensic science as analytical standards for the detection of marijuana use.

Several developments during the past few years have contributed to the current resurgence in interest in this area. The identification of the first cannabinoid receptor (CB1) in rat brain[7] was a major advance. The identification of a second, peripheral, receptor subtype in splenocytes (CB2),[8] as well as the discovery of arachidonylethanolamine (anandamide) as the endogenous ligand for CB1,[9] has made the story much more interesting. Involvement of the pharmaceutical industry has resulted in synthesis and evaluation of large numbers of analogs, and in the discovery of the first receptor antagonist. The chemistry which is associated with these discoveries will not be discussed in this chapter. Instead, the focus will be more eclectic, going back approximately ten years or so, and limited to the synthesis of tricyclic compounds of the natural series. The chemistry which addresses the functionalization of the aliphatic sidechain is for the most part straightforward, and will not be included in this discussion.

Methods Making Use of Cuprates

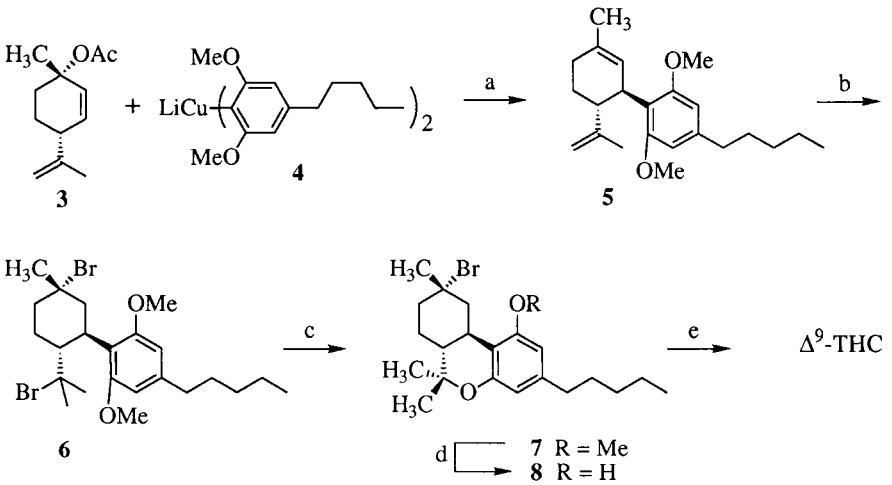
The obvious retrosynthetic disconnection of the tricyclic cannabinoid skeleton leads to olivetol and an appropriately functionalized monoterpene unit. The absolute sense of stereochemistry of the monoterpene can be used to control the stereochemistry of the final product. There is a serious drawback associated with the simplest application of this strategy, which is indicated in eq 1.[10]



(a) $MgSO_4$ anh, CH_2Cl_2, r.t.; (b) $BF_3{\cdot}Et_2O$, 0 °C, 1.5 h; (c) $NaHCO_3$ anh; ca. 31% Δ^9-THC, 15% **2** and other products.

Acid catalyzed condensation of *p*-menthadienol **1** (mixture of *cis* and *trans* isomers) with olivetol under carefully optimized conditions led to a mixture of the desired product, Δ^9-THC, along with substantial quantities of byproduct **2**, referred to as the "abnormal" isomer, in which hydroxyl and *n*-pentyl groups are formally transposed. The lack of regiospecificity for the cyclization compromises the efficiency of this approach. The proportion of "abnormal" isomer, which results from C-C bond formation at C4 of olivetol, rather than at C2, can be minimized by choice of reaction conditions (*vide infra*).

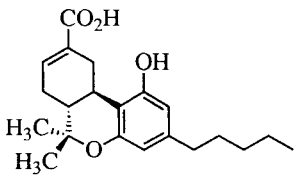
Scheme 1



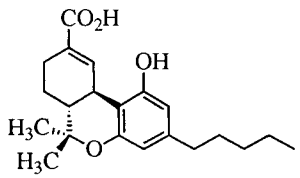
(a) 1 equiv **3**, 2 equiv **4**, 3.5 equiv $BF_3{\cdot}Et_2O$, -76 °C, 5 h, 78%; (b) CH_2Cl_2, satd HBr (gas), -20 °C, 100%; (c) r.t. 5 h, 100%; (d) 9 equiv BBr_3, -76 °C to r.t., 7 h, 58%; (e) 1.2 equiv *t*-BuOK, PhH, 5 °C 1h, 65 °C 10 min; ca. 75% overall from **5**.

One way to overcome the problem is to bias the desired reaction overwhelmingly by metallation at C2. Rickards and Rønneberg devised an ingenious solution to this problem and applied it to the synthesis of Δ^9-THC (Scheme 1).[11] Homocuprate **4** was prepared from dimethoxyolivetol by regiospecific directed lithiation at C2, followed by exposure to half an equivalent of cuprous bromide. The reaction with (1*S*,4*R*)-*p*-mentha-2,8-dien-1-yl acetate **3**[12] was catalyzed by boron trifluoride etherate to produce (-)-*trans*-cannabidiol dimethyl ether **5**. The *anti* stereochemistry of **5** is that which is expected for the cuprate coupling, and is reinforced in this case by the adjacent bulky isopropenyl group. This appears to be the first example of a Lewis acid catalyzed S_N2' substitution reaction involving a homocuprate. In this reaction there is no possibility for attack at C4 of olivetol. The requirement for two equivalents of homocuprate per equivalent of **3**, i.e. four equivalents of olivetol dimethyl ether for each equivalent of acetate detracts somewhat from the appeal of this route. All attempts to simultaneously demethylate and cyclize **5** by exposure to boron tribromide gave complex reaction mixtures containing only small

amounts of Δ^9-THC. Therefore it was necessary to protect the terpenoid C-C double bonds before cleaving the methyl ether. Exposure of **5** to dichloromethane saturated with hydrogen bromide gas gave the unstable dibromide **6** in quantitative yield. Remarkably, upon standing at room temperature for 5 h, **6** underwent spontaneous mono-demethylation and ring closure to **7** in quantitative yield. Olivetol dimethyl ether does not react with hydrogen bromide under these conditions, therefore this remarkable reactivity has been attributed to relief of strain in the dihydrobromide. Exposure of **7** to boron tribromide led to bromophenol **8** in 58% yield. Treatment with potassium *tert*-butoxide led to a mixture of (-)-Δ^9-THC, contaminated with <10% (-)-Δ^8-THC. For preparative purposes, it was unnecessary to separate and purify the unstable brominated intermediates. Instead, **5** was carried through the sequence of reactions in Scheme 1 to produce in 75% overall yield the mixture of Δ^9 and Δ^8-THC isomers, along with <5% of $\Delta^{9(11)}$-THC.



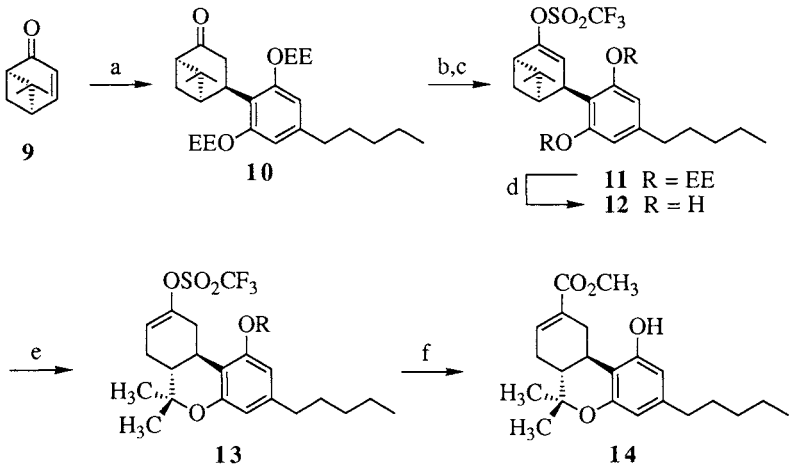
Δ^8-THC-acid



Δ^9-THC-acid

The cuprate approach to the synthesis of cannabinoids is versatile, and lends itself to the synthesis of the oxidized human urinary metabolites of Δ^9-THC. The major metabolic pathway for Δ^9-THC administered in smoke to humans involves the microsomal oxidation of the 9-methyl group to the corresponding carboxylic acid, which is subsequently converted to a mixture of glucuronides which are excreted in urine.[13,14] 11-Nor-Δ^8-THC-carboxylic acid (Δ^8-THC acid) is used in a rapid immunoassay for the detection of THC metabolites in human urine.[15] The 5'-trideuterio 11-nor-Δ^9-THC-carboxylic acid is used as an internal standard for the unambiguous confirmation and quantification, by GC/MS, of positive results from the immunoassay screen. Both the Δ^8 and the Δ^9-THC acids are important in forensic science.[16-20] Early syntheses of these oxidized metabolites were marred by the acid catalyzed isomerization of Δ^9 to Δ^8 and by the facile oxidation of the phenol. These two problems are responsible for the extremely low yields which have been reported for some of the early syntheses of these metabolites.[21-24] We became interested in this problem a few years ago, and have developed efficient, enantioselective syntheses to both of these important materials. The Δ^8-THC acid synthesis is summarized in Scheme 2.[25]

Scheme 2

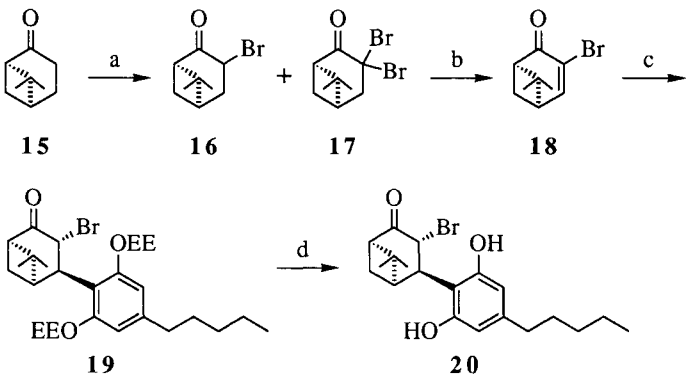


(a) transfer 1 equiv of 2-lithio-*bis*-(1-ethoxyethyl)olivetol to 1 equiv lithium 2-thienyl-cyanocuprate; to cuprate add 1/1 solution in THF of **9** and BF_3·Et_2O, -78 °C, 66%; (b) $KN(SiMe_3)_2$, THF, 0 °C; (c) $PhN(SO_2CF_3)_2$; (d) PPTS, MeOH, CH_2Cl_2, 25 °C, 65% overall for 3 steps; (e) BF_3·Et_2O, CH_2Cl_2, 25 °C, 87%; (f) 10% $PdCl_2(PPh_3)_2$, K_2CO_3, CO, MeOH, THF, 25 °C, 72%.

The terpene fragment for this work, (+)-apoverbenone **9**, was prepared from cheap and readily available (-)-β-pinene according to Grimshaw's procedure.[26] Olivetol was converted to the bis-ethoxyethyl (EE) ether, and was regiospecifically metallated at C2 with *n*-butyllithium in tetrahydrofuran (THF). The *lithio*-olivetol was converted to the mixed, higher-order cuprate with lithium 2-thienylcyanocuprate.[27] The advantage of using the mixed cuprate, rather than the homocuprate, is the more efficient utilization of the olivetol. Neither the aryl cuprate, nor the enone is particularly reactive, therefore boron trifluoride etherate is required in order to activate the enone toward Michael addition. Attack of the cuprate takes place *trans* to the bridge bearing the *gem*-dimethyl group for steric reasons. This step establishes the stereochemistry at C10a. Enolization of **10** and trapping with N-phenyltriflimide leads to vinyl triflate **11**, which is deprotected in the next step. Exposure of **12** to Lewis acid at room temperature results in a spectacular reaction, in which the tricyclic ring is formed simultaneously with transposition of the double bond to produce exclusively Δ^8 product **13** in excellent yield. Unlike most enol ethers, vinyl triflates are chemically robust. For steric reasons, the rearrangement reaction cannot be a concerted process. The reaction takes place in a stepwise fashion, facilitated by the relief of ring strain which attends the cleavage of the four-membered ring. In the final step of the sequence, palladium catalyzed carbonylation of **13** in THF/methanol leads in 72% yield to crystalline methyl ester of Δ^8-THC acid **14**. The synthesis of **14** is accomplished in only five steps from apoverbenone in 27% overall yield. This is a practical and highly efficient, preparatively useful

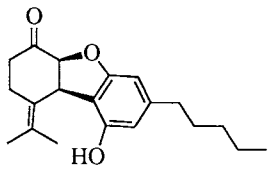
synthesis. It is significant that no ring-junction isomers of **13** or **14** are formed, since other workers have shown that their separation cannot be easily accomplished.

Scheme 3

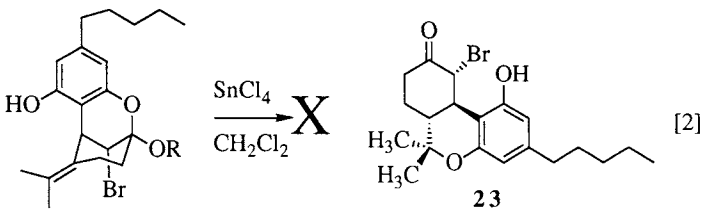


(a) NBS, cat $(PhCO_2)_2$, CCl_4, 75 °C; **16/17** = 2/3, 55%; (b) Li_2CO_3, LiBr, DMF, 130 °C, 76%; (c) **18**/BF_3·Et_2O, 1/1, added to mixed, higher order cuprate in THF, 55-60%; (d) PPTS, MeOH, CH_2Cl_2, 25 °C, 90%.

After our success in completing the synthesis of the Δ^8-THC acid, we turned our attention to the more challenging problem posed by the Δ^9 isomer. In order to apply the cuprate strategy to this problem, it is necessary to introduce a leaving group in the terpene fragment which will end up on C10 in the cyclized material. Elimination of this leaving group then leads to the desired Δ^9 unsaturation in the final product. This concept was examined in a preliminary study which is summarized in Scheme 3.[28] (1*R*)-(+)-Nopinone **15** was brominated to produce a mixture of mono- and dibromides **16** and **17**. Dibromide **17** was converted to α-bromoenone **18**; mixed, higher order cuprate addition took place as in Scheme 2 to produce **19**, which was deprotected in excellent yield to resorcinol **20**. All attempts to rearrange **20** to a tricyclic cannabinoid failed. Two of the reactions provided some insight into the source of the problem. Exposure of **20** to an excess of titanium tetrachloride in chloroform at 60 °C provided benzofuran **21** in 42% yield (unoptimized). Treatment of **20** with *p*-toluenesulfonic (tosic) acid in chloroform and methanol or ethanol provided the bridged ketal **22** in good yield (eq 2). Under no circumstance could **22** be converted to **23**. This was surprising, because the conversion of the corresponding *non*-halogenated cannabinoid methyl ketal had been successfully cyclized to the ketone with the *cis* ring junction stereochemistry.[29] Furthermore, the reaction of **24**, a substrate lacking only the bromine substituent of **20** proceeded in excellent yield (eq 3).



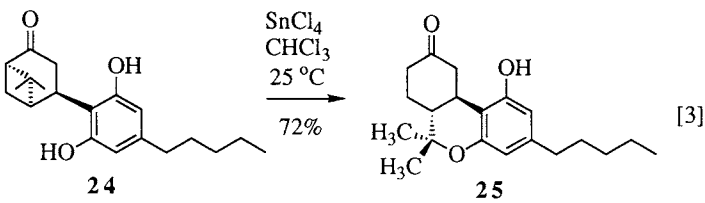
21



22 R = Me or Et

23

[2]



24 **25**

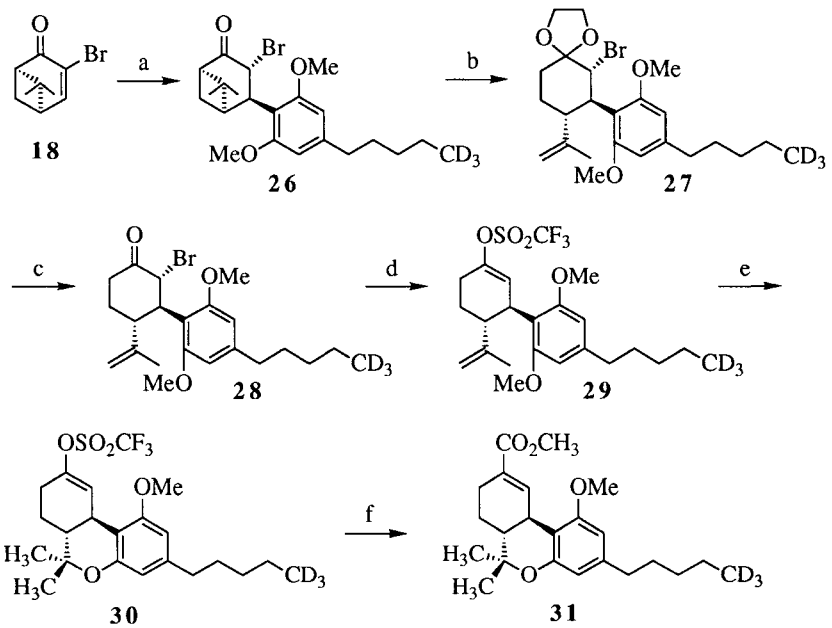
[3]

From these results it is obvious that the electronic effect of the bromine substituent adversely influences the cationic cyclization process by *destabilizing* the ketone carbonyl group in α-bromoketone **20**. This prompted a rethinking of the problem. Clearly, in the *absence* of the dihydrobenzopyran ring, the α-bromine is incompatible with a free phenolic hydroxyl. Depending upon reaction conditions, it either undergoes nucleophilic displacement (**21**), or it leads to the formation of a very stable ketal (**22**). Consequently, the solution to the problem is to chose a protecting group for the phenolic hydroxyls which is robust enough to survive the conditions for the acid catalyzed cleavage of the cyclobutane ring in an intermediate like **20**. The methyl ether group was chosen for this purpose, since it is extremely stable, but is readily cleaved by thiolate anions in a polar, aprotic solvent, such as N,N-dimethylformamide (DMF).

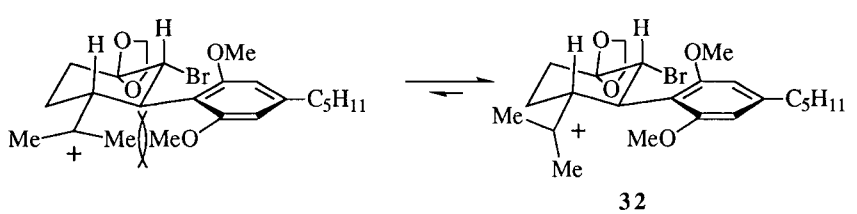
Our synthesis of 5'-trideuterio 11-nor-Δ^9-THC-carboxylic acid is summarized in Scheme 4.[28] α-Bromoenone **18** was converted to **26** via the mixed, higher order cuprate, as before. In this work, 5'-($^2H_3$)olivetol was used. Cleavage of the four-membered ring of **26** was accomplished with 1,2-*bis*(trimethylsilyloxy)ether and one equivalent of trimethylsilyl triflate. When catalytic silyl triflate was used, only ketalization of **26** took place. The ring cleavage could then be induced by adding more silyl triflate. This reaction is also noteworthy because it produces exclusively 2-bromo-4-isopropenyl cyclohexanone-1-ethylene ketal **27**; no isopropylidene isomer

could be detected in the crude product. This, in all likelihood, is due to a stereoelectronic effect exercised by the C4 aryl substituent: in the carbocationic intermediate, the steric bulk of the aryl group prevents the alignment of the 2p orbital on C6 with the C6a-H bonding orbital. Consequently, proton loss takes place from the methyl group in **32** (eq 4), leading to the isopropenyl group of the product.

Scheme 4

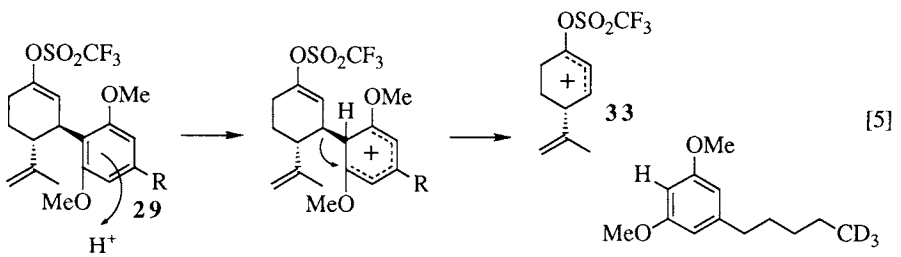


(a) **18**/BF_3-Et_2O, 1/1, added to mixed, higher order cuprate in THF, 83%; (b) 1,2-*bis*(trimethylsilyloxy) ether, 1 equiv Me_3SiOTf, CH_2Cl_2, 65%; (c) 50% aq $HClO_4$, acetone, 40 °C, 66-71%; (d) $LiCuMe_2$, Et_2O, 0 °C; $PhN(SO_2CF_3)_2$, DME, 0 °C, 75-81%; (e) Me_3SiI, $CHCl_3$, 22 °C, 47%, 17/1 *trans/cis*; (f) cat. $Pd(OAc)_2$, PPh_3, CO, MeOH, DMF, Et_3N, 82%.



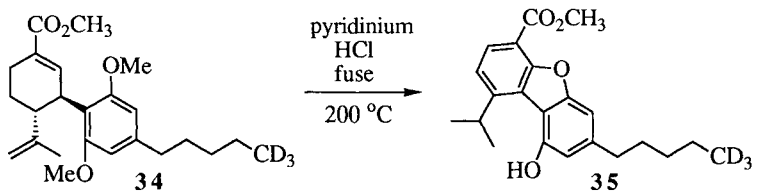
[4]

Hydrolytic cleavage of the ethylene ketal group in **27** was a facile process. There are a number of reagents which will reduce α-halo ketones; in the case of **28**, lithium dimethylcuprate was very effective, regiospecifically leading to the $\Delta^{9(10)}$ enolate, which was trapped with N-phenyltriflimide to produce **29**. Exposure of **29** to an excess of trimethylsilyl iodide at room temperature led to tricyclic vinyl triflate **30** as a 17/1 *trans/cis* isomeric mixture in 47% yield. This tandem monodemethylation-cyclization deserves comment. All attempts to improve the yield of this process by using the various methods for generating trimethylsilyl iodide in situ, such as sodium iodide or lithium iodide with trimethylsilyl chloride, or allyltrimethylsilane and iodine, or hexamethyldisilane and iodine, either gave lower yields of **30**, or a higher percentage of the undesired *cis* ring fusion isomer. A major reaction byproduct was 5'-($^2H_3$)olivetol dimethyl ether, which must arise from initial proton transfer to the electron-rich aromatic ring, followed by C-C bond cleavage with generation of allylic carbocation **33** (eq 5). It is somewhat surprising that the triflate group does not destabilize **33** sufficiently to inhibit its formation. The C-C bond cleavage is driven by the relief of strain in **29**. The undesired process of equation 5 as well as the desired conversion of **29** to **30** require catalysis by hydroiodic acid: no reaction took place when trimethylsilyl iodide was used in the presence of an acid scavenger, such as pyridine or methylcyclohexene, or when an excess of allyltrimethylsilane or hexamethyldisilane relative to iodine was used.



[5]

The presence of a few percent of the *cis* ring fusion isomer of **30** must be due to proton transfer processes which generate an isopropylidene intermediate. Separation of the two ring junction isomers was difficult, therefore the mixture was carried through to the last step. The palladium catalyzed carbonylation of **30** proceeded in excellent yield, and the final product was isolated as a single (*trans*) isomer in 82% yield following preparative HPLC. Although the yield for the cyclization step was disappointing, this synthesis produces material of high optical purity, and has proven to be preparatively very useful. The optical purity of the final product is an important issue, if the materials are to be valuable for forensic purposes.



[6]

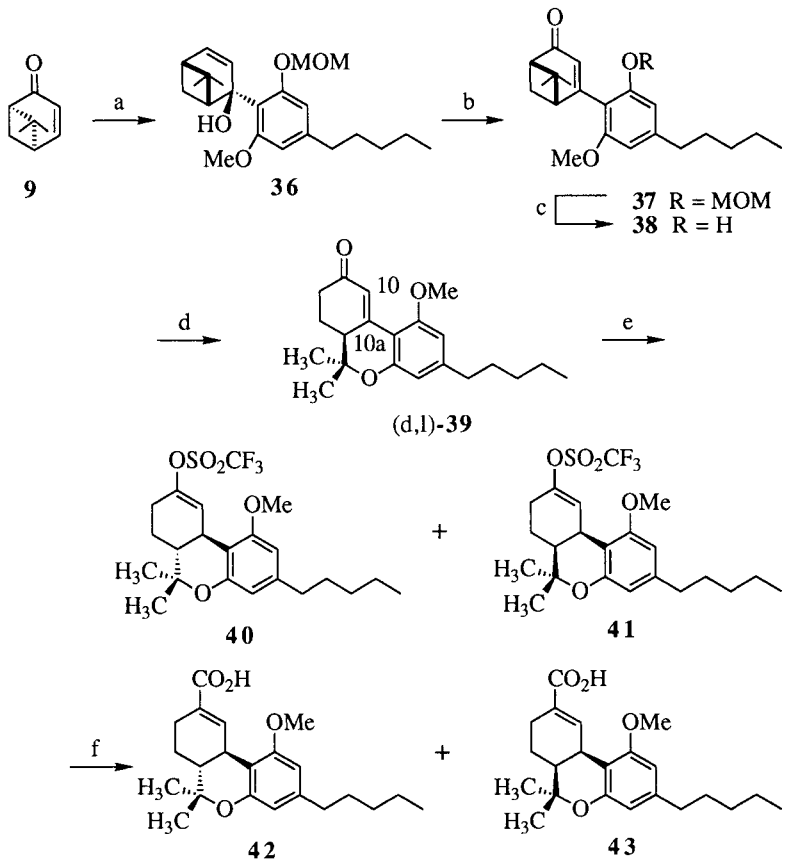
A single attempt was made to improve the cyclization process (eq 6). Carbonylation of **29** produced methyl ester **34** in 80% yield. Methyl ether cleavage in **34** by fusing with pyridinium hydrochloride resulted in a clean conversion to the cannabifuran analog **35**. This again shows that in the Δ^9 series, a free phenolic hydroxyl will add as a nucleophile to C10 whenever there is an electron-withdrawing group at C9.

Methods Making Direct Use of 2-(Lithio)olivetol Derivatives

This strategy for assembling the tricyclic nucleus of the natural cannabinoids is similar to the cuprate approach. Apoverbenone again serves as the terpenic reaction partner, but instead of performing a conjugate addition to the enone, the lithiated olivetol is added to the carbonyl carbon. The convenience of avoiding the cuprate which this strategy affords is offset by the low yields for the subsequent manipulations. An example of the application of this approach to a racemic synthesis of Δ^9-THC acid is summarized in Scheme 5.[30,31]

Lithiation of 1-methoxy-3-(methoxymethyl)-5-pentylbenzene was accomplished with *n*-butyllithium in THF at room temperature. The anion was added to (+)-apoverbenone **9**, and the tertiary, allylic alcohol was oxidized with rearrangement to enone **37**. Selective cleavage of the MOM (MOM = methoxymethyl) was accomplished in excellent yield with trimethylsilyl bromide to give **38**. Exposure of the reaction product to refluxing chloroform containing ethanol and tosic acid led in 86% yield to tricyclic enone **39** in 86% yield. Even though the starting material for this reaction was optically active, **39** was found to be racemic! There are several points at which racemization could have taken place; the authors suggest that this may have happened via hydride shifts during the acid-catalyzed cyclization, or through acid catalyzed enolization of the product. One need not postulate hydride shifts; sequential protonation-deprotonations would also explain this result. The observation that upon scaleup of this reaction variable amounts of the isopropylidene byproduct **44** were obtained makes this the likelier scenario. Inclusion of ethanol in the reaction mixture was found to be necessary for optimal yields. The postulated role of the ethanol was to intercept the tertiary cation *before* proton loss could lead to **44**, followed by an intramolecular S_N1 reaction with the phenol to form the dihydrobenzopyran ring.[32] It is unlikely that **44** can serve as an intermediate for **39**, since its attempted cyclization gave as the sole isolable product 3-(2-hydroxy-6-methoxy-4-pentylphenyl)cyclohexenone **45** via a retro-aldol process. The facility of this reaction is yet another example of a process which is driven by the relief of strain between the two bulky substituents in a bicyclic precursor.

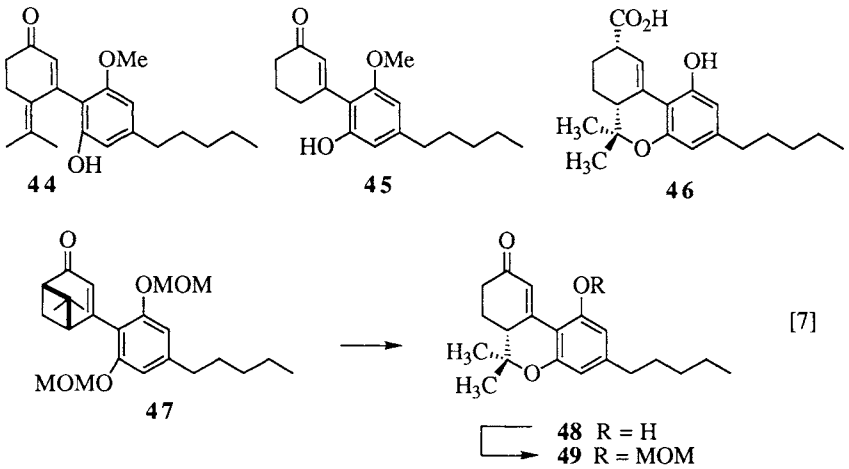
Scheme 5



(a) **9** added to 2-lithio anion of 1-methoxy-3-(methoxymethoxy)-5-pentylbenzene, THF; (b) PCC, CH_2Cl_2, 2 h, r.t., 81% for two steps; (c) Me_3SiBr, CH_2Cl_2, -30 to 0 °C, 91%; (d) $CHCl_3$, EtOH, *p*-TsOH, 86%; (e) Li, NH_3 liq, THF, -78 °C; $PhN(SO_2CF_3)_2$, THF, 0 °C, 84%, 3/1 **40/41**; (f) cat. $Pd(OAc)_2$, PPh_3, CO, HCO_2H, DMF, Et_3N, 82%, 3/1 **42/43**.

Racemization during the cyclization step leading to **39** is unfortunate, as it diminishes the practical utility of this synthesis. The conclusion of this synthesis is, however, instructive and will be discussed. Intermediate **39** bears $\Delta^{10(10a)}$ unsaturation, and it is necessary to reduce the double bond so as to establish the *trans* stereochemistry of the ring junction. Birch reduction of **39**, followed by trapping of the regiospecifically generated enolate with N-phenyltriflimide, led to a 3/1 mixture of vinyl triflates **40** and **41**. The product mixture, containing the undesired *cis* ring fused product **41**, was carried through the palladium catalyzed carbonylation step to produce in excellent yield a 3/1 mixture of acids **42** and **43**. Both sets of stereoisomers were inseparable by conventional chromatography. All attempts to cleave the phenolic methyl ether in racemic trans carboxylic acid **42** with sodium thiopropoxide in DMF led only to rearranged acid **46**.

Consequently, the reactions of Scheme 5 failed to provide racemic Δ^9-THC acid. Since it was not possible to cleave the phenolic methyl ether at the end of the sequence, the obvious solution was to use a less robust protecting group from the outset.

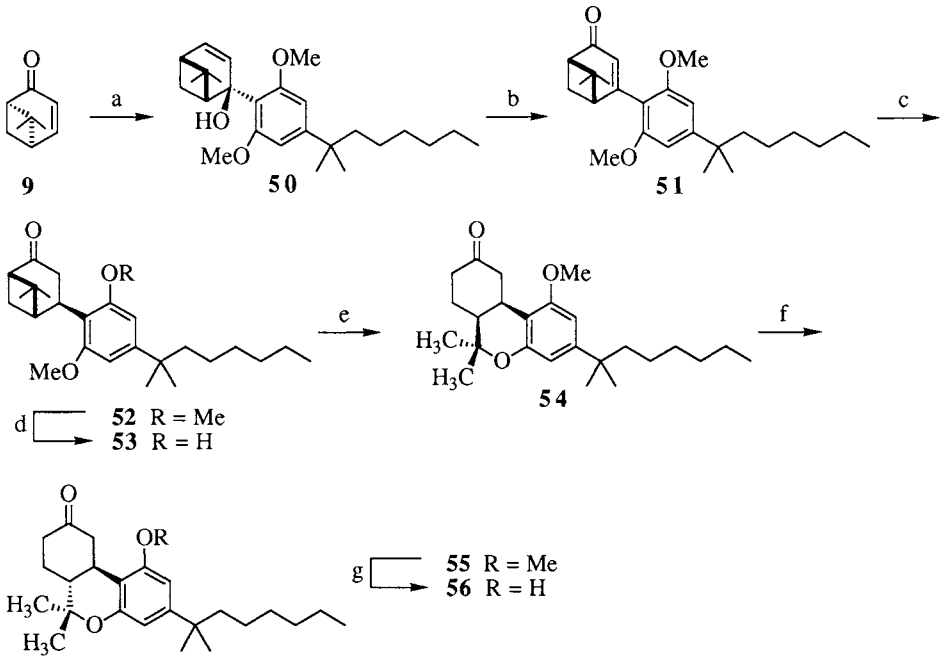


[7]

Equation 7 summarizes the key steps in the successful route.[30,31] The bis-MOM ether of olivetol was lithiated, added to (+)-apoverbenone, and the product oxidized in 79% overall yield to give enone **47**. Reflux in chloroform-ethanol in the presence of tosic acid led to racemic "Roche phenol"[33,34] **48** in 78% yield. Cleavage of both MOM groups in **47** takes place during this reaction, neccessitating a reprotection step of the phenol. Phenol **48** was converted to ether **49** by exposure to chloromethyl methyl ether and diisopropylethylamine in dichloromethane in 92% yield. Enone **49** was carried through the reactions of Scheme 5 to produce the MOM ether of racemic Δ^9-THC acid. Cleavage of the MOM protecting group in the last step was accomplished in 35% yield with pyridinium tosylate in refluxing 2-butanone to produce the racemic phenolic acid. The failure of this approach to control absolute stereochemistry of the final product or relative stereochemistry of the ring junction detracts from its appeal.

Huffman has applied this strategy much more successfully to an enantiodivergent synthesis of the enantiomers of the antinauseant drug nabilone (Scheme 6).[35] Since nabilone has the 1',1'-dimethylheptyl aromatic sidechain, the appropriate dimethoxy aryllithium was added to (+)-apoverbenone **9** to produce tertiary allylic alcohol **50**. Rearrangement-oxidation with pyridinium dichromate (PDC) led to enone **51** in 62% overall yield for the two steps. Birch reduction leads to the saturated ketone with the stereochemistry indicated in structure **52**. The stereochemistry is determined during the protonation step, with the proton attacking the molecule from the side opposite the *gem*-dimethyl bridge. Selective cleavage of one of the two aryl methoxyl groups was easily accomplished by treatment with sodium thiopropoxide.

Scheme 6

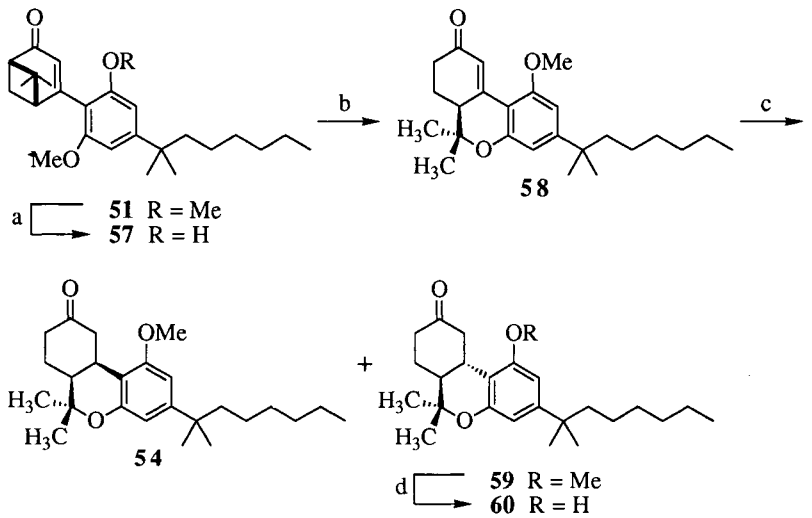


(a) **9** added to 2-lithio aryl anion, THF; (b) PDC, CH_2Cl_2, 2 h, r.t., 62% for two steps; (c) Li, NH_3 liq, THF, -78 °C, 76%; (d) *n*-PrSNa, DMF, r.t., then 120 °C, 3 h, 76%; (e) $SnCl_4$, $CHCl_3$, r.t., 18 h, 80%; (f) $AlCl_3$, CH_2Cl_2, r.t., 12 h, 78%; (g) *n*-PrSNa, DMF, r.t., then 120 °C, 3 h, 57%, based on starting material consumed.

Cleavage of the four-membered ring in **53** with formation of the tricyclic carbon skeleton was accomplished with stannic chloride at room temperature in 80% yield. The ring junction stereochemistry of the product is exclusively *cis*. Isomerization to the *trans* ring fused compound (**55**) was catalyzed by aluminum trichloride at room temperature. This key step had been described by Archer and coworkers in the first published synthesis of nabilone.[29] It is significant that the ring junction isomerization takes place without racemization. This implies that the mechanism for the process involves complexation of the Lewis acid with the dihydrobenzopyran oxygen atom, generation of the tertiary C6 carbocation, followed by *reversible* loss of the proton on C6a. In this instance bond cleavage at C10a cannot take place, as it did in **29** (eq 5), nor is there a mechanism for facile cleavage of the C6-C6a bond, as was seen in the transformation of **44** to **45**. Under more vigorous treatment with strong Lewis acid the product would likely have undergone racemization through the reversible formation of an *ortho*-quinone methide (*vide infra*). Cleavage of the phenolic methyl ether of **55** in the last step led to nabilone **56** in 57% yield, based on starting material consumed. This synthesis succeeds much better than the Δ^9-THC acid synthesis of Scheme 5 which was frustrated by the presence of the extra unit of

unsaturation in the aliphatic carbocyclic ring. Whereas the Birch reduction of **39** (Scheme 5) leads to a mixture of ring junction isomers, the Birch reduction of **51** (Scheme 6) takes place stereospecifically.

Scheme 7

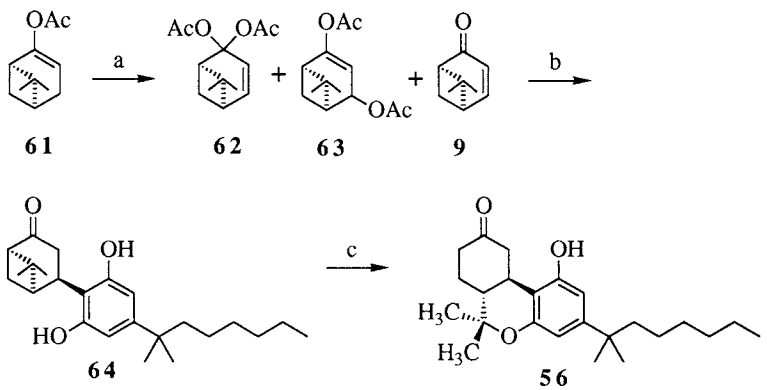


(a) *n*-PrSNa, DMF, r.t., then 120 °C, 3 h, 71%; (b) 3 equiv $AlCl_3$, CH_2Cl_2, r.t., 12 h, 55%; (c) Li, NH_3 liq, THF, -78 °C, 34% **54**, 50% **59**; (d) *n*-PrSNa, DMF, r.t., then 120 °C, 3 h, 72%.

The enantiomer of nabilone presumably could be obtained in the same way starting from (-)-apoverbenone. This material, in turn, could be prepared from (+)-β-pinene, which is available from the isomerization of commercially available (+)-α-pinene.[36] The neccessity of going through several steps in order to prepare the optically active starting material detracts from this approach. Huffman devised an elegant procedure to circumvent this problem by simply altering the sequence of reaction steps (Scheme 7). Intermediate **51** is common to both enantiomeric series. Prior cleavage of the phenolic methyl ether group led to phenol **57** which underwent aluminum trichloride catalyzed rearrangement-cyclization to **58** in 55% yield. Birch reduction of **58** led to the saturated ketones **54** and **59** in 34% and 50% yield, respectively. The major product of the Birch reduction was converted to (+)-nabilone **60** in 72% yield, in the usual way. Temperature influenced the ratio of products from the Birch reduction. At -33 °C no selectivity for the desired *trans* isomer could be observed, whereas at -80 °C a 2/1 ratio of **59** to **54** was observed. Performing the Birch reduction *subsequent* to the cyclization reaction makes it possible to obtain (+)-nabilone from the common intermediate **51**, but at the expense of diminished yields due to the formation of ring junction stereoisomers.

For the synthesis of cannabinoids which incorporate a *tert*-alkyl group at C1', the issue of "abnormal" isomers (cf. eq 1) is moot: the steric bulk of the alkyl group effectively forces C-C bond formation to take place at C2. Consequently, for the synthesis of nabilone, and for cannabinoids derived from nabilone, it is *unnecessary* to perform the extra steps which are associated with the directed metallation of the aromatic residue. A very effective synthesis, which uses an improved version of the Lilly strategy, is summarized in Scheme 8.[37]

Scheme 8



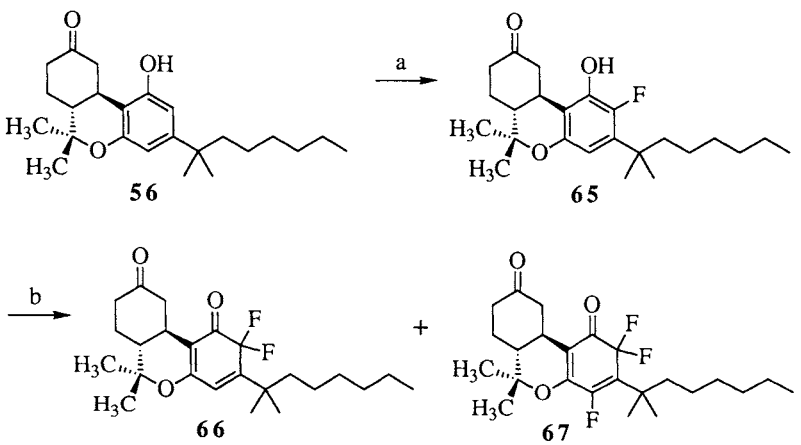
(a) $Pb(OAc)_4$, PhH, gentle reflux, 2 h; (b) 1,3-dihydroxy-5-(1,1-dimethylheptyl)-benzene, aq TsOH, $CHCl_3$, r.t., 1 d, 76% overall for two steps; (c) CH_3NO_2, 1.5 equiv Me_3SiOTf, 0 °C to r.t., 5 h, 98%.

Enol acetate **61** was prepared according to the Lilly procedure, and was oxidized with lead tetraacetate to a 65/30/5 mixture of diacetates **62** and **63**, and apoverbenone **9**. This reaction was also described in the original procedure, however, the key differences are, (a) to conduct the oxidation under the mildest conditions possible, and (b) not to distil the reaction product. Distilling the reaction product led to sharply diminished yields. If the oxidation is conducted at higher temperature, the proportion of apoverbenone **9** in the product increases. Distilling the reaction product has the same effect. The acid catalyzed condensation of 1,3-dihydroxy-5(1,1-dimethylheptyl)-benzene with diacetates **62** and **63** leads to the product **64**, whereas the apoverbenone does not react under these conditions. Diacetates **62** and **63** presumably ionize to a common allylic carbocation which is intercepted by C2 of the resorcinol. The overall yield of **64** for the two steps is 76%. Optimum conditions for the ring cleavage and cyclization of **64** to (-)-nabilone **56** called for treatment with a moderate excess of trimethylsilyl triflate in nitromethane. This lead to an isolated yield for nabilone of 98% in excellent optical purity. The role of the silyl triflate may be to provide a source of anhydrous triflic acid, which is the likely catalyst for this reaction. The overall yield for the three steps of Scheme 8 is 74%. No protecting groups were

used in our work. The Lilly group reported a 24% overall yield,[29] whereas the overall yield for Scheme 6 is 13% for seven steps.

A more efficient method for the synthesis of nabilone was desired in order to probe the effects of aromatic halogen substitution on receptor binding. The phenolic hydroxyl is known to be an important determinant of the activity in both the classical and the non-classical cannabinoid series, suggesting a hydrogen bonding interaction with the receptor. Replacing the *ortho* hydrogen in nabilone with a fluorine atom would then be expected to modify the ligand-receptor interaction. The *ortho*-fluorination of (-)-nabilone was accomplished in 88% yield by simply treating the unprotected ketophenol with *N*-fluoropyridinium triflate at room temperature in dichloromethane (Scheme 9).

Scheme 9



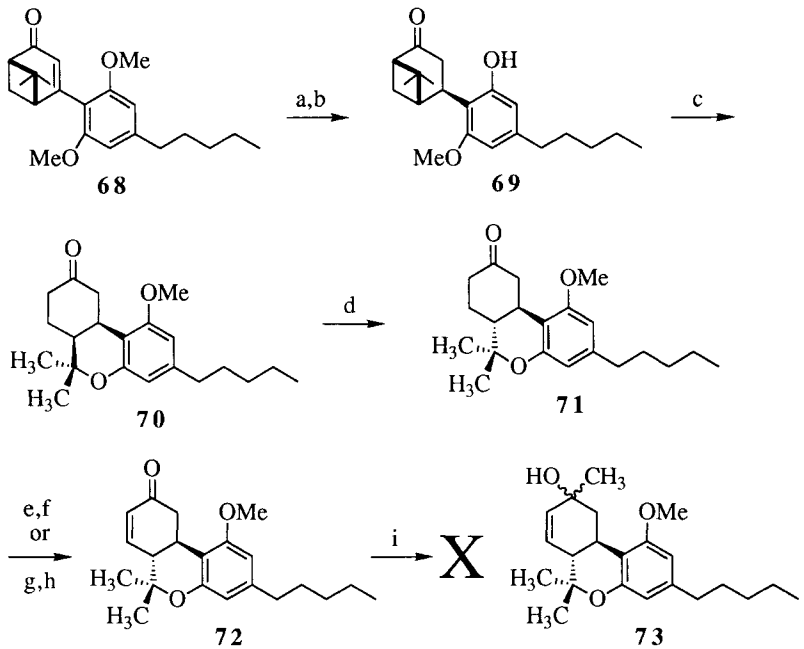
(a) 1 equiv *N*-fluoropyridinium triflate, CH_2Cl_2, r.t., 88%; (b) sealed tube, excess *N*-fluoropyridinium triflate, CH_2Cl_2, 90 °C, 80%, **66/67** = 3/1.

When either 2-fluoronabilone **65** or nabilone itself were treated with an excess of reagent at higher temperature, a 3/1 mixture of di- and trifluorinated products **66** and **67** were isolated in excellent yield. When **66** was isolated and treated with *N*-fluoropyridinium triflate, no fluorination to produce **67** took place, therefore **66** cannot be a precursor to **67**. 2,4-Difluoronabilone, the presumed intermediate for **67**, was not isolated from the reaction. All three fluorinated products showed diminished binding to the CB1 receptor compared with nabilone (for **65**, K_i = 40 nM; for **67**, K_i = 6159 nM).

The general strategy which was outlined in Schemes 5 and 6 has been used for the stereoselective synthesis of the epimeric Δ^7-THCs. These compounds were targeted as a result of an earlier computational study which had predicted diminished activity for compounds bearing C9 substituents pointing to the α-face of the molecule.[38] In order to determine whether the lack

of activity was solely due to the presence of Δ^7 unsaturation, or the α-substituent, both isomers of Δ^7-THC were prepared (Scheme 10).[39]

Scheme 10

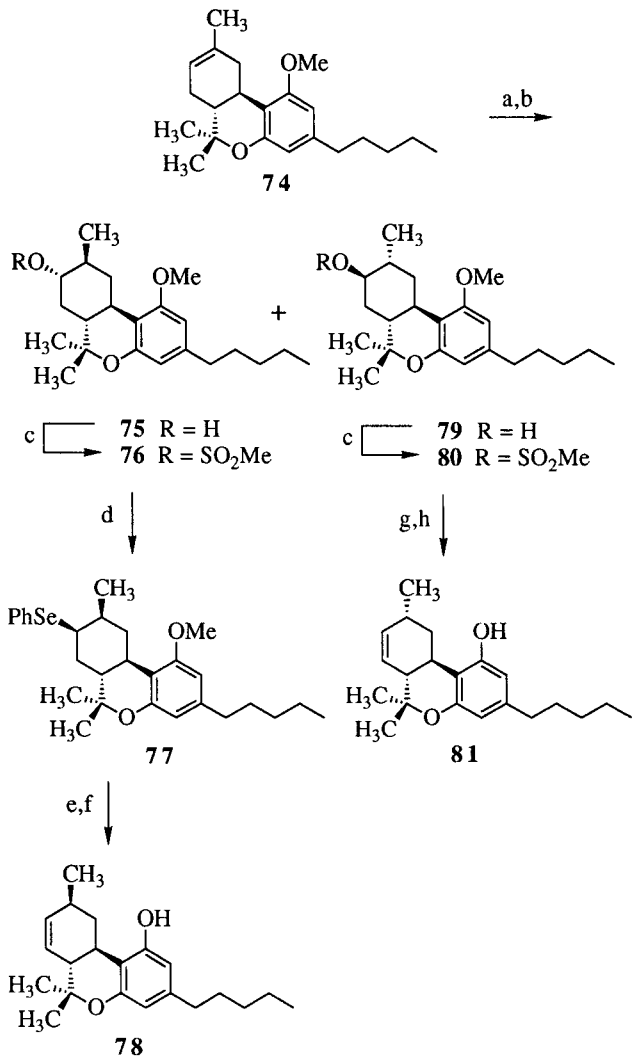


(a) Li, NH_3 liq, THF, -60 °C; (b) *n*-PrSNa, DMF, 120 °C, 71% for two steps; (c) $SnCl_4$, $CHCl_3$, r.t., 70%; (d) $AlCl_3$, CH_2Cl_2, r.t., 75%; (e) *t*-$BuMe_2SiOTf$, Et_3N, CH_2Cl_2, 25 °C; (f) $Pd(OAc)_2$, CH_2Cl_2, 40 °C, 51% for two steps; (g) PhSeCl, EtOAc, 25 °C; (h) H_2O_2, 0 °C, 44% for two steps; (i) see text.

This sequence of reactions parallels those described in Scheme 6, the major difference being in the aliphatic sidechain. Enone **68** was derived from (+)-apoverbenone, as before, and was first reduced stereospecifically, then one of the two phenolic hydroxyls was cleaved to produce **69**. Cleavage of the four-membered ring and cyclization to *cis* ring junction isomer **70** took place in 70% yield. Aluminum trichloride catalyzed isomerization of the ring junction stereochemistry led to natural *trans* ketone **71**. Introduction of $\Delta^{7(8)}$ unsaturation was accomplished either by treatment of the *tert*-butyldimethylsilyl enol ether with palladium acetate, or through a phenylselenylation-oxidative deselenation sequence, to produce enone **72**. All attempts to perform the nucleophilic addition of a methyl group to the carbonyl carbon of **72** failed. With methylmagnesium halides, methyllithium or methylcerium dichloride either starting material or complex reaction mixtures were recovered. The failure of this reaction was attributed to complexation of the phenolic ether oxygen to the metal of the reagent, followed by enolization.

Although the authors do not emphasize the point, this is potentially a very valuable observation, as it suggests an alternative approach to Δ^9-THC acid. It was also postulated that the the desired product, a tertiary allylic alcohol, may undergo decomposition under forcing conditions. Consequently, a non-stereospecific approach starting with the methyl ether of Δ^8-THC was adopted (Scheme 11).

Scheme 11



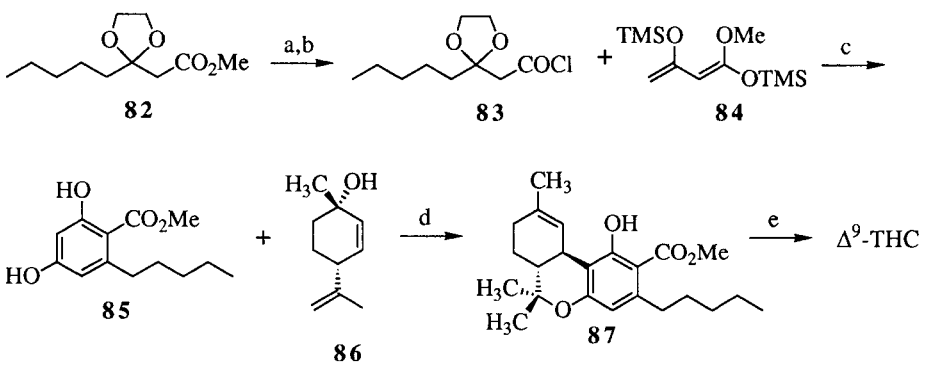
(a) BH_3, THF, 0 °C; (b) H_2O_2, NaOH, 25 °C, 77% for two steps; (c) $MeSO_2Cl$, Et_3N, CH_2Cl_2, -40 to -5 °C; 42% **76** + 35% **79**; (d) PhSeSePh, $NaBH_4$, EtOH, 25 °C; (e) H_2O_2, $NaHCO_3$, THF, 60% for two steps; (f) *n*-PrSNa, DMF, 120 °C, 68% ; (g) *t*-AmOK, *t*-AmOH, 102 °C, 46%; (h) *n*-PrSNa, DMF, 120 °C, 69%.

Hydroboration-oxidation of **74** took place in high yield to produce an epimeric mixture of alcohols **75** and **79** which were converted to the corresponding mesylates **76** and **80**. Mesylate **76** was treated with phenyl selenide anion, generated *in situ*, to produce selenoether **77**. Oxidation-elimination of phenylselenium was followed by phenolic methyl ether cleavage to produce β-methyl Δ^7-THC **78**. Treatment of mesylate **80** with potassium *tert*-amylate, followed by phenolic ether cleavage led to the α-methyl isomer **81**. Both isomers of Δ^7-THC were evaluated *in vitro* according to their ability to displace [^{3}H]CP-55,940 from a rat brain membrane preparation. As predicted by the earlier computational study, the K_i for isomer **78** was comparable to Δ^9-THC (71.5 nM vs. 41 nM for Δ^9-THC) whereas for isomer **81** K_i = 304 nM.

Biomimetic Approaches to the Cannabinoids

The biomimetic approach to the classical cannabinoids is complicated by the intervention of a competing pathway for cyclization which leads to the "abnormal" products discussed earlier in this chapter. Notwithstanding, this strategy has seen use both for Δ^9-THC synthesis, and also for the preparation of Δ^9-THC acid. A most ingenious solution to the problem is due to Chan (Scheme 12).[40]

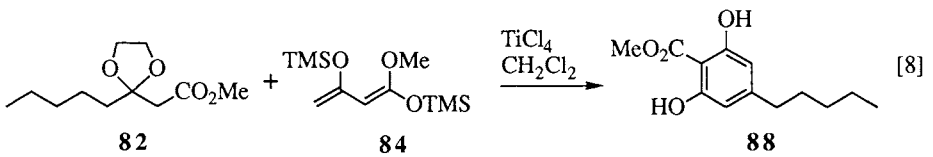
Scheme 12



(a) aq MeOH, NaOH; (b) $(ClCO)_2$, PhH; 100% for two steps; (c) CH_2Cl_2, $TiCl_4$, 55%; (d) see text, 55%; (e) 25% NaOH, MeOH, 78%.

Chan's synthesis of Δ^9-THC starts with protected β-ketoester **82** which is hydrolyzed to the carboxylic acid and converted to acid chloride **83**. A Mukaiyama aldol process of the acid chloride with 1,2-*bis*-(trimethylsiloxy)-1-methoxybutadiene **84** is catalyzed by titanium tetrachloride and leads directly to 6-carbomethoxyolivetol **85** in 55% yield. The carbomethoxy group in **85** blocks one of the two sites which are responsible for the "abnormal" condensation products, and also deactivates the aromatic ring toward electrophilic attack to a certain extent. The critical condensation reaction between **85** and one equivalent of (+)-*trans*-p-mentha-2,8-dien-1-ol

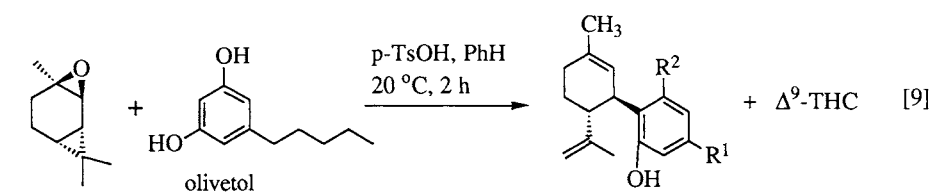
86 took place under the conditions which had been defined by Razdan in his pioneering synthesis (eq 1)[10]: the two reagents were mixed together for 20 min in dichloromethane in the presence of anhydrous magnesium sulfate. The solution was cooled to 0 °C, boron trifluoride etherate was added, and the mixture stirred for 1.5 h. Solid sodium bicarbonate was added, and the reaction mixture filtered and concentrated. Under these conditions **87** was isolated in 55% yield. Razdan's paper is instructive, for his thorough study and optimization of the corresponding reaction of **86** with olivetol makes it very clear that the reaction is successful only under a very narrowly defined set of conditions. In the final step of Chan's synthesis, base-mediated ester hydrolysis and decarboxylation produced Δ^9-THC in 78% yield.



82 **84** **88** [8]

It is noteworthy that the titanium tetrachloride catalyzed condensation of methyl ester **82** with diene **84** gives rise to 2-carbomethoxyolivetol **88** (eq 8) in 72% yield, and that neither aromatic cyclization product is contaminated with the other.[40] Ester **88** is potentially valuable as a starting material for cannabinoids through an alternative strategy (*vide infra*). The regiocontrol which is observed in the cycloaromatization reaction indicates that the reactivity order for the initial condensation step is acid chloride > ketal > ester.

The terpenic partner in these biomimetic syntheses need not have the menthane carbon skeleton. Other terpenes can enter the same reaction manifold through prior acid catalyzed rearrangement. For example, Crombie has shown that (+)-*trans*-2-carene epoxide also undergoes acid catalyzed condensation with olivetol (eq 9).[41,42]



(+)-*trans*-2-carene epoxide

olivetol

89 $R^1 = C_5H_{11}$, R^2 = OH

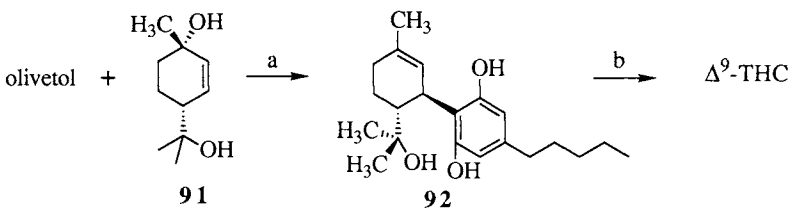
90 R^1 = OH, $R^2 = C_5H_{11}$ [9]

The reaction with tosic acid in benzene produces a 1/1/2 mixture of "ortho" and "para" cannabidiols **89** and **90** and Δ^9-THC. Traces of Δ^8-THC are also formed in this reaction. After 24 h under the reaction conditions, there were no cannabidiols detectable by glc. Exposure of (+)-trans-2-carene epoxide to tosic acid in benzene at 50 °C led to *cis*-p-mentha-2,8-dien-1-ol and other products. Therefore, it is likely that the reactions of equations 1 and 9, as well as the

condensation of Scheme 12, proceed through the same allylic carbocation intermediate. There is no need to postulate an alternative mechanism which involves attack of one of the phenolic oxygen atoms on the carbon bearing the *gem*-dimethyl group in carene epoxide.

Mechoulam has optimized the synthesis of cannabidiol **89** from the acid catalyzed condensation of olivetol with (+)-*cis*-p-mentha-2,8-dien-1-ol.[43] The reaction is conducted with a small excess of olivetol using boron trifluoride on basic alumina as the catalyst. The starting materials were added to a refluxing suspension of the catalyst and the reaction was quenched within 10 sec by adding 10% aqueous sodium bicarbonate. These conditions, which were evidently derived following a careful optimization scheme, give rise to **89** in 41% yield, along with 14% of the "abnormal" condensation product **90**.

Scheme 13



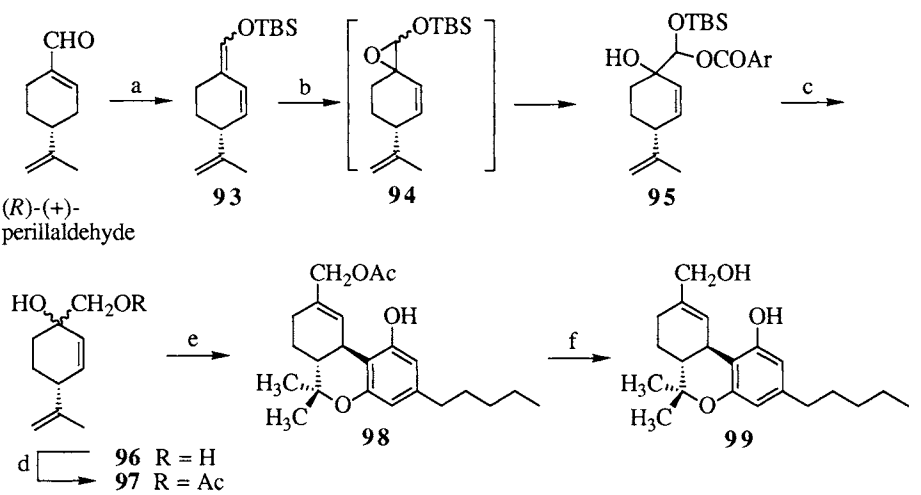
(a) CH_2Cl_2, p-TsOH, 24 h, r.t., 68%; (b) CH_2Cl_2, $ZnBr_2$, 3Å molecular sieves, reflux, 24 h, 72%.

A major improvement over Razdan's pioneering synthesis of Δ^9-THC has been disclosed by Stoss (Scheme 13).[44] In this work, olivetol was condensed with *cis*-p-menth-2-ene-1,8-diol **91**, under mild acid catalyzed conditions. The bicyclic product **92** was isolated in 68% yield as a solid which was purified by recrystallization. Purification of **92** is a critical step which confers a major advantage to this approach: it appears that the subsequent condensation leading to Δ^9-THC is far more successful when pure material is used. Exposure of **92** to anhydrous zinc bromide in refluxing dichloromethane leads to Δ^9-THC in 72% yield. This method is operationally simple, it can be applied to large scale, and it avoids the generation of significant quantities of the "abnormal" isomer, Δ^8-THC or *bis*-adducts of terpene to olivetol. It is not obvious why having **91**, rather than *p*-menthadienol **1**, react with olivetol should minimize the formation of the "abnormal" product, although it may be related to a relative increase in the unfavorable steric interaction between the tertiary alcohol and the *n*-pentyl sidechain, relative to isopropenyl, which *disfavors* the "abnormal" product.

The biomimetic strategy has been applied very successfully to the synthesis of Δ^9-THC acid.[45] This requires an optically active terpenic fragment which incorporates oxidation in C11. An obvious choice is (*R*)-(+)-perillaldehyde, which is now an article of commerce. A very convenient preparation from (+)-limonene oxide which is amenable to large scale synthesis has

also been described.[46] A brief synthesis of (-)-11-*nor*-Δ^9-THC-9-methanol **99** is summarized in Scheme 14. In the first step (+)-perillaldehyde was converted to the *tert*-butyldimethyl silyl (TBS) enol ether **93**. This has the effect of moving the C-C double bond within the six-membered ring, and also of activating the enol ether toward oxidation in the next step. Epoxidation in a two-phase ether/saturated aqueous sodium bicarbonate system led to the labile silyloxy epoxide **94** which was not isolated. Under the reaction conditions, this material underwent epoxide ring cleavage by *m*-chlorobenzoate leading to **95** (Ar=*m*-chlorophenyl). Reduction with lithium aluminum hydride produced a diastereoisomeric mixture of diols **96** in 66% overall yield from perillaldehyde. All attempts to condense olivetol with diol **96** failed. This result should be contrasted with the ease of cyclization of olivetol with *p*-mentha-2,8,dien-1-ol, which differs from **96** only in the presence of the primary hydroxyl group. Presumably, the primary hydroxyl group of **96** destabilizes the allylic carbocation sufficiently to suppress the desired reaction. By contrast, monoacetate **97** which was derived from **96** underwent boron trifluoride etherate catalyzed condensation with olivetol to provide tricyclic product **98** in 30% yield. Reductive removal of the primary acetate with lithium aluminum hydride gave (-)-11-*nor*-Δ^9-THC-9-methanol **99** in 94% yield. In spite of the low yield for the condensation-cyclization step, the overall yield of **99** from perillaldehyde was 19%.

Scheme 14

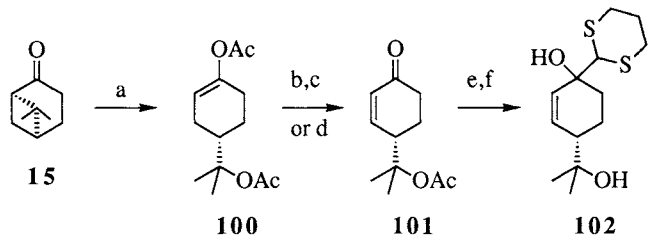


(a) *t*-BuMe$_2$SiOTf, Et$_3$N, CH$_2$Cl$_2$, 0 °C; (b) *m*-CPBA, Et$_2$O, satd aq NaHCO$_3$, 25 °C; (c) LiAlH$_4$, THF, 0 °C; work up with fluoride; 66% overall from perillaldehyde; (d) Ac$_2$O, pyridine, 100%; (e) olivetol, CH$_2$Cl$_2$, BF$_3$·Et$_2$O, 0 °C, 2 h, 30%; (f) LiAlH$_4$, THF, 94%.

The success of monoacetate **97** in the cyclization reaction can be ascribed to anchimeric assistance in the ionization of the tertiary, allylic alcohol through an acetoxonium ion. Acetate **98**

was successfully converted to Δ^9-THC acid through the following straightforward sequence of steps. The phenolic hydroxyl group was protected as the *tert*-butyldimethylsilyl ether, and the primary acetate was cleaved with lithium aluminum hydride. Swern oxidation of the C11 alcohol was followed by further oxidation to the carboxylic acid with sodium chlorite. In the final step the silyl ether protecting group was cleaved with fluoride to provide the Δ^9-THC acid in very good overall yield. This route is amenable to scaleup.

Scheme 15



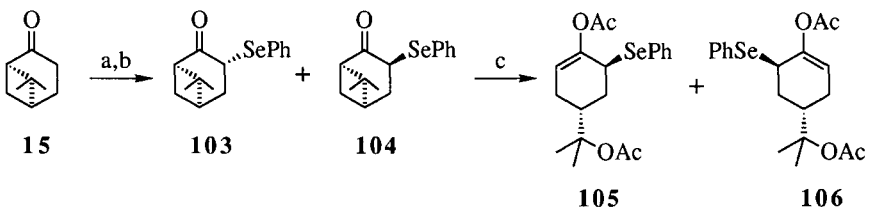
(a) $Zn(OAc)_2$, $BF_3{\cdot}Et_2O$, Ac_2O, 70-85%; (b) PhSeBr, $AgOSO_2CF_3$, PhH; (c) H_2O_2, THF, H_2O, r.t.; (d) $Pd(OAc)_2$, $Ph_2PCH_2CH_2PPh_2$, $H_2C{=}CHCH_2OCO_2Et$, Bu_3SnOMe; (e) excess 2-lithiodithiane, THF; (f) $LiAlH_4$, 82%.

A closely related synthesis of the C-11 oxygenated cannabinoids is due to Razdan.[47] Scheme 15 summarizes the synthesis of the optically active terpenic fragment which was used. The individual steps are illustrated starting from (*1R*)-(+)-nopinone, which is more readily available than its enantiomer, which is required in order to obtain the desired absolute stereochemistry of the cannabinoid final products. (1*R*)-(+)-Nopinone **15** was cleaved to diacetate **100** in high yield according to Yoshikoshi's procedure.[48,49] Oxidation of the enol acetate following Tsuji's protocol,[50] or more traditionally, by phenylselenylation-oxidative deselenation, gave acetoxy enone **101** with no loss of optical purity from nopinone. Exposure of the enone to an excess of 2-lithiodithiane, followed by reductive cleavage of the acetate with lithium aluminum hydride, led to dithiane diol **102** in 82% yield. The overall yield of **102** from nopinone was 82%. The same sequence of reactions was repeated starting with (*S*)-(-)-nopinone. The similarity of **102** and **97** (Scheme 14) should be noted. Both molecules are analogous to *p*-menthadienol, but incorporate oxidation at C11.

It would have been most convenient, and also much cheaper, to have been able to access the natural enantiomeric series starting from (1*R*)-(+)-nopinone. An unsuccessful attempt to do so is summarized in Scheme 16.[51] (1*R*)-(+)-Nopinone **15** was converted to a 70/30 mixture of α-phenylselenoketone diastereoisomers **103** and **104**. The α-heteroatomic substituent was introduced so as to be able to generate unsaturation in the product following ring cleavage specifically. The major isomer **104** was treated to the Yoshikoshi conditions for rearrangement,

leading to a roughly equimolar *mixture* of allylic selenides **105** and **106**. The facile [1,3] sigmatropic migration of phenylselenium in effect racemizes the product by scrambling the position of the double bond in the product.

Scheme 16



15 **103** **104** **105** **106**

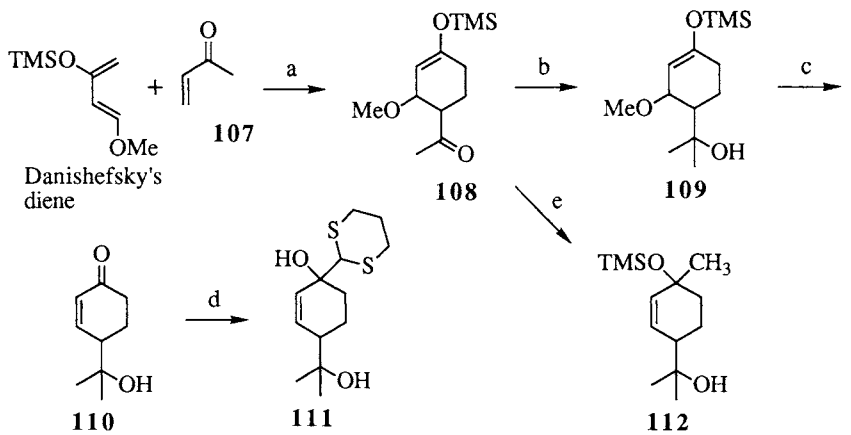
(a) LDA, THF; (b) PhSeBr, 91%, **103/104** = 30/70; (c) $Zn(OAc)_2$, $BF_3{\cdot}Et_2O$, **105/106** ca. 1/1.

A racemic synthesis of **102** (see Scheme 15) has also been disclosed.[51] The Diels-Alder reaction of Danishefsky's diene with methyl vinyl ketone **107** (Scheme 17) led to cycloadduct **108**, which was treated with methylmagnesium bromide to produce tertiary alcohol **109** in very high yield. Gentle acid catalyzed rearrangement of **109** led to hydroxyenone **110**. The conditions for this reaction must be chosen with care, otherwise dehydration of the teriary alcohol group in the product leads to a conjugated diene. Exposure of **110** to an excess of 2-lithiodithiane produces the racemic product **111** in excellent overall yield. It is interesting also that exposure of **108** to a large excess of methylmagnesium bromide leads to tertiary silyl ether **112**, of potential value for the synthesis of Δ^9-THC. The mechanism for this unexpected transformation probably involves Lewis acid catalyzed ionization of the methoxy group to form an oxoallylic carbocation, followed by nucleophilic trapping by the methyl Grignard reagent. The reactions of Scheme 17 have been applied very successfully to large scale synthesis. Recent advances in the methodology for performing enantioselective Diels-Alder cycloadditions suggests a role for this approach in the future.

The syntheses of 11-*nor*-Δ^9-THC-9-methanol and Δ^9-THC acid which were carried out using **111** are interesting because of the way they underscore the subtle nuances in the reactivity of this system (Scheme 18). Boron trifluoride catalyzed condensation of **111** with olivetol produced only the two benzofuran isomers **113** and **114**, resulting from "abnormal" and "natural" attack of the olivetol on **111**, respectively.[51] When tosic acid was used to catalyze the condensation, the tricyclic nucleus of the natural cannabinoids was formed, however, the "abnormal" isomer **115** was the major reaction product. The "natural" isomer **116** was isolated in 19% yield. The *cis* and the *trans* isomers of **111** each led to the same mixture of products **115** and **116**, therefore both isomers lead to the same intermediate, presumably the allylic carbocation which results from acid catalyzed ionization of the C9 hydroxyl. Significantly, no Δ^8 isomers were formed in this process. The Δ^8 isomer of **115** was prepared from the Δ^8 aldehyde and 1,3-

propanedithiol/boron trifluoride etherate, and was subsequently subjected to the same conditions which were used for the preparation of **115** (tosic acid in benzene at reflux). No isomerization to **115** was observed. This suggests that the Δ^9 isomers from the cyclization reaction are not thermodynamically favored. This, in turn, implies that the dithiane group destabilizes a C9 carbocation, and in this way prevents the usual isomerization of the Δ^9 to Δ^8 series. Mercury (II) catalyzed dithiane hydrolysis led to α,β-unsaturated aldehyde **117** in excellent yield. Carbonyl reduction with lithium aluminum hydride in the last step leads in satisfactory yield to racemic 11-*nor*-Δ^9-THC-9-methanol **99**.

Scheme 17



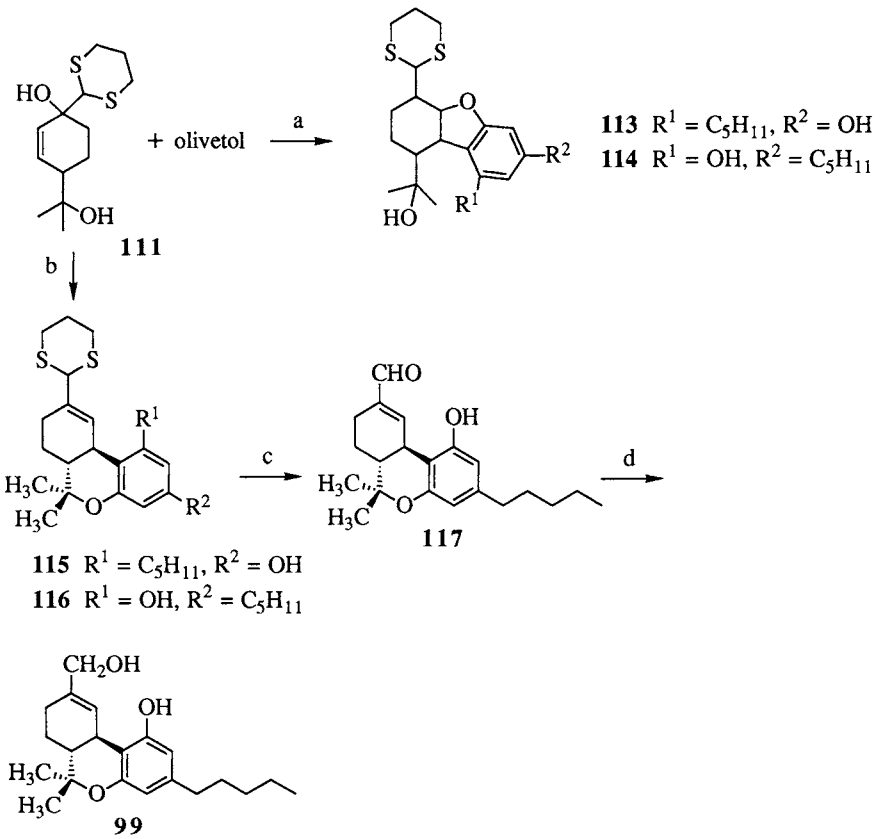
(a) PhH, reflux, ca. 20 h; (b) MeMgBr, Et_2O, 0 °C, 95% for two steps; (c) Cl_3CCO_2H, CCl_4, reflux, 5 h 71% for three steps; (d) excess 2-lithiodithiane, THF, 4/1 diasteroisomers, 66%; (e) large excess MeMgBr.

The condensation of **111** with 5-(1,1-dimethyl)heptyl resorcinol was also carried out. In this case, formation of the "abnormal" isomer was suppressed as a consequence of the steric effect which has been discussed earlier in this chapter. The desired isomer was isolated in 43% yield.

The conversion of **116** to Δ^9-THC acid was accomplished very effectively (Scheme 19).[52] Protection of the phenolic hydroxyl group in **116** with *tert*-butyldimethylsilyl chloride produced silyl ether **118**. Hydrolytic cleavage of the dithiane group was accomplished by treatment with an excess of iodomethane in aqueous DMF and potassium carbonate in 60% overall yield for the two steps. The α,β-unsaturated aldehyde was oxidized using a variant of Pellegata's procedure.[53] This procedure had been used in an earlier synthesis of the Δ^9-THC acid, and had been shown to proceed readily and without isomerization to the Δ^8 series. It was critical to use a large excess of 2-methyl-2-butene during the sodium chlorite oxidation. This is

probably necessary for the effective scavenging of chlorine, which would otherwise oxidize the electron-rich aromatic ring. Many alternative conditions for performing this step were tried unsuccessfully: silver oxide, potassium

Scheme 18

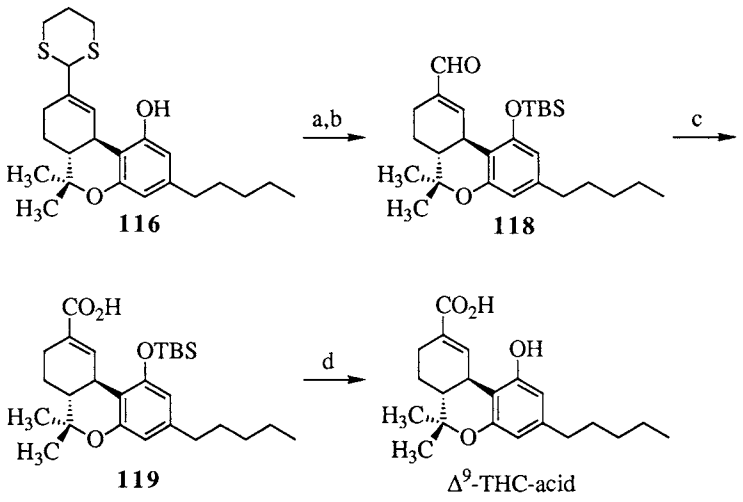


(a) $BF_3{\cdot}Et_2O$; (b) *p*-TsOH, PhH, 85 °C, 30% **115**, 19% **116**; (c) $BF_3{\cdot}Et_2O$, aq THF, HgO, r.t., 1 h, 80%; (d) $LiAlH_4$, Et_2O, r.t., 30 min, 53%.

permanganate and pyridinium chlorochromate. When manganese dioxide in the presence of sodium cyanide and methanol was used, a mixture of Δ^8 and Δ^9 methyl ester isomers were obtained. When the phenolic hydroxyl group in **118** was protected as the acetate rather than the silyl ether, yields of the acid were much lower, presumably because the acetate underwent partial hydrolysis during the reaction. This work demonstrates that even a procedure as simple as the oxidation of an aldehyde to an acid is far from straightforward in the cannabinoid system which can undergo double bond isomerizations and undesired oxidative processes involving the aromatic

ring. Fluorodesilylation of **119** with tetra-*n*-butylammonium fluoride in THF completed the synthesis of racemic Δ^9-THC acid. The reactions of Scheme 19 were duplicated with the (+)- and the (-)-series.

Scheme 19

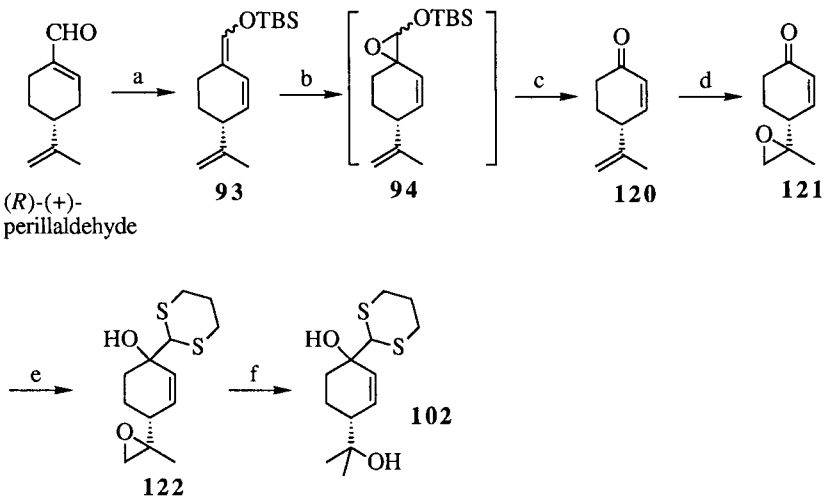


(a) *t*-$BuMe_2SiCl$, imidazole, THF, r.t.; (b) MeI, DMF, K_2CO_3, H_2O, 60% for two steps; (c) *t*-BuOH, H_2O, 2-methyl-2-butene, $NaClO_2$, NaH_2PO_4, 86%; (d) *n*-Bu_4NF, THF, 96%.

In order for the Δ^9-THC acid to be of use as a forensic tool, it is necessary that it be optically pure. Therefore, it is of interest to discuss the means by which the reactions of Schemes 18 and 19 were applied to the enantioselective synthesis of Δ^9-THC acid. This exercise reduces to the synthesis of optically enriched or optically pure **111** (Scheme 17), which was accomplished through a trivial modification of the reactions of Scheme 14. (*R*)-(+)-Perillaldehyde was converted to the *tert*-butyldimethylsilyl enol ether **93**, which was epoxidized to **94** with *m*-chloroperoxybenzoic acid in a two-phase system, as in Scheme 14 (Scheme 20). If the epoxidation of the enol ether is not stopped immediately after the disappearance of the starting material, which can be ascertained by tlc, then addition of *m*-chlorobenzoic acid to the product leads to **95**. Treatment of silyloxy epoxide **94** with sodium periodate and hydrofluoric acid in aqueous acetonitrile, or treatment of **95** under the same conditions for a longer period of time, leads to enone **120** in 58% overall yield for the three steps. Under these reaction conditions, **94** or **95** undergoes initial hydrolysis to an α-hydroxy aldehyde, which is immediately cleaved by periodate to the ketone in the usual manner, through the intermediacy of the hydrate. Performance of the hydrolysis and oxidative cleavage steps sequentially results in substantially lower yields, presumably due to the lability of the α-hydroxy aldehyde. Regioselective monoepoxidation of **120** takes place at the non-conjugated double bond to produce a 1/1 mixture of epoxide

diastereoisomers **121**. Addition of 2-lithiodithiane to the ketone carbonyl group, followed by reductive cleavage of the epoxide with lithium aluminum hydride leads to **102**, as a mixture of *cis* and *trans* isomers, in excellent overall yield. Proceeding from **102**, the optically active Δ^9-THC acid was prepared, as well as the analog with the 1',1'-dimethylheptyl sidechain.

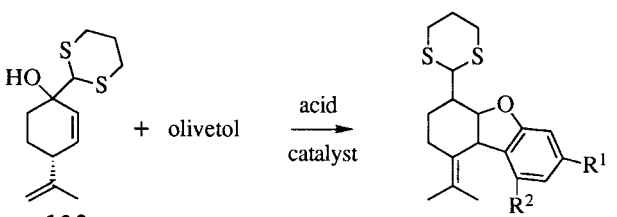
Scheme 20



(a) *t*-$BuMe_2SiOTf$, Et_3N, CH_2Cl_2, 0 °C, 100%; (b) *m*-CPBA, Et_2O, satd aq $NaHCO_3$, 30 min, 25 °C; (c) aq CH_3CN, HF, $NaIO_4$, 58% for three steps; (d) *m*-CPBA, CH_2Cl_2, 10 h, 85%, 1/1 isomers; (e) 2-lithiodithiane, THF, r.t., 60%; (f) $LiAlH_4$, Et_2O, 0 °C to r.t., 1 h, 78%.

The primary difference between the terpenoid products of Schemes 14 and 20 is in the "lower half" of the molecule: in **102** there is a 2-hydroxyisopropyl, rather than an isopropenyl substituent. The introduction of this hydroxyl group required two extra steps, the epoxidation and the reductive cleavage of the epoxide. The reason for the extra work can be understood from equation 10. Condensation of olivetol with **123** the terpenic fragment with an isopropenyl group, with either boron trifluoride etherate, tosic acid or trichloroacetic acid as catalyst, gave only 3 to 4% of the desired tricyclic product **116** (see Scheme 19). The major product was tentatively identified as a mixture of regioisomers **124** and **125** (eq 10). The conclusion to be drawn from this is that the tertiary hydroxyl group in the "bottom half" of the **102** facilitates the formation of the heterocyclic ring, relative to analog **123** which bears an isopropenyl substituent. This is certainly true for the reaction conditions which were used (however see also Scheme 21). With both substrates the first steps are the same, namely, acid catalyzed ionization of the tertiary alcohol at C9 to generate an allylic carbocation, followed by C-C bond formation with olivetol. Nevertheless, at first glance this is a puzzling result, since acetate **97** (Scheme 14) was used very

successfully. Evidently, the dithiane group in **123** and **102** (Scheme 20) is doing more than just inhibiting the Δ^9 to Δ^8 isomerization. Razdan suggests[51] that the inhibition of the isomerization occurs because the dithiane destabilizes the β-carbocation (at C9), and that the dithiane also stabilizes the γ-carbocation (at C10), which results in the formation of the benzofuran isomers **124** and **125**. Razdan suggests that this is surprising and unexpected, since sulfur usually stabilizes β-cations over γ-cations, by the favored formation of three-membered, over four-membered sulfonium rings. This is certainly true, however, in these allylic systems there is a plausible mechanistic pathway (eq 11) which accounts for the observations of equation 10 and Scheme 18. Equation 11 shows the product **126** of the initial condensation between olivetol and the allylic carbocation derived from ionization of **123**. Under the reaction conditions (tosic acid in refluxing benzene) reversible proton transfer to one of the two sulfur atoms of the dithiane can take place. This can result either in the concerted process shown, or in the (reversible) formation of another allylic carbocation which is substituted with sulfur at its one terminus. In either case, attack of the phenolic oxygen at C10 takes place to form benzofuran **127**. The sulfur here does not directly stabilize a γ-carbocation, but through this indirect process, perhaps involving the generation of an allylic carbocation. The enol sulfide functionality in intermediate product **127** can also undergo reversible protonation; the carbocation intermediate is intercepted by the adjacent sulfur so as to restore the six-membered ring of the dithiane. Proton loss leads to **128**. If one assumes, as Razdan does,[51] that the formation of the dihydrobenzopyran ring is slow in the isopropenyl series, then the faster step which converts **126** to **127** leads to the observed benzofuran products. In the 2-hydroxyisopropyl series the situation is reversed: formation of the dihydrobenzopyran ring is the fast step.

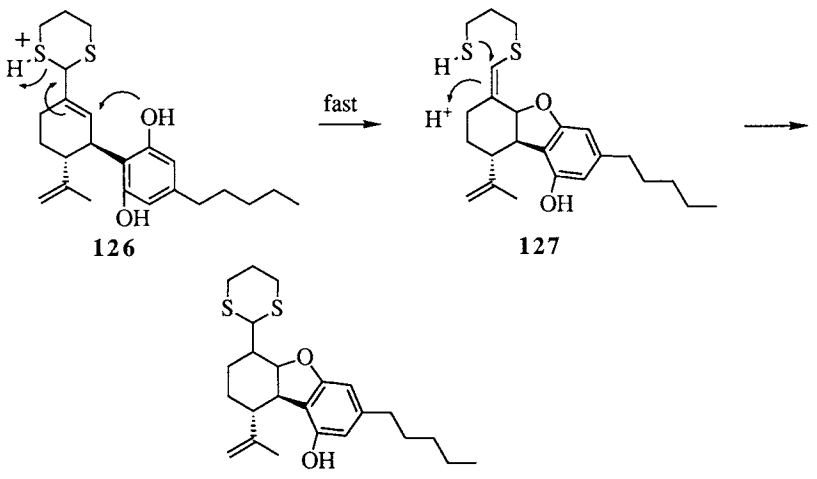


123

[10]

124 $R^1 = C_5H_{11}$, $R^2 = OH$

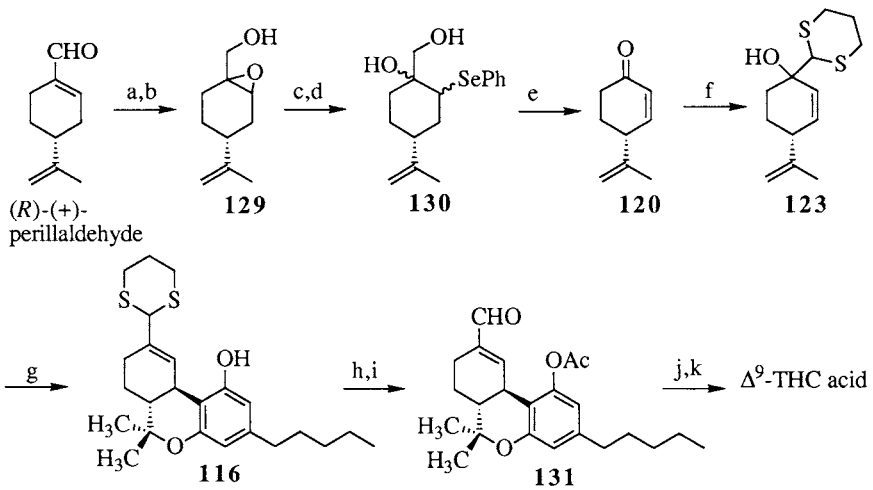
125 $R^1 = OH$, $R^2 = C_5H_{11}$



[11]

An optimized version of Razdan's synthesis of Δ^9-THC acid has been described (Scheme 21).[54] The starting material once again was (*R*)-(+)-perillaldehyde,[46] which was reduced to perilla alcohol and selectively epoxidized to **129**. Since the enantiomers of perilla alcohol are now articles of commerce, synthesis of the starting material is no longer necessary. Acetylation of the primary alcohol, followed by regioselective epoxide ring opening with phenyl selenide produced selenide diol **130**. Exposure of **130** to sodium periodate in aqueous acetonitrile led to dienone **120** (see Scheme 20). Addition of 2-lithiodithiane to the ketone gave tertiary alcohol **123** which was condensed with olivetol under very mild conditions to produce tricyclic cannabinoid **116** in 43% yield. This is a significant result, because it shows that through the choice of milder reaction conditions, the undesired reaction manifold leading to benzofuran products (cf. eq 10) can be avoided. The phenolic hydroxyl group of **116** was protected as the acetate, and the dithiane ring was hydrolyzed through a mercuric ion catalyzed reaction leading to **131** in 51% overall yield for the two steps. The choice of acetate as the protecting group for the phenol may not have been optimal, considering Razdan's earlier observations. Oxidation of the α,β-unsaturated aldehyde functionality of **131** with manganese dioxide in methanol in the presence of cyanide and acid, followed by hydrolytic cleavage of the phenolic acetate and the methyl ester, led to Δ^9-THC acid. No yield was specified for the last two steps. Notwithstanding, this work represents an optimization of Razdan's original synthesis; it can probably be improved further by choosing a different protecting group for the phenol.

Scheme 21



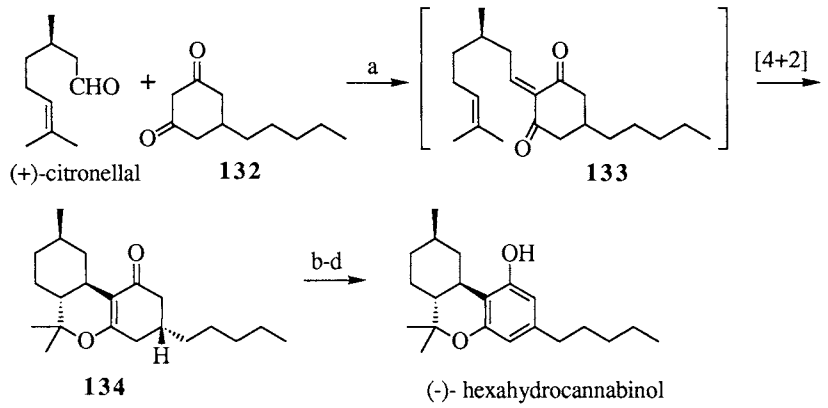
(a) $LiAlH_4$, Et_2O, r.t., 1 h; (b) $VO(acac)_2$, t-BuOOH, $PhCH_3$, 2 h, r.t., 90% for two steps; (c) Ac_2O, pyridine, overnight, r.t., 94%; (d) PhSeSePh, $NaBH_4$, EtOH, 24 h, r.t., 88%; (e) $NaIO_4$, aq CH_3CN, 2 h, r.t., 44%; (f) 2-lithiodithiane, THF, 45 °C, 56%; (g) olivetol, $BF_3{\cdot}Et_2O$, anh $MgSO_4$, CH_2Cl_2, 1 h, -63 °C, 43%; (h) Ac_2O, pyridine, overnight, r.t.; (i) $HgCl_2$, HgO, aq CH_3CN, 1.5 h, reflux, 51% for two steps; (j) NaCN, MnO_2, HOAc, MeOH; (k) aq KOH, EtOH.

Cycloaddition Reactions for the Synthesis of Cannabinoids

In total synthesis it is often true that the most efficient approach to a particular structure is by means of a Diels-Alder cycloaddition. The efficiency of the process derives from being able to form multiple C-C bonds simultaneously, often with very good control of stereochemistry. Consequently, it should come as no surprise that strategies making use of a cycloaddition reaction as the key step were used early on to prepare cannabinoids. Scheme 22 summarizes Tietze's contribution to this area.[55] 5-*n*-Pentyl-1,3-cyclohexanedione **132** was condensed at 100 °C with (*R*)-(+)-citronellal in a tandem aldol-dehydration-cycloaddition process to give tricycle **134** in 65% yield as a ca. 1/2 mixture of diastereoisomers. Diketone **133** is the postulated intermediate which undergoes an intramolecular hetero Diels-Alder reaction. The stereochemistry of the ring junction is controlled by the C9 methyl group, and results from a chair-cyclohexane transition state for the incipient ring, in which the C9 methyl is equatorial.[56] The conversion of **134** to (-)-hexahydrocannabinol (HHC) took place in three steps: enolization of the ketone, phenylselenation and oxidative deselenation. The overall yield of aromatic product was 70% and 50% from each of the two isomers of the phenylselenoketone. The synthetic utility of this synthesis is marred by the need for an oxidative aromatization step. Also, 5-*n*-pentyl-1,3-cyclohexanedione is less available than olivetol, therefore the better approach would have been to devise a method which utilizes

olivetol. Such a synthesis would also require fewer steps. Several such approaches have been demonstrated successfully, and are discussed in what follows.

Scheme 22

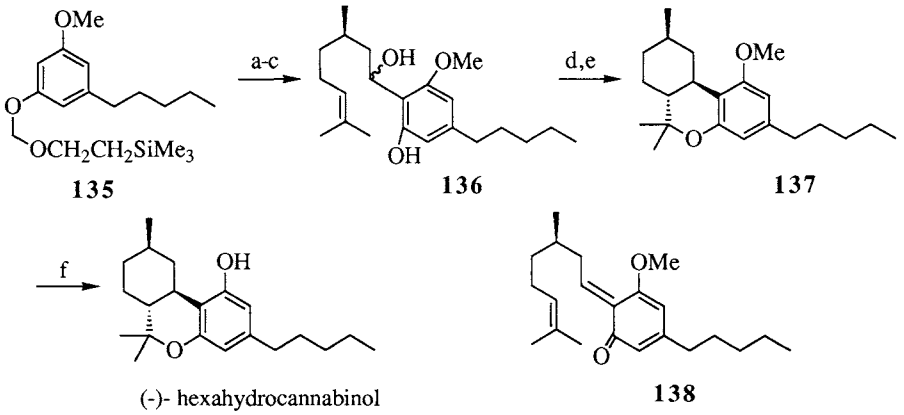


(a) 100 °C, DMF, 65%; (b) LDA, THF; (c) PhSeCl, 65% for two steps; (d) *m*-CPBA, 3,5-dimethoxyaniline, -40 to 25 °C, 70% from β-phenylselenoketone, 56% from α-phenylselenoketone.

An obvious retrosynthetic disconnection of HHC leads to a quinone methide. A number of similar syntheses of HHC which proceed through such an intermediate have been described. Marino has described a brief synthesis which proceeds from the 2-(trimethylsilyl)ethoxymethyl (SEM) ether **135** (Scheme 23).[57] Regiospecific metallation at C2 of **135** with *tert*-butyllithium, followed by addition of (*R*)-(+)-citronellal and cleavage of the SEM group led to **136**, which was isolated in 40% overall yield from **135**. Persilylation of **136** took place with trimethylchlorosilane and pyridine in refluxing benzene. The entire reaction mixture was added to an excess of cesium fluoride in refluxing acetonitrile to produce the methyl ether of HHC in 80-90% yield. Selective cleavage of the phenol silyl ether with elimination of the benzylic trimethylsilyloxide was postulated to generate intermediate *o*-quinone methide **138** which undergoes the intramolecular cycloaddition reaction to generate **137**. Cleavage of the methyl ether in the last step with sodium thioethoxide leads to (-)-HHC.

The stereochemistry of the cycloaddition reaction again follows from a consideration of the most stable chair cyclohexane transition state. Transition state **139** which has the C9 methyl group equatorially disposed in the developing chair form of the six-membered carbocycle accounts for the observed stereochemistry.

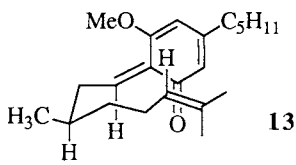
Scheme 23



135 **136** **137**

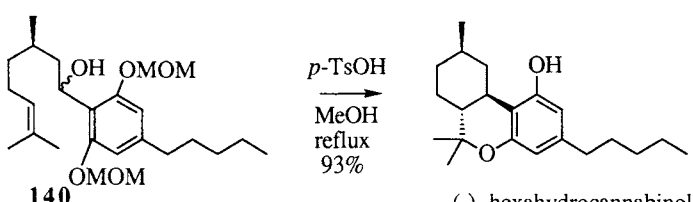
(-)- hexahydrocannabinol **138**

(a) *t*-BuLi, hexane; (b) (+)-citronellal, THF; remove solvent; (c) *n*-Bu_4NF, THF, reflux, 40% for three steps; (d) Me_3SiCl, pyridine, PhH, reflux; (e) 7 equiv CsF, CH_3CN, reflux, 40 h, 80-90% for two steps; (f) NaSEt.

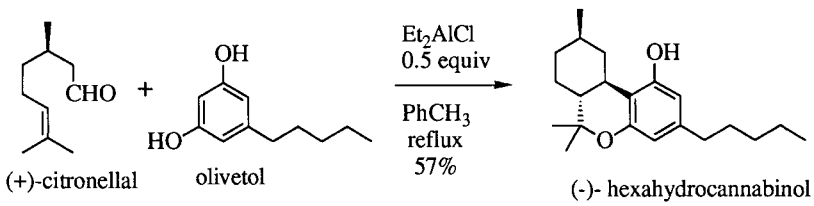


139

It is not obvious why the two oxygen atoms of olivetol in **135** were required to have different protecting groups. A more closely related approach to HHC which is more efficient is summarized in equation 12.[58] The *bis*-(methoxy)methyl ether of olivetol was metallated with *n*-butyllithium and N,N'-tetramethylethylene diamine (TMEDA), and the anion was condensed with (*R*)-(+)-citronellal to produce **140** in 75% yield. Simply heating **140** at reflux in methanol for 6 h in the presence of half an equivalent of tosic acid led to (-)-HHC in 93% yield. Acid catalyzed cleavage of both MOM groups is followed by ionization of the secondary benzylic alcohol to produce a quinone methide in what is probably a reversible step. The cycloaddition to form (-)-HHC takes place irreversibly. In terms of number of steps as well as overall yield, the synthesis of equation 12 is superior to that of Scheme 23. An even more efficient synthesis of (-)-HHC has been described.



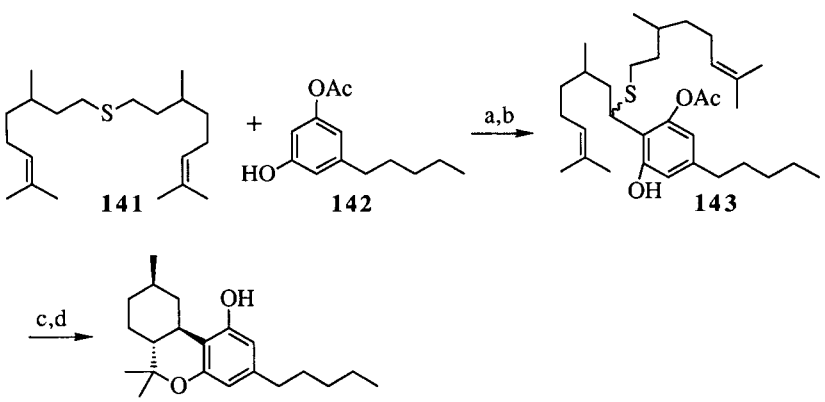
140 (-)- hexahydrocannabinol [12]



(+)-citronellal olivetol (-)- hexahydrocannabinol [13]

Simply mixing (*R*)-(+)-citronellal with olivetol in toluene with half an equivalent of diethylaluminum chloride, and heating the mixture to reflux leads to (-)-HHC in 57% isolated yield (eq 13).[59,60] This transformation can be thought of as taking place through an initial Lewis acid catalyzed reaction at C2 of olivetol, followed by Lewis acid catalyzed elimination of a water molecule to form an *o*-quinone methide which cyclizes to (-)-HHC. The equivalent of water which is generated during the reaction is scavenged by the diethylaluminum chloride. In this instance the elusive goal of a one-step synthesis of a natural product, starting from commercially available starting materials, is realized! One should note that the regiospecific metallation of the aromatic is unnecesary, phenolic protecting groups are unneeded, and elimination of the benzylic hydroxyl requires no activation.

Scheme 24



141 **142** **143**

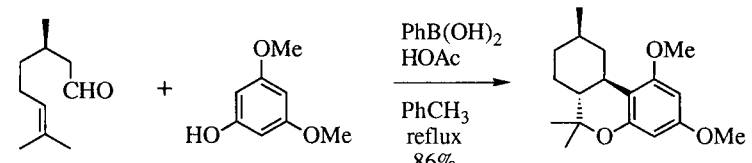
hexahydrocannabinol

(a) SO_2Cl_2, pyridine, CH_2Cl_2; (b) Et_3N, cyclohexane, 25% for two steps, + 23% "abnormal" product; (c) $NaBH_4$, EtOH, 50 °C, 18 h; (d) 0.5 equiv $BF_3{\cdot}Et_2O$, PhH, r.t., 2 h, 71% for two steps.

There are several other related approaches to HHC which have been reported which are less efficient than that of equation 13, but which are of heuristic interest, and will be mentioned. Scheme 24 shows one such approach which makes use of a thioether **141** to alkylate olivetol monoacetate **142**.[61] The thioether is chlorinated with sulfuryl chloride and pyridine and allowed to react with **142** to form an intermediate phenoxysulfonium ylide which undergoes [2,3]

sigmatropic rearrangement to form the C-C bond at C2 of the olivetol fragment. The yield of **143** was 25% and a nearly equimolar quantity of the "abnormal" isomer was also isolated from the reaction. Reductive cleavage of the acetoxy group in **143**, followed by treatment with boron trifluoride etherate, led to HHC in 71% yield.

Another approach to the HHC system which avoids the aryl metallation step makes use of phenylboric acid as a catalyst. Equation 14 summarizes the salient features of this reaction.[62] Exposure of equimolar quantities of (*R*)-(+)-citronellal, 3,5-dimethoxyphenol and phenylboric acid in dry toluene to an excess of glacial acetic acid at reflux for 20 to 40 h under a Dean-Stark water separator led to tricyclic compound **144** in 86% yield. The ring fusion stereochemistry was *trans*, however, **144** was present as a 90/10 equatorial/axial mixture of isomers at C9 (cannabinoid numbering). The erosion of stereocontrol in this cyclization may be attributable to the rather vigorous conditions for generation of the *o*-quinone methide. The phenylboric acid is thought to catalyze the initial C-C bond making reaction by first forming the 3,5-dimethoxyphenyl borate ester with loss of a water molecule, then acting as a Lewis acid toward the carbonyl oxygen atom of the citronellal. The utility of this protocol for cannabinoid synthesis will depend upon whether the "abnormal" isomer can be avoided during the cyclization. Regrettably, none of the reactions which have been reported reveal the regiochemical preference, since the phenols have had identical substituents at C3 and C5.

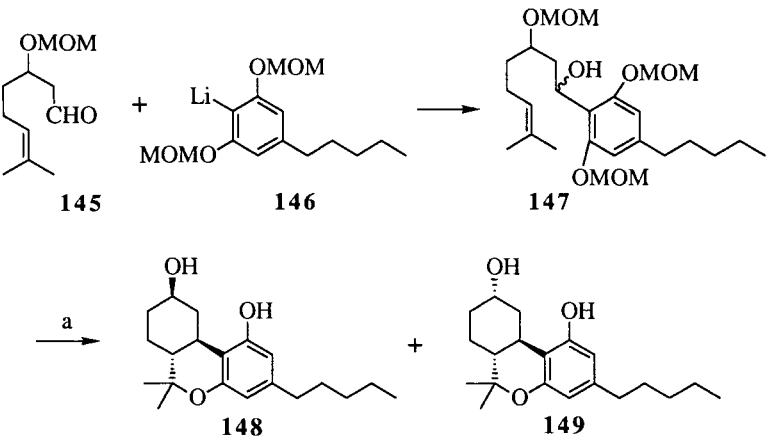


(+)-citronellal 3,5-dimethoxyphenol **144** [14]

Scheme 25 describes the synthesis of racemic 9-nor-9-hydroxyhexahydrocannabinol and provides yet another example of the idiosyncratic nature of much of this chemistry.[63] The racemic aldehyde **145** was prepared in four steps from commercially available 5-hepten-2-one: (1) regioselective carbomethoxylation with dimethyl carbonate and sodium hydride; (2) ketone reduction with sodium borohydride; (3) conversion of the hydroxyl to the MOM ether with dimethoxymethane and phosphorus pentoxide; (4) reduction of ester to aldehyde with diisobutylaluminum hydride. The *bis*-MOM ether of olivetol was metallated by treatment with *n*-butyllithium in ether/hexane in the presence of TMEDA, and was added to aldehyde **145**. The yield for the addition step was 40%. Adduct **147** was heated to reflux in anhydrous methanol in the presence of tosic acid for 3 h to produce in 76% yield a mixture of equatorial and axial C9 hydroxyl isomers **148** and **149** in 76% yield. In contrast to the cases discussed previously, the major product from this reaction is the axial alcohol. This result is difficult to rationalize, but it seems to suggest that an intramolecular hydrogen bonding interaction involving the C9 hydroxyl

may play an important role in determining the preferred transition state geometry for cyclization. This example illustrates the point which has been made earlier, namely that even seemingly minor perturbations in structure can have profound influences on the reactivity of these systems. This is one of the reasons that it is so challenging to devise truly general efficient synthetic approaches to the cannabinoids bearing C9 or C11 functionality.

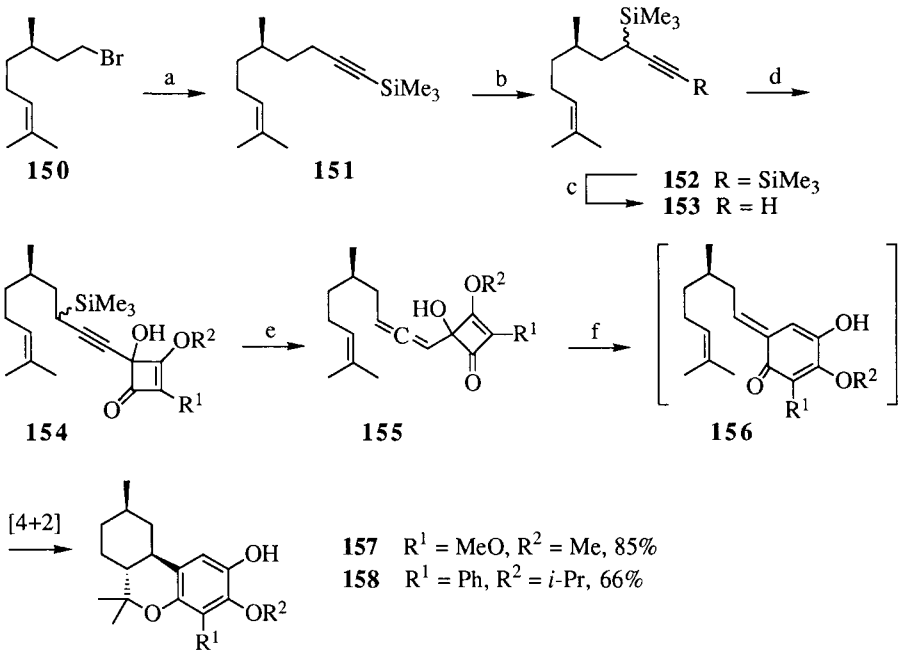
Scheme 25



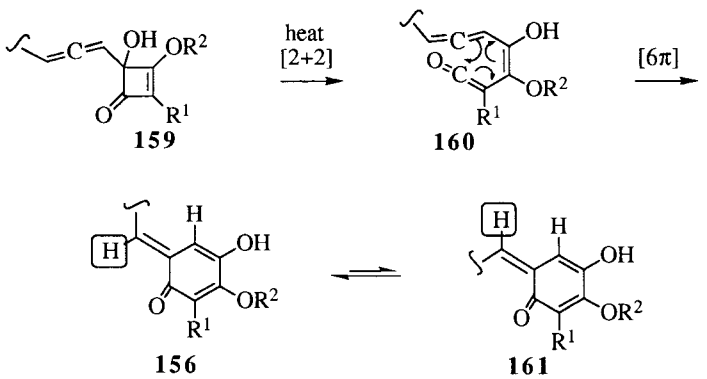
(a) anhydrous MeOH, p-TsOH, reflux, 3 h, 76%, **148/149** = 35/65.

A very different approach to HHC analogs through *o*-quinone methide cycloadditions is due to Moore.[64] In Moore's approach, a cascade of electrocyclic processes results in the assembly of the cannabinoid skeleton (Scheme 26). Commercially available (*R*)-(+)-citronellyl bromide **150** was treated with the lithium trimethylsilyl acetylide to produce silyl acetylene **151**. Deprotonation of **151** with *tert*-butyllithium took place to give a propargyl anion which was trapped with trimethylsilyl chloride to give **152**. Silver assisted desilylation of the acetylenic trimethylsilyl group led to **153** in moderate overall yield for the two steps. Removal of the acetylenic hydrogen with *n*-butyllithium, followed by addition of the lithium acetylide to a cyclobutene dione, led to adduct **154** in good yield. The regiochemistry for acetylide anion addition follows from a consideration of the relative electronegativities of the two carbonyl groups in the cyclobutene dione: the carbonyl group which is slow to react with the nucleophile, and which survives intact in the product, is part of a vinylogous ester. Fluoride ion induced rearrangement of **154** gave the allene **155** which was gently warmed in benzene to produce HHC analogs **157** and **158** in excellent overall yield. Allene cyclobutenone **155** presumably rearranges to *o*-quinone methide **156**, which cyclizes in the usual manner. The mechanism for the conversion of **155** to **156** is shown in equation 15.

Scheme 26



(a) $Me_3SiC{\equiv}CLi$, THF, DMSO, 0 °C, 70%; (b) *tert*-BuLi, Me_3SiCl, -23 °C; (c) EtOH, KCN, $AgNO_3$, 45% for two steps; (d) *n*-BuLi, THF, -78 °C; add cyclobutene dione, 71-80%; (e) *n*-Bu_4NF, THF, 80-84%; (f) PhH, 50 °C, 36 h for **157**, 40 °C, 7 h for **158**.

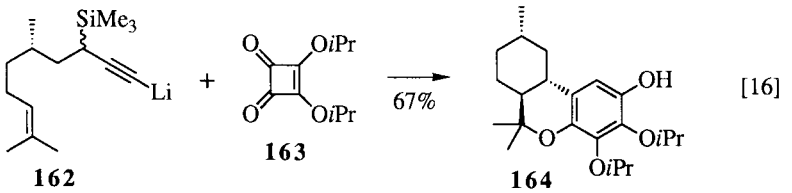


[15]

A retro [2+2] cycloaddition cleaves the four-membered ring in **159**. The conrotation takes place torquoselectively with the hydroxyl group rotating outward to form ketene **160**. This is followed by a [6π] electrocyclic process which generates *o*-quinone methide **156**. The [4+2] cycloaddition reaction which forms the dihydrobenzopyran ring of the final product can only take place from isomer **156** which is predicted to be favored over its geometrical isomer **161**: the steric

interactions between the rest of the molecule and the ketene carbonyl oxygen are more important than the interactions with the ring hydrogen. As a consequence, during the conversion of **160** to **161** rotation of the allene takes place in such a way as to place the encircled hydrogen *syn* to the carbonyl oxygen.

The reactions of Scheme 26 were also carried out starting from (*S*)-(-)-citronellyl bromide to produce **164** (eq 16). Lithioacetylide **162** was allowed to react with cyclobutenedione **163**, and the adduct was converted in 67% overall yield to cannabinoid analog **164** which belongs to the enantiomer series.[65] The stereochemistry for the cyclizations which lead to **157** and **158** (Scheme 26) and which lead to **164** (eq 16) is again determined by the preference for a chair cyclohexane transition state in which the C9 methyl group adopts the equatorial position.



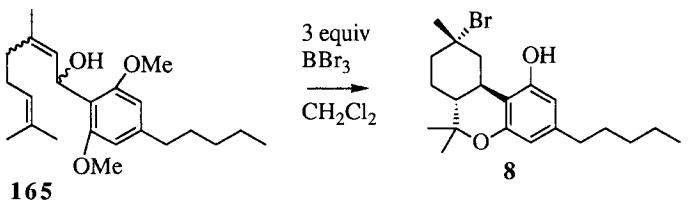
162 **163** **164** [16]

This approach to cannabinoid synthesis is refreshingly novel, but it will probably be challenging to modify it for the synthesis of the natural series of compounds, which incorporate an aromatic hydroxyl group at C1. In order to do so, oxygen would have to be introduced during the rearrangement of **154** to **155** (Scheme 26). The allenic hydrogen atom in **154** closest to the four-membered ring would have to be replaced by an alkoxy group.

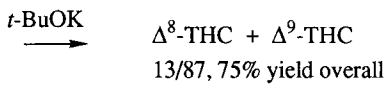
Up to this point, the discussion of cycloaddition strategies has focussed on the synthesis of cannabinoids lacking Δ^8 or Δ^9 unsaturation. The same general scheme has been applied to the preparation of cannabinoids bearing unsaturation in the aliphatic carbocycle, however, since C9 is sp^2 hybridized in this case, there is no easy way to adapt this for enantioselective synthesis.

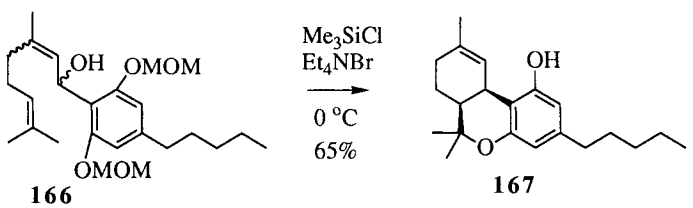
Equations 17 and 18 summarize Rickards' synthesis of the THCs through a cycloaddition reaction.[66] The extremely unstable allylic benzylic alcohol **165** (eq 17) was prepared as a 1/2 E/Z isomeric mixture from citral and 2-lithio dimethoxyolivetol in 80% yield. Treatment of **165** with 3 equivalents of boron tribromide, followed by column chromatography on acidic silica gel produced Δ^8-THC in 40% yield, elimination of hydrogen bromide having taken place during the course of the chromatographic purification. The boron tribromide mediates the cleavage of the two phenolic methyl ethers, and it also catalyzes the generation of the reactive intermediate which undergoes cyclization. Chromatography of the same reaction mixture on Florisil led to a 3/2 mixture of Δ^8- and Δ^9-THC in 57% yield. The ratio could be improved to 1/1 (66% yield) by conducting the chromatography on basic alumina. A much more effective way to maximize the proportion of the Δ^9 isomer was to isolate the reactive tertiary bromide **8** by mplc, and conduct the elimination in a separate step with potassium *tert*-butoxide. Under these conditions the ratio of Δ^9- to Δ^8-THC was 87/13. This is still not as good as the 95/5 ratio of isomers which Razdan

reported for a similar elimination from the tertiary chloride, using potassium *tert*-amylate as the base. In Razdan's reaction, and also in this instance, the observed regiochemical preference for elimination to the Δ^9 isomer is a consequence of an intramolecular process, in which the phenoxide anion abstracts the proton from C10. The erosion of the regiochemical preference in the case of the bromide is a consequence of the greater solvolytic lability of the bromide versus the chloride. An obvious way to circumvent this problem would be to use boron trichloride in place of the tribromide in the cyclization step. All attempts to do so failed to provide cyclized material.



[17]



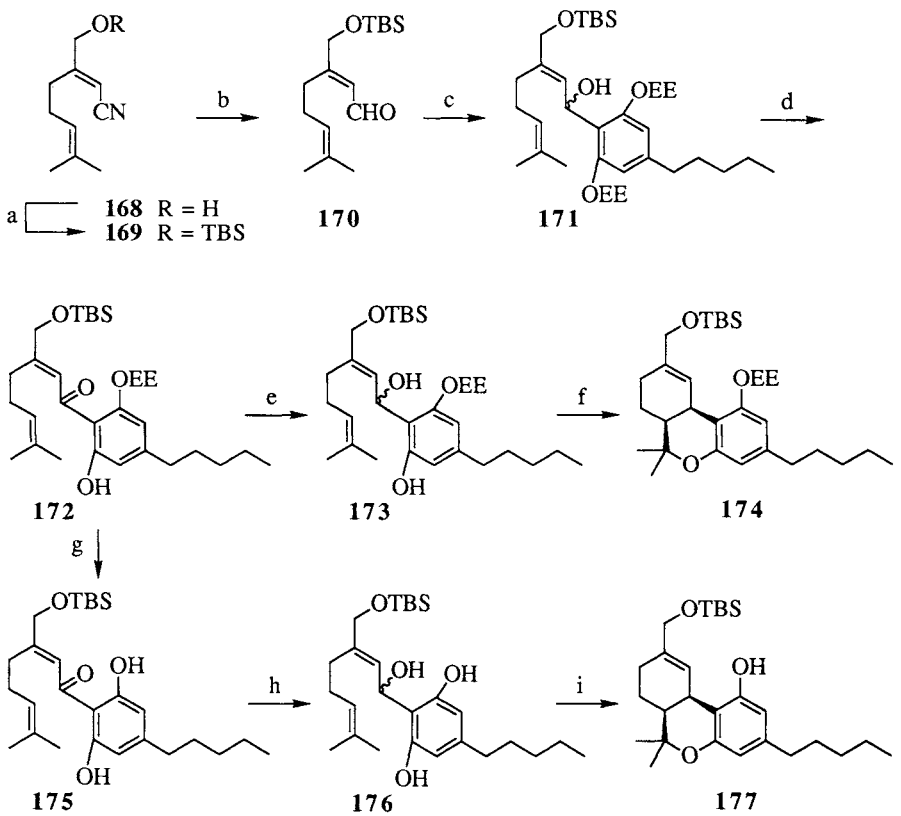


[18]

Whereas the boron tribromide mediated reactions of **165** (eq 17) produced compounds with *trans* ring junction stereochemistry, it was also possible to enter the *cis* manifold through a minor modification of the procedure.[66] Alcohol **166** was prepared from citral in 79% yield (eq 18). Exposure of **166** to trimethylsilyl bromide, generated *in situ* from trimethylsilyl chloride and tetraethylammonium bromide, produces cis-Δ^9-THC **167** in 65% yield. The divergence of the stereochemical outcomes of the two reactions has been rationalized as follows.[66] In the reaction of equation 17, ionization of the alcohol takes place in the first step to generate an allylic benzylic carbocation. The *trans* ring fusion is then formed either during the cyclization step itself, or in a subsequent isomerization of the *cis* isomer, a process which is catalyzed by boron tribromide. The isomerization step presumably takes place through opening and reclosing of the dihydrobenzopyran ring. A similar allylic benzylic carbocation is formed from the reaction of trimethylsilyl bromide and **166** (eq 18). In this case, loss of $H_3COCH_2^+$ would generate an *o*-quinone methide which undergoes the intramolecular hetero Diels-Alder cycloaddition which leads in this case to the *cis* ring fusion stereochemistry. Isomerization to the *trans* ring fusion isomer

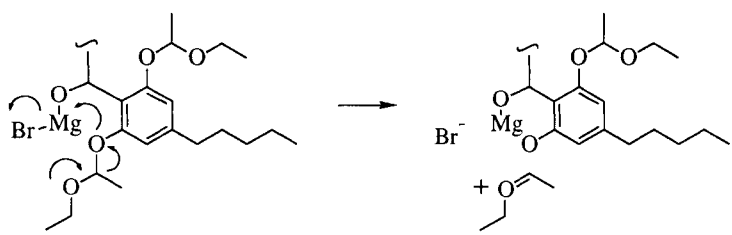
does not take place in the reaction of equation 18 because the trimethylsilyl bromide does not catalyze the re-opening of the dihydrobenzopyran ring. Isomerization from Δ^9 to Δ^8 also does not take place because of the mildness of the reaction conditions, and also because in the *cis* ring junction isomer, the Δ^9 is thermodynamically favored over Δ^8. The unanswered question here is *why* the *o*-quinone methide cycloaddition leads to *trans* ring fused products in the saturated series, whereas it leads only to *cis* ring fused products in the unsaturated series. As will be seen by the example of the following paragraph, the trend appears to be general, and it suggests that there is a fundamental difference between the transition state geometries for the cyclization in each case.

Scheme 27



(a) *t*-$BuMe_2SiCl$, DMF, imidazole, >95%; (b) *i*-Bu_2AlH, Et_2O, -5 °C, 96%; (c) 2-lithio *bis*-ethoxyethyl olivetol, THF, 68%; (d) azodicarbonyl dipiperidine, *t*-BuOMgBr, 79%; (e) $NaBH_4$, 94%; (f) CF_3CO_2H, anh $CHCl_3$, 0 °C, 69%; (g) HOAc, THF, H_2O (1/1/1), 25 °C, 12 h, 60%; (h) $NaBH_4$, MeOH, THF, 0 °C, 64%; (i) CF_3CO_2H, anh $CHCl_3$, 0 °C, 58%

A cycloaddition strategy has been used to prepare racemic nor-Δ^9-*cis*-6a,10a-THC-9-carboxylic acid (Scheme 27).[67] The terpenic fragment was prepared from 5-hepten-2-one, which was converted to α,β-unsaturated nitrile **168** in a single step according to the published procedure.[68] Protection of the primary hydroxyl as the TBS ether took place in nearly quantitative yield to produce **169**, which was reduced to enal **170** with diisobutylaluminum hydride in 96% yield. Aldehyde **170** was next condensed with the lithio anion derived fron *bis*-ethoxyethyl olivetol to produce **171** in 68% yield. Exposure of **171** to Lewis or protic acids under a variety of conditions led to complicated reaction mixtures containing only minor quantities of cyclic products. The failure of this reaction appears to be related to the presence of the ethoxyethyl groups, therefore their removal prior to the cyclization was indicated. This is not a trivial transformation, because survival of the allylic benzylic alcohol function during the acidic conditions for removal of the phenolic protecting groups would hardly be expected. Selective removal of one of the two phenolic protecting groups was accomplished in an unusual manner. Oxidation of the benzylic alcohol to the ketone under Mukaiyama's conditions,[69] azodicarbonyldipiperidine and bromomagnesium *tert*-butoxide, led to ketophenol **172** in 79% yield. The probable mechanism through which one of the ethoxyethyl groups is lost is illustrated by equation 19. Proton transfer from **171** to the bromomagnesium alkoxide leads to the bidentate chelate involving one of the two phenolic oxygens; fragmentation takes place with loss of the stable oxocation. The fragmentation has been shown to take place from the alcohol; the fragmentation could also take place following the oxidation to the phenone through a similar mechanism. Fragmentation of the second acetal protecting group does not take place because of the stability of the magnesium phenoxide.



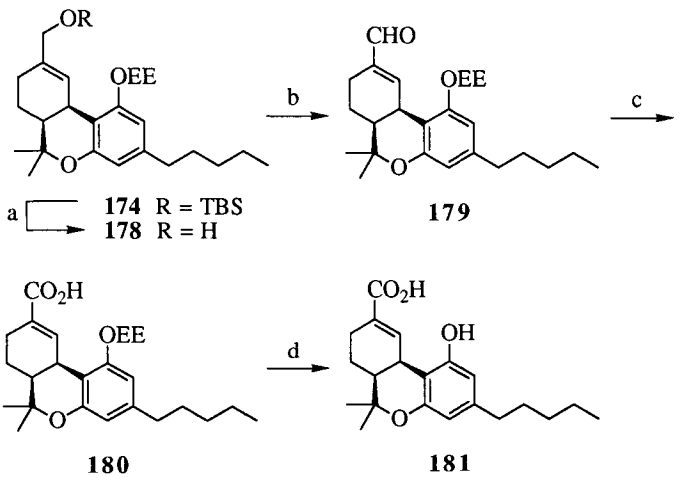
[19]

Reduction of the ketone functionality in **172** leads to diol **173** in 94% yield. Cyclization to **174** took place in 69% yield upon treatment with trifluoroacetic acid in chloroform. Tricyclic cannabinoid **174** was formed as a 6/1 mixture of *cis* and *trans* ring junction isomers. It is perhaps worth noting at this juncture that ring junction stereochemistry is easily determined by ^{1}H NMR. In **174** the proton attached to C10a appeared at δ 3.61 as a broad singlet ($w_{1/2}$ 12 Hz). By comparison, in Δ^9-THC acid, which has the *trans* ring junction stereochemistry, the proton attached to C10a appears as a doublet of doublets (J = 10.5, 1.5 Hz) at δ 3.37. These trends are general.

The remaining ethoxyethyl protecting group in ketophenol **172** was hydrolytically cleaved in good yield to provide ketoresorcinol **175**. Reduction of the carbonyl group in **175** with sodium borohydride produced triol **176** which was cyclized to **177** under the same conditions which were used for **173**. Cannabinoid **177** was formed in 58% yield, again as a 6/1 mixture of *cis* and *trans* isomers.

The conversion of **174** to racemic nor-Δ^9-*cis*-6a,10a-THC-9-carboxylic acid **181** is summarized in Scheme 28.[67] Selective cleavage of the silyl ether protecting group exposes the C11 primary allylic alcohol which is oxidized in 79% yield according to Mukaiyama's procedure to aldehyde **179**. Pellegata oxidation[53] of the aldehyde to the acid proceeded in 79% yield. Hydrolytic removal of the phenolic ethoxyethyl group was accomplished by brief exposure to pyridinium tosylate (PPTS) in methanol to give **181** in 76% yield.

Scheme 28

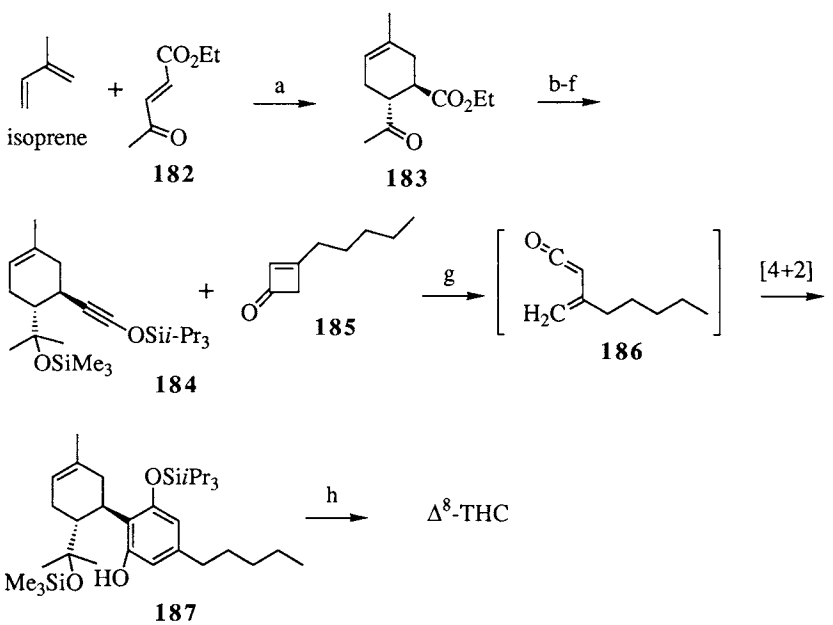


(a) *n*-Bu_4NF, THF, -25 °C, 65%; (b) azodicarbonyl dipiperidine, *t*-BuOMgBr, 79%; (c) *t*-BuOH, H_2O, 2-methyl-2-butene, $NaClO_2$, NaH_2PO_4, 79%; (d) pyridinium tosylate, MeOH, r.t., 76%.

Kowalski has applied a conceptually different approach to the synthesis of racemic Δ^8-THC based on successive cycloaddition reactions (Scheme 29).[70] The starting point is the Diels-Alder cycloaddition of isoprene with ketoester **182** to produce in high yield the aliphatic carbocyclic ring of the final product. The following five steps were conducted in a single operation, without isolation of intermediates, and follow the general outline of earlier work by Kowalski. Selective addition of methyllithium to the ketone carbonyl group of **183** could be accomplished at -90 °C. Addition of dibromomethyllithium to the ester carbonyl led to an intermediate α,α-dibromomethyl ketone which was treated at low temperature with *n*-butyllithium. Metal-halogen exchange generates a metallocarbene which rearranges to an

acetylene alkoxide. Trapping with triisopropylsilyl chloride generates the silyloxy acetylene functionality in **184**. Trapping of the tertiary alkoxide with trimethylsilyl chloride ends the sequence. The sterically demanding triisopropylsilyl group is only transferred to the unencumbered oxygen of the alkoxyacetylene, which makes it possible to differentiate the two oxygens of **184**. These steps took place in 52% overall yield. The silyloxyacetylene group can participate in a [4+2] cycloaddition reaction with vinylketene **186** which is formed during thermal decomposition of cyclobutenone **185**, which was easily prepared in two steps from the addition of *n*-pentylmagnesium bromide to 3-ethoxycyclobutenone, followed by hydrolysis of the product with aqueous hydrochloric acid.[71,72] The ketene cycloaddition reaction follows the general methodology which was developed by Danheiser for the annelation of regiospecifically substituted phenols and resorcinols.[73] Exposure of cycloadduct **187** to refluxing ethanolic hydrochloric acid leads to racemic Δ^8-THC in 61% overall yield for the two steps. The overall yield from **182** was 29%. Since steps b through f (Scheme 29) were conducted in a single operation, in essence this represents a four step synthesis of Δ^8-THC. Conceptually, Kowalski's synthesis is unique among the methods which have been discussed in this chapter, although it bears some similarity to Moore's approach. Adapting Kowalski's protocol for the enantioselective synthesis of the Δ^9 series would be a very interesting exercise indeed.

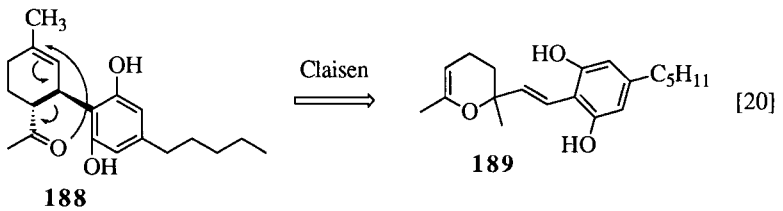
Scheme 29



(a) $TiCl_4$, CH_2Cl_2, r.t., 93%, 20/1 isomeric mixture; (b) MeLi, THF, -90 °C; (c) $LiCH_2Br$, -78 °C; (d) *n*-BuLi, -78 °C to r.t.; (e) 7 equiv *i*-Pr_3SiCl, -78 °C to r.t.; (f) 10 equiv Me_3SiCl, -78 °C to r.t., 52% for five steps; (g) $PhCH_3$, 80 °C, 1 h; (h) HCl, EtOH, reflux, 61% for two steps.

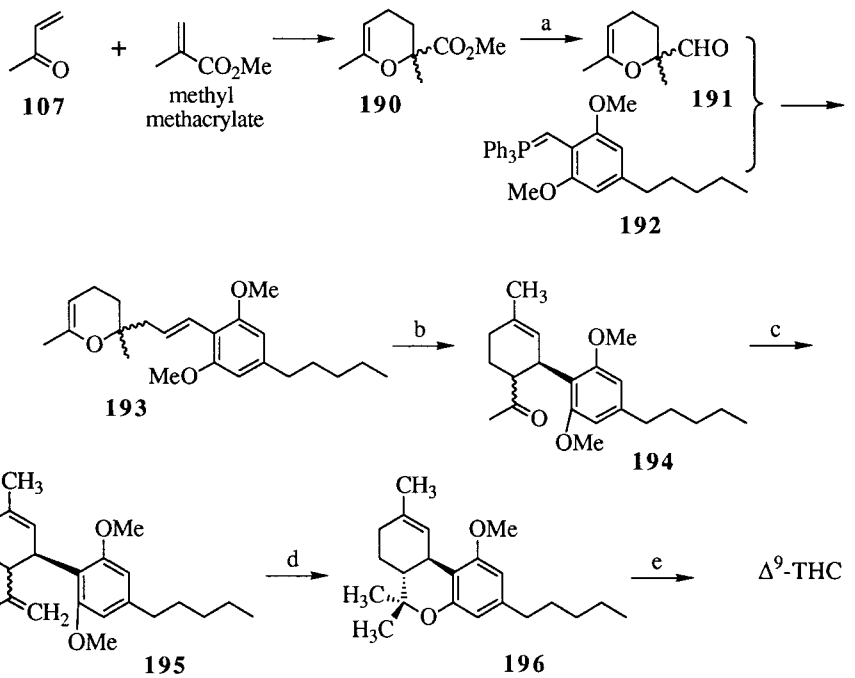
Miscellaneous Strategies for Cannabinoid Synthesis

An interesting and conceptually novel synthesis of racemic Δ^9-THC has been reported by Pinnick and Childers.[74] The distinguishing feature of this work was their recognition of the retrosynthetic disconnection shown in equation 20. A Claisen disconnection can be applied to **188**, leading to dihydropyran **189**. The application of this retrosynthetic strategy is summarized



[20]

Scheme 30



(a) *i*-Bu_2AlH; (b) Claisen reaction, r.t., 94%, 1/1 *cis/trans*; (c) $Ph_3P{=}CH_2$, 80%; (d) Me_3SiI, r.t., 94%; (e) NaSEt, DMF, 78%.

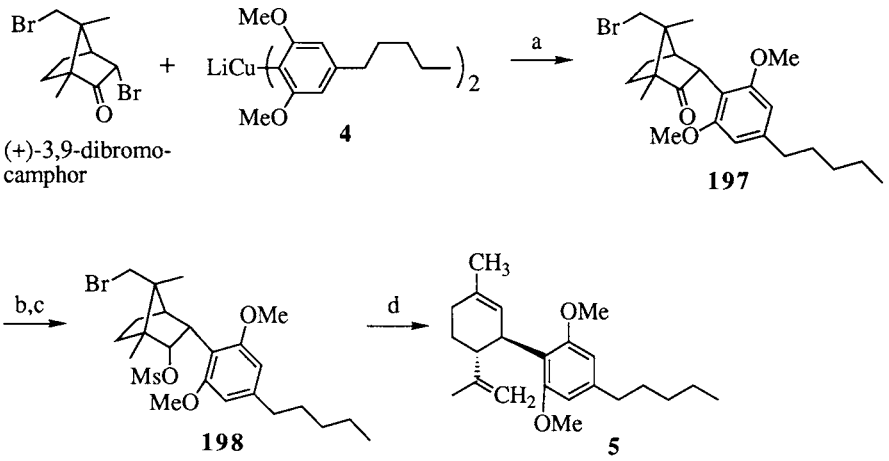
in Scheme 30. The hetero-Diels-Alder reaction between methyl vinyl ketone **107** and methyl methacrylate leads to cyclic ether **190**. Selective reduction of the ester group in **190** with

diisobutylaluminum hydride produces aldehyde **191**, which is combined with the olivetol-derived phosphorus ylide **192**. The product **193** is isolated as a mixture of E and Z geometric isomers. The Claisen rearrangement, which is required to proceed through a cyclohexane boat transition state because of steric constraints, takes place at room temperature in 94% yield to produce a 1/1 mixture of *cis* and *trans* isomers of methyl ketone **194**. Methylene triphenylphosphorane converted the acetyl group in **194** to an isopropenyl group in 80% yield. Exposure of **195** to trimethylsilyl iodide at room temperature produced **196**, the methyl ether of Δ^9-THC in 94% yield as a single isomer. Since the starting material was an isomeric mixture, isomerization of C6a must have taken place at some point on the reaction pathway. This is somewhat surprising, since Rickards had shown (eq 18) that in a similar system trimethylsilyl *bromide* failed to catalyze isomerization of the *cis* to the *trans* ring fusion stereochemistry. Cleavage of the methyl ether group in **196** led to Δ^9-THC in 78% yield. This is a very efficient synthesis: the overall yield of Δ^9-THC from **190** was 37%. This can be adapted for enantiospecific synthesis, as well.

A stereo- and enantioselective synthesis of **5** proceeding from (+)-3,9-dibromocamphor has been described.[75] Since **5** serves as the starting material for other cannabinoids, this work suggests that camphor can be used as the terpene partner and the source of asymmetry in cannabinoid synthesis in a more general context. The terpenic starting material can be made conveniently on large scale and in high yield from (+)-camphor in two steps.[76] The reaction with homocuprate **4** took place only under carefully defined conditions. In pure ether, only reduction of the 3-bromine took place, and the sole reaction product was 9-bromocamphor. The reaction was also found to be very sensitive to air, as well as to any excess of the base which was used to generate the cuprate. In 1/1 ether dimethylsulfoxide (DMSO), or in 1/1 ether/DMF, the desired substitution reaction leading to **197** took place in 71% yield. The stereochemistry of this substitution reaction is irrelevant, since the product can be equilibrated to the thermodynamically favored *endo* isomer. Reduction of the ketone carbonyl group to the *endo* alcohol with diisobutylaluminum hydride, followed by mesylation led to mesylate **198** in 93% overall yield for the two steps. Bromomesylate **198** is set up for a Grob-type fragmentation reaction. Exposure of **198** to five equivalents of sodium naphthalenide resulted in reductive cleavage and the formation of **5**, the dimethyl ether of cannabidiol in 71% yield.

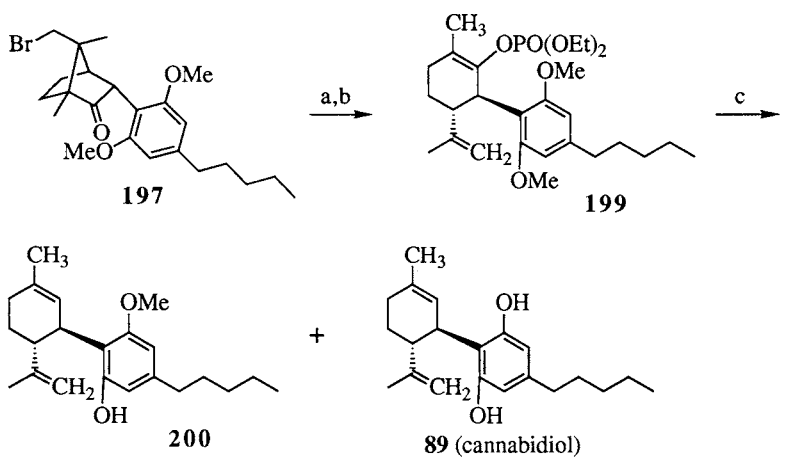
Intermediate **197** can also be manipulated through a slightly different sequence (Scheme 32).[76] Exposure of **197** to sodium naphthalenide leads to a reductive ring cleavage with the formation of a ketone enolate which is trapped with diethyl chlorophosphate to produce **199** in 89% yield. Reductive removal of the phosphate by treatment with lithium in methylamine leads to partial cleavage of the two methyl ethers, producing cannabidiol **89** in 35% yield and its monomethyl ether **200** in 43% yield. This is an unusual entry into the cannabinoid skeleton. The approach is very efficient, however, the utility of the process will be greater if it can be shown that oxygenation at C11 can be incorporated into the scheme.

Scheme 31



(a) THF, DMSO, 1/1, 0 °C to r.t., 18 h, 71%; (b) 2 equiv *i*-Bu_2AlH, 0 °C, 45 min; (c) $MeSO_2Cl$, pyridine, DMAP, r.t., 18 h, 93% for two steps; (d) 5 equiv Na-naphth, -78 °C to r.t., 71%.

Scheme 32

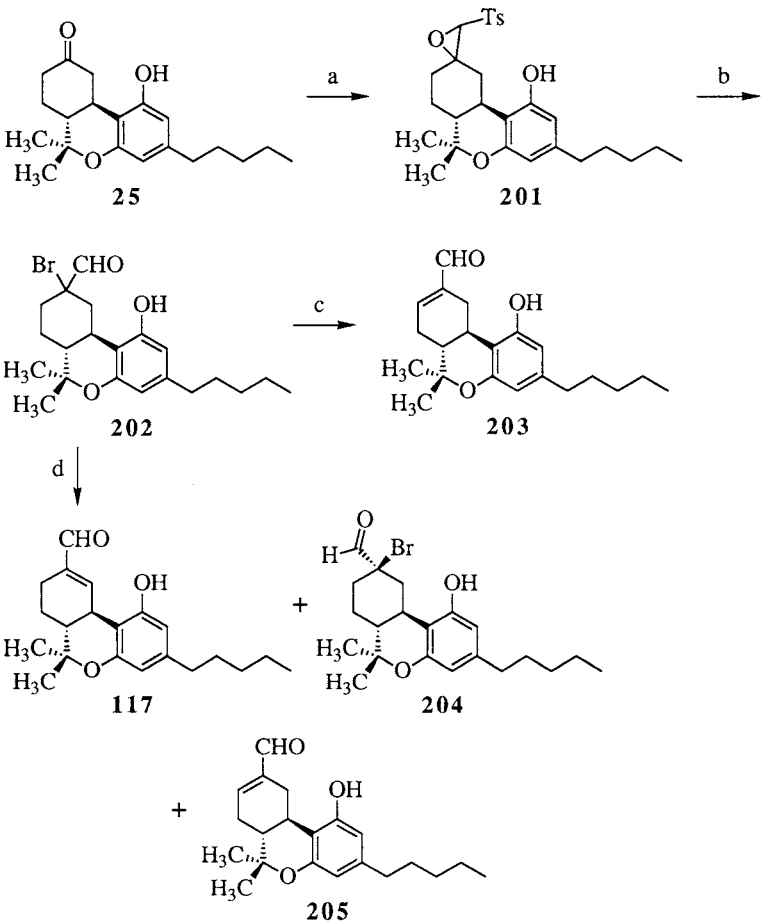


(a) Na-naphth; (b) $ClPO(OEt)_2$, 89% for two steps; (c) Li, $MeNH_2$, -10 °C, 43% **200**, 35% **89**.

Functional Group Manipulations in the Aliphatic Carbocycle

In this section some of the reactions which have been used to alter functionality in the aliphatic portion of the cannabinoid skeleton will be examined. Much of this work has again been motivated by the desire for an easier access to the C11 oxygenated series.

Scheme 33

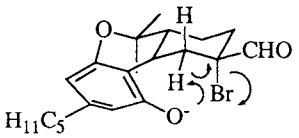


(a) $TsCH_2Cl$, $PhCH_2NEt_3Cl$ cat., CH_3CN, 50% aq NaOH, 96%; (b) $MgBr_2$, THF, 2 d, r.t., 73%; (c) Li_2CO_3, LiBr, DMF, 100 °C, 6 h, 90%; (d) *sym*-collidine, 4Å molecular sieves, PhH reflux, 72 h, 26.3% **117**, 48.6% **204**, 24.3% **205**.

Scheme 33 summarizes ApSimon's approach to the C11 functionalized THC metabolites.[77] Ketone **25** was converted in nearly quantitative yield to tosyl epoxide **201** (Ts = *p*-toluenesulfonyl) by reaction with chloromethyl tolyl sulfone under phase-transfer catalytic

conditions. Anhydrous magnesium bromide, prepared *in situ* from 1,2-dibromoethane and magnesium, catalyzed the rearrangement of the epoxide to a mixture of α-bromoaldehydes **202**. Exposure of this material to a mixture of lithium carbonate and lithium bromide in DMF at 100 °C for 6 h led to Δ^8 aldehyde **203** in 90% yield. Gaining access to the thermodynamically disfavored Δ^9 isomer **117** was considerably less successful through this route. Treatment of **202** with *sym*-collidine in refluxing benzene for 72 h led to a mixture containing 26.3% of the desired Δ^9 aldehyde **117**, 24.3% of Δ^8 aldehyde **203**, and 48.6% of the β-bromo isomer of the starting material, which failed to undergo elimination under the reaction conditions.

The strategy embodied by Scheme 33 was to adapt the earlier work which had shown that good yields of Δ^9-THC could be prepared from the intramolecular dehydrohalogenation of a C9 bromide to the synthesis of the C11 oxygenated series.[78,34] In another example of the oftentimes frustrating and idiosyncratic nature of the chemistry in this series, the key elimination step was disappointing, both under the conditions which have been specified in Scheme 33, and also when sodium hydride or sodium *tert*-amylate was used for the intramolecular dehydrobromination. The failure of these reactions has been attributed to the fact that the ideal *trans*-anti geometry for the intramolecular elimination cannot be achieved in either of the isomers of **202**. This is illustrated for the reactive α-bromo isomer **206** in equation 21. The question which is not answered is why the intramolecular elimination should work so much better in the case of Δ^9-THC itself, which also cannot achieve the proper geometry for elimination.

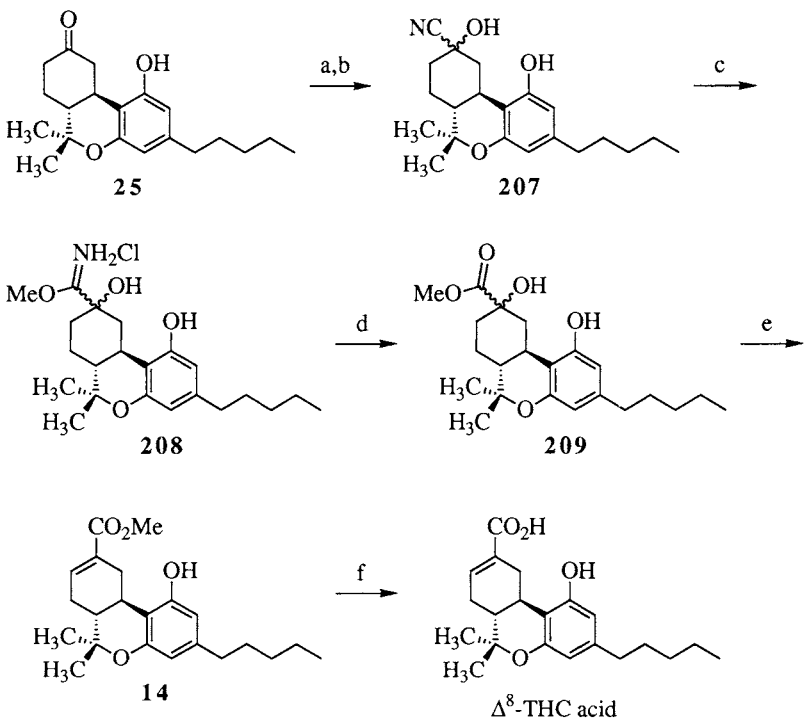


206 [21]

If the Δ^8 C11 oxygenated is desired, it can be readily accessed either through ApSimon's approach (Scheme 33), or through the methodology of Schwartz and Madan (Scheme 34).[79] The approach is not applicable to the preparation of the more challenging Δ^9 series. Ketone **25** again served as the starting material in Schwartz's synthesis. Conversion in the usual way to the cyanohydrin diastereoisomers **207** took place in nearly quantitative yield. Exposure of the cyanohydrin to gaseous hydrochloric acid in methanol led to imino ether hydrochloride **208** which was converted to the diastereomers of α-hydroxy methyl ester **209** in 75% overall yield for the two steps. Treatment of **209** with thionyl chloride in pyridine at room temperature for 1 h led in 50% to 73% yield to the methyl ester **14** of Δ^8-THC acid. This dehydration step had caused some difficulties. Dehydration did not take place with phosphorus oxychloride in pyridine. Instead, the 1-phosphate ester was isolated in 50% yield. Elimination of the phosphate ester to **14** could not be accomplished. Exposure of **209** to Burgess' reagent resulted in dehydration only to the extent of 10%.[80] This may not be surprising, since the phenolic hydroxyl was unprotected, and probably reacted with the reagent. Hydrolysis of the methyl ester was

accomplished in aqueous methanolic sodium hydroxide with continuous bubbling of argon gas for 2 h in >95% yield. The bubbling of argon gas was intended to inhibit the rapid, base catalyzed oxidation of the phenolate by adventitious oxygen. This synthesis was performed on racemic material, however, it could just as easily be performed on the optically pure series.

Scheme 34

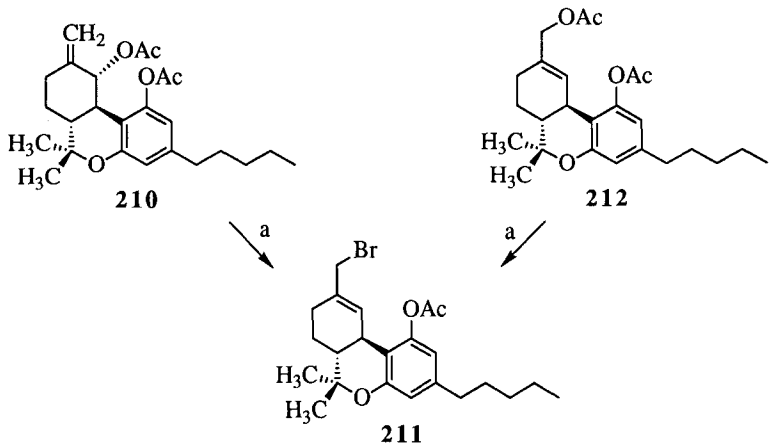


(a) NaCN, MeOH, H_2O, 2 h, 25 °C; (b) HOAc, MeOH, HCl gas, >95% for two steps; (c) HCl gas, MeOH, 3 °C, 1.25 h, then -20 °C, 72 h; (d) 6N HCl; concentrate, then suspend in 50% aq MeOH, 75% for two steps; (e)$SOCl_2$, pyridine, r.t., 1 h, 50-73%; (f) NaOH, MeOH, H_2O, reflux, 2 h, Ar gas bubbling, >95%.

In the original approach to the synthesis of the C11 oxygenated cannabinoids, allylic acetate **210** was converted to rearranged allylic bromide **211** with hydrobromic acid in acetic acid.[24] This harsh reaction led to a mixture of products which was used in the next step without chromatographic purification, resulting in a very low yield of the final product after acetolysis and hydrolysis of **211**. Markedly improved conditions for this allylic rearrangement have been developed by Seltzman (Schemes 35 and 36).[81] Exposure of **210** to a small excess of trimethylsilyl bromide in the presence of catalytic zinc iodide in dichloromethane led in 97% yield to the rearranged allylic bromide **211**. In the absence of zinc iodide the reaction was very

sluggish. For example, treatment of **210** with five equivalents of trimethylsilyl bromide and no zinc iodide led to **211** in 61% yield after 48 h at reflux in dichloromethane. Allylic acetate **212** was also converted to **211** in high yield under the same reaction conditions.

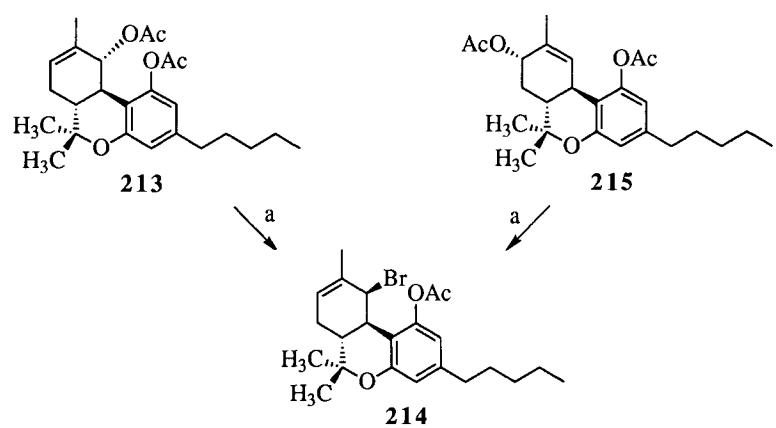
Scheme 35



(a) 2-3 equiv Me_3SiBr, 5 mol % ZnI_2, CH_2Cl_2, r.t., 4 h, 97% from **210**, 87% from **212**.

Allylic acetates **213** and **215** were converted to bromide **214** in high yield under the same reaction conditions (Scheme 36). Both sets of experiments suggest that the allylic bromide products are formed under thermodynamic control from a common intermediate in each case. In Scheme 35, the most stable product is the trisubstituted alkene. In Scheme 36 the most stable product is the Δ^8 alkene.

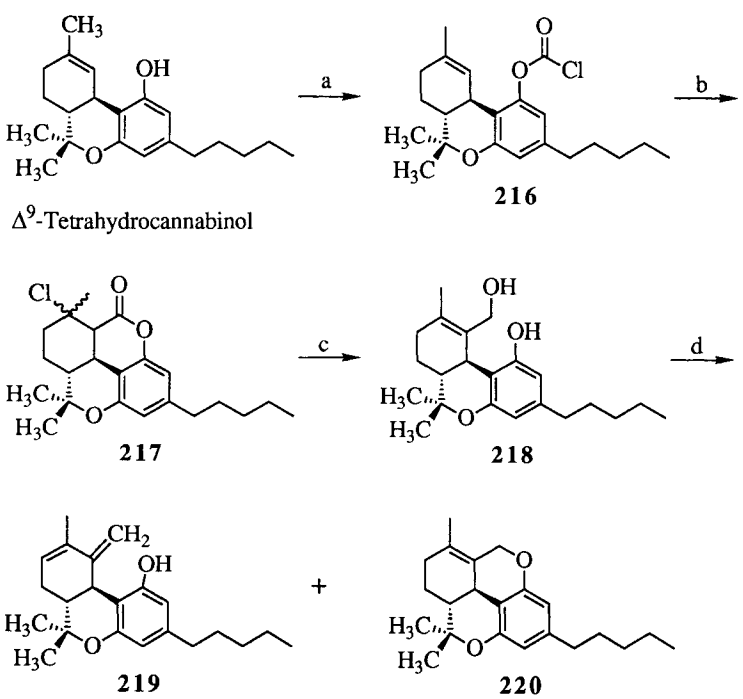
Scheme 36



(a) 2-3 equiv Me_3SiBr, 5 mol % ZnI_2, CH_2Cl_2, r.t., 4 h, 79% from **213**, 64% from **215**.

A pair of rotationally restricted THC ethers were prepared in order to test the hypothesis that the psychotropic effects of the cannabinoids are associated in part with the orientation of the lone pairs on the phenolic hydroxylic oxygen. The synthesis of one of these is detailed in Scheme 37.[82] Direct approaches to **220** via the Lewis acid catalyzed fragmentation of the phenolic 2-methoxyethoxymethyl (MEM) ether led to extensive degradation of the starting material under a variety of reaction conditions. This led to the adoption of a modified approach. Δ^9-THC was converted in high yield to chloroformate **216** which underwent an intramolecular aluminum trichloride catalyzed Darzens-Nenitzescu condensation to give **217**. Exposure of **217** to lithium aluminum hydride led to sequential dehydrohalogenation and lactone reduction to produce diol **218** in modest yield. Diol **218** could be converted to the desired ether **220** in low yield by treatment with tosyl chloride in pyridine. The major reaction byproduct was diene **219**, which could be isolated from **218** as the sole reaction product after heating in dimethylsulfoxide. The intramolecular functionalization of C10 via the Darzens-Nenitzescu reaction also suggests a potentially useful alternative pathway to Δ^9-THC acid.

Scheme 37

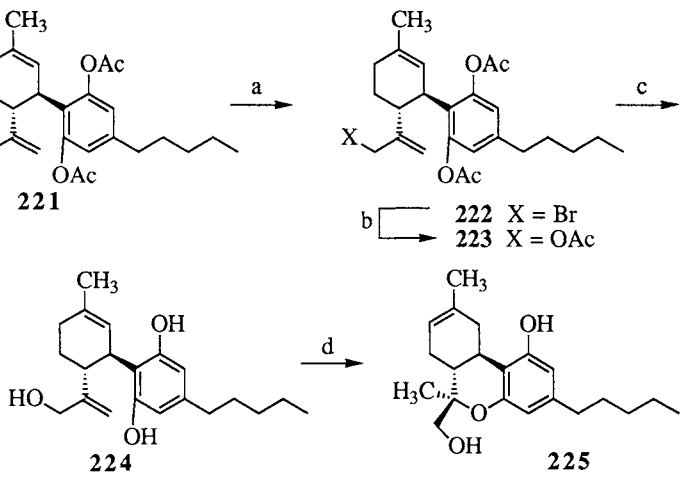


(a) Cl_2CO, $PhNMe_2$, $PhCH_3$, 0 °C, 94%; (b) $AlCl_3$, CH_2Cl_2, then aq K_2CO_3, 51%; (c) $LiAlH_4$, Et_2O, 42%; (d) 3 equiv TsCl, pyridine, 12% **219**, 18% **220**; 60% **219** from 132 °C, DMSO, 1 h.

Functional Group Manipulations in the Dihydrobenzopyran Ring

Very little work has been reported for functionalizations in this portion of the cannabinoid skeleton. Razdan was the first to introduce oxygen functionality into the C6 β-equatorial methyl group.[83] A small quantity of this material had been isolated as a byproduct of the epoxidation of cannabidiol. A synthesis directed specifically to this target is outlined in Scheme 38.

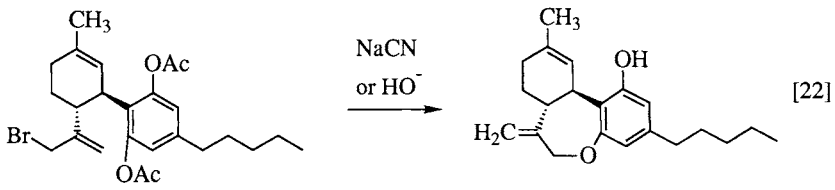
Scheme 38



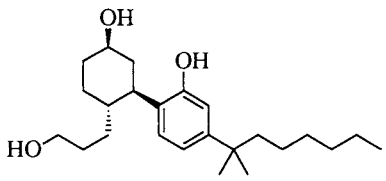
(a) NBS, THF, dark, 0 °C, 2 h, 81%; (b) AgOAc, HOAc, 20 °C, 72 h; (c) Na_2CO_3, MeOH, H_2O, 20 °C, 86% for two steps; (d) *p*-TsOH, PhH, reflux, 48%.

The diacetate of cannabidiol (**221**), was converted to allylic bromide **222** in high yield by treatment with N-bromosuccinimide (NBS) in the dark at 0 °C. The phenolic acetates serve to deactivate the aromatic ring toward electrophilic bromination. Evidently, no appreciable competition by the Δ^9 double bond for the halogenating agent took place. Silver ion assisted solvolysis of the halide produced allylic acetate **223** which was hydrolyzed to triol **224** in 86% yield for the two steps. Exposure of **224** to tosic acid in refluxing benzene produced **225** in 48% yield, acid catalyzed isomerization to the Δ^8 isomer having taken place during the reaction. The reason for the preference of the reaction for the β-equatorial hydroxymethyl isomer is not clear. Certainly there is insufficient difference in energy between **225** and its α-axial hydroxymethyl diastereoisomer to account for this result. This implies that the preference for **225** is kinetic in nature, which seems equally improbable, given the reaction conditions.

Allylic bromide **222** was also converted to enol oxepin derivative **226** upon treatment with sodium cyanide or hydroxide (eq 22).[84]



222 226 [22]



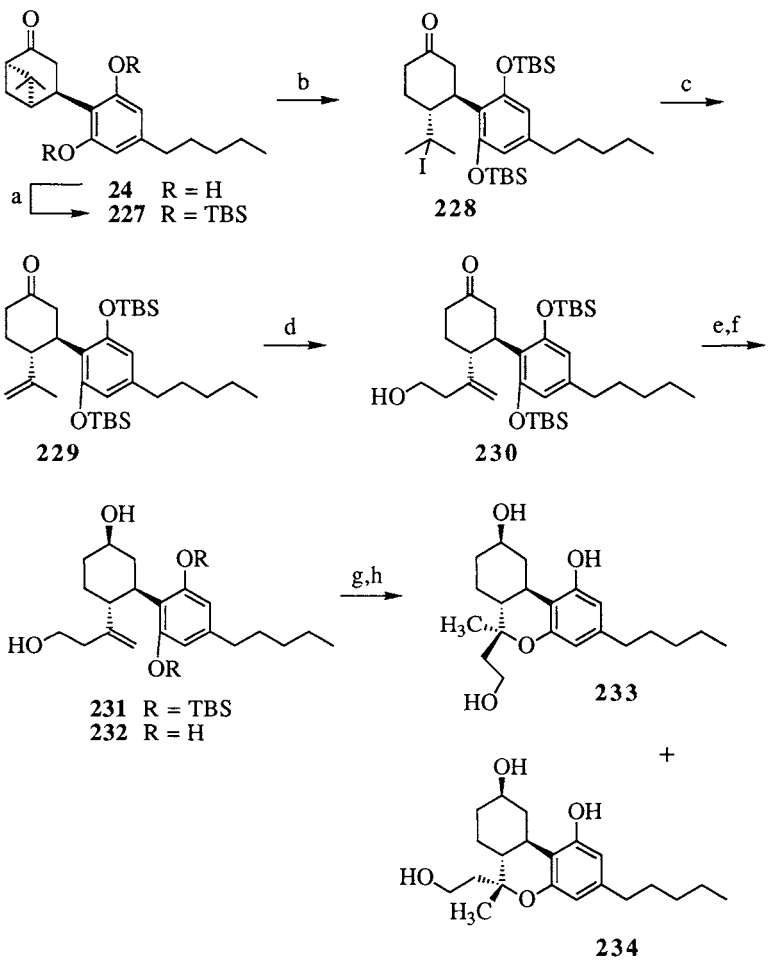
CP-55,940 [23]

We were able to demonstrate good levels of stereochemical control of hydroxyalkyl substituents at C6. Our work was prompted by a desire to prepare a series of cannabinoid analogs which combine structural elements of HHC and the potent, non-classical cannabinoid CP-55,940 (eq 23). This synthetic analog is an analgesic, the potency being attributed in part to the introduction of the hydroxypropyl binding component. Analogs **233** and **234** (Scheme 39) were designed to be conformationally restricted analogs of CP-55,940.[85] The major challenge posed by these structures concerns the control of stereochemistry at C6. The starting material for this synthesis was ketoresorcinol **24** (see eq 3). The phenolic hydroxyl groups were protected as the TBS ethers, and the four-membered ring was cleaved with trimethylsilyl iodide under mild reaction conditions to produce unstable tertiary iodide **228**. This material was immediately dehydrohalogenated by exposure to 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in benzene at room temperature. The overall yield for the two steps was 52%. The next task formally required appending a hydroxymethyl group to the isopropenyl methyl group of **229**. This was accomplished by means of an ene reaction of formaldehyde using Yamamoto's methylaluminum bis(2,6-diphenylphenoxide)-formaldehyde reagent (MAPH)[86] in 50-55% yield. Protection of the ketone carbonyl group was unneccesary during this reaction. Reduction of the carbonyl group with sodium borohydride led to equatorial alcohol **231**, which was deprotected by brief exposure to tetra-*n*-butylammonium fluoride to tetraol **232**

The stereochemically determining step is the ring closure which forms the dihydrobenzopyran ring. Exposure of **232** to tosic acid in refluxing toluene was completely non-stereospecific, and led to a 1/1 mixture of **233** and **234**. Protonation of the isopropenyl group evidently led to a tertiary carbocation which was intercepted by one of the phenolic hydroxyl groups with a complete lack of stereochemical bias. This result is difficult to interpret in light of the stereospecificity of Razdan's reaction (see Scheme 38, conversion of **224** to **225**). The problem was solved by using a different electrophile to catalyze the ring closure. Treatment of

232 with mercuric acetate in THF, followed by reductive demercuration with sodium borohydride, led to a 86/14 mixture of **233** and **234** in 75% yield for the two steps.

Scheme 39



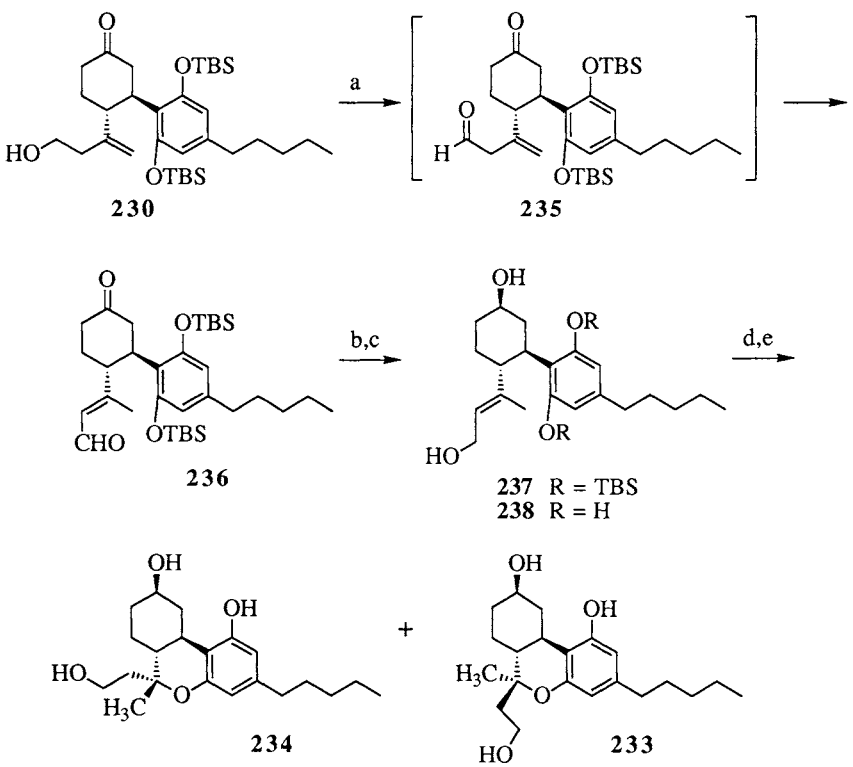
(a) *t*-BuMe$_2$SiCl, imidazole, DMF, 23 °C, 85%; (b) H$_2$C=CHCH$_2$I, I$_2$, CCl$_4$, 0 °C; (c) DBU, PhH, 23 °C, 52% for two steps; (d) MAPH, *sym*-trioxane, CH$_2$Cl$_2$, 0 °C, 50-55%; (e) NaBH$_4$, THF/*i*-PrOH, 9/1, 23 °C, 88%; (f) *n*-Bu$_4$NF, THF, 23 °C, 90-96%; (g) Hg(OAc)$_2$, THF, 0 °C; (h) NaBH$_4$, aq NaOH, 75% for two steps, **233/234** 86/14.

A minor variation in the procedure made it possible to prepare **234** as the major product (Scheme 40).[85] Swern oxidation of homoallylic alcohol **230** presumably led to β,γ-unsaturated aldehyde **235** which underwent spontaneous isomerization to α,β-unsaturated aldehyde **236**

under the reaction conditions. Reduction of both carbonyl groups in **236** with sodium borohydride and cleavage of the two phenolic silyl ether protecting groups led to tetraol **238** in high yield. Mercuration followed by reductive demercuration as described for **232** led to a 85:15 mixture of **234** and **233** in 80% yield.

This demonstrated that each of the two diastereoisomers could be prepared selectively, however, it did not shed much light on the origin(s) of this surprising selectivity. Since the acid catalyzed ring closure at high temperature had produced an equimolar mixture of **233** and **234**, we surmised that the selectivity of the mercuration-demercuration reaction was kinetic in origin. Since the major product in each instance was derived from the axial organomercurial, the stereochemical preference could be the result of an anomeric effect of the developing mercurinium ion in the transition state. This hypothesis was supported by an independent series of experiments.[87]

Scheme 40



(a)DMSO, $(ClCO)_2$, CH_2Cl_2, -78 °C, 85%; (b) $NaBH_4$, THF/*i*-PrOH, 9/1, 23 °C, 72%; (c) *n*-Bu_4NF, THF, 23 °C, 92%; (d) $Hg(OAc)_2$, THF, 0 °C; (e) $NaBH_4$, aq NaOH, 80% for two steps, **234/233** 85/15.

Isomers **233** and **234** were strikingly different in their affinities for the CB1 receptor.[85] Of the two, **233**, in which the hydroxyethyl group has a β-equatorial relative configuration, was shown to possess considerable affinity for the receptor (IC_{50} = 100 nM) whereas **234** exhibited much weaker affinity (IC_{50} >10 μM). This data supports the hypothesis that the receptor binding affinity of CP-55,940 is influenced in part by a specific interaction involving the primary aliphatic hydroxyl.

Epilogue

Some solutions to the problems posed by the synthesis of the classical cannabinoids have been discussed. The difficulties posed by this relatively simple structure have been outlined, as well as some of the more successful stereo- and enantioselective syntheses from recent years. It should be clear that fully optimal solutions to the synthetic problems have not been defined in all cases, and that significant challenges remain. In the future, much of the synthesis is likely to be directed toward a better description, at the molecular level, of the ligand-receptor interactions which are fundamental for an understanding of the pharmacology of the cannabinoids.

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3. Dibenzopyran numbering has been used throughout this chapter.

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32. If both reactions, the one leading to **39** and the one leading to **44**, proceed through the same tertiary cation, as postulated by Huffman, it is not clear why intercepting this cation as the ethyl ether should offer any advantage.

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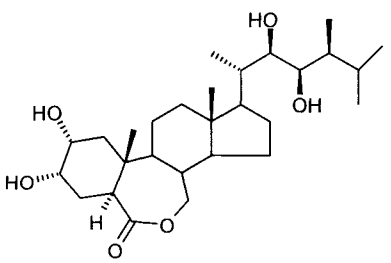
Methods for Construction of Sidechain of Brassinosteroids and Application to Syntheses of Brassinosteroids

Biao Jiang, Liangfu Huang, Weishen Tian and Weishan Zhou

1. Introduction

Brassinolide [(22R, 23R, 24S)-2α, 3α, 22, 23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one (**1**)] isolated from the pollen of rape (*Brassica napus*) is a plant growth promoting steroid with a seven-membered B-ring lactone and four successive chiral centres in the sidechain.[1] For high activity, both the B-ring lactone and the configuration at C-24 were found to be important. [2] Brassinolide promotes cell division, cell elongation, and plant growth. Brassinolide possesses a broad spectrum of biological activities compared with the known plant hormones. Since the discovery of brassinolide, a number of related compounds have been isolated from plants and form a new class of plant growth promoters, which are named as brassinosteroid.



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Brassinolide has practical applications in agriculture. Its remarkable biological activities and novel chemical structure have led many laboratories to develop its synthesis. A difficult task in the construction of the sidechain is to control the stereochemistry of C22, C23 and C24. Recently, we accomplished several new methods for the construction of brssinosteroid sidechain, which are quite stereoselective and with a high yield.

We employed hyodeoxycholic acid for the first time as the starting material, which has very rich resources in China. When hyodeoxycholic acid was used as starting material, we first degraded it to the 20-carbaldehyde steroid as ergosterol or stigmasterol, then stereoselectively constructed the sidechain of brassinosteroids. In the view of the greater economy and convenience, we also adopted

directly the intact sidechain of hyodeoxycholic acid as starting material for the synthesis of brassinosteroids. We were successful in applying Sharpless's osmium-catalyzed asymmetric dihydroxylation for the first time to the (22E)-24-alkyl steroidal unsaturated sidechain, providing the natural (22R,23R)-22,23-diol as the major products. Furthermore we also were successful in applying this elegant method to the 22(E)-methyl hyodeoxycholate and other unsaturated steroidal sidechains.

In the meantime we have synthesised a great number of new brassinosteroids which exhibit moderate to high biological activity.

2. Occurrence

Since the discovery of brassinolide (**1**) as the first steroidal plant growth promoter in the *rape pollen* in 1979,[1] the occurrence of brassinolide and related steroids in other plant tissues has been successively reported[2,3,4,5,6] and it represents a new class of plant growth promoters. As shown in **Table 1,** Brassinosteroids were found not only in higher plants but also in lower plants. Generally

Table 1. Occurrence of Brassinosteroids

Plant	Brassinosteroids	ref.
1*Alnus glutinosa L.*(pollen of European alder)	**1, 6**	6
2 *Apium graveolens*(seeds of celery)	**32**	8
3.*Banksia grandis* (pollen)	**1, 6**	2,3
4.*Beewax*	**5**	7
5.*Brassica napus L.*(rape pollen)	**1**	1
6.*Brassica compestris L.* var. pekinensis (sheaths, seeds of Chinese cabbage)	**1, 3, 4, 6, 8, 11**	2,3,
7.*Castanea crenata Sieb* et Zucc (gall, shoots, leaves, flower buds of chestnut)	**1, 3, 4, 6, 8, 11**	2,3,6
8.*Castanea spp.*(galls)	**1, 6**	3,6
9.*Catharanthus roseus L.* (galls)	**1, 6**	3,6
10. *Cistrus hirusutum* (pollen)	**1, 6**	2,3
11.*Citrus unshiu Marcov.* (pollen)	**1, 6, 19, 21**	2,3
12.*Citrus sinensis Osbeck* (pollen)	**1, 6**	2,3
13.*Chrysanthemum golden rod s.*altissima	**6**	6
14.*Cryptomeria japonica D. Don*(pollen, anthers of cedar)	**1, 6**	6
15.*Diospyros kaki* Thunb (seeds of persimmon)	**6**	6

16.*Distylium racemosum* sieb.et Zucc(galls, leaves)	**1, 3, 4, 6, 8, 11**	6
17.*Dolichos lablab L* (seed)	**1, 2, 3, 6, 9, 10 , 23, 24**	2,3,6
18.*Echium piantagineum* (pollen)	**1, 6**	2,3
19.*Equisetium arvense* L(strobilus)	**4, 6, 9, 11**	6
20.*Eriobotrya japonica Lindl*(flower buds of loquat)	**6**	2,3
21.*Eucalyptus marinata*(pollen)	**9**	2,3
22.*Eucalyptus calophylla* (pollen)	**1**	2,3
23.*Fagopyrum esculentum Mpench* (pollen of buckwheat)	**1, 6**	6
24.*Heliathus annuus L.* (pollen of sunflower)	**1, 6, 11**	6
25.*Hydrodictyon reticulatu*(Green alga)	**7, 8**	6
26.*Lilium elegans Thumb.*(the pollen of lily)	**1, 6, 19, 21**	2,3
27.*Lilium longiflorum cv.Georgia* (pollen, anthers of lily)	**33**	4
28.*Oryza sativa* (rice shoots)	**1, 3, 4, 8, 11**	2,3,6
29.*Pharbitis purpurea Voigt*(immature seeds)	**6, 11**	6
30.*Phaseolus vulgaris L* (immature seeds)	**1, 9, 12, 20,23, 24, 25, 30, 31**	2,3,6
31.*Picea sitchenesis Bong Carr* (shoots of sitka spruce)	**6, 9**	2,3,6
32.*Piophocarpus tetragonolobus*(immature seeds)	**1, 6, 8, 25, 26**	2,3
33.*Pisum sativum L.cv.Holland*(shoots)	**6**	2,3
34.*Pinus silverstris*(cambial scrapings of Scots pine)	**1, 6**	2,3
35.*Pinus thunbergii Parl*(pollen of Japanese black pine)	**6, 19**	2,3,6
36.*Raphanus sativus var.Remo*(seed)	**1, 6**	6
37.*Secale cereale*(seeds)	**34**	5
38.*Solidago altissima* (stems)	**1**	2,3
39.*Thea sinensis L.* (leaves of green tea)	**1, 3, 4, 6, 8, 11, 19, 21**	2,3,6
40.*Triticum aestivum L.*(Immature seeds of wheat)	**1, 6, 8**	2,3
41.*Typha latifolia L.*(pollen of cat-tail)	**19, 21**	2,6
42.*Tulipa gesneriana* (pollen of tulip)	**19**	2,3
43.*Vicia faba L.* (pollen of broad bean)	**1, 5, 6, 11**	2,3,6
44.*Zea mays L.*(immature seeds of corn)	**6,19,21**	2,6

they occur in the pollen, immature seeds, leaves, sheaths and shoots of plant as well as insect gall. 24-Epibrassinolide (**5**) has also been found in beewax.[7] Among the plants so far investigated, brassinolide (**1**) and castasterone (**2**) occur most frequently. In most plants, several brassinosteroids were often found in the same plant. In the immature seeds of *Phaseolus vulgaris* more than 30

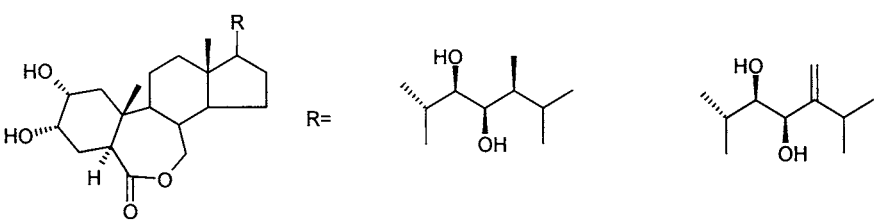
brassinosteroids including unknown compounds (partial structures being determined by GC-MS analysis) occurred.[2,3,6] Among all natural brassinosteroids, brassinolide and castasterone are the most important members due to their wide distribution and their potent biological activity.

3. Structure

The structure of brassinolide is (22R, 23R, 24S)-2α, 3α, 22, 23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one. The naturally-occurring brassinosteroids can be considered to be derivatives of 5α-cholestane (**Figure 1**). According to B ring oxidation stage, brassinosteroids can be divided into 7-oxalactone (**1-5**), 6-ketone (**6-22, 27-28, 30-32, 33-34**) and non B-ring oxidized brassinosteroids (**23-26, 29**). In general, 7-oxalactone brassinosteroids have higher activity than 6-ketone congeners, non-oxidized brassinosteroids reveal almost no activity in the bean internode assay and very weak activity in the rice lamina inclination assay. Brassinosteroids, which possess various orientation of vicinal hydroxyl groups at C-2 and C-3 in ring A, have been found in mature plants (**13-18, 27-29, 31**), the 2α,3α-dihydroxyl group containing brassinosteroids having stronger bioactivity. With respect to the sidechain of brassinosteroids, all natural ones have the same 22R,23R-vicinal hydroxyl groups, but the substitution at C-24 is different, such as there is no alkyl substitutent (**4, 11**) at C-24, a methyl (**1, 5-7, 13, 15-17, 19, 21, 23, 27-29, 33-34**), a methylene (**2, 9, 12, 14, 18, 20, 24-26, 30-32**), an ethyl (**8**), or an ethylene (**10, 25**) substitutent at C-24. At C-25, most brassinosteroids do not have additional methyl substitutent and only a few of them have a methyl substitutent (**14, 18, 20, 22, 26, 30-32**). The C-24 configuration in the most of the brassinosteroids with 24-alkyl groups is S (a methyl or ethyl), with the three exceptions of 24-epicastasterone (**7**), 3, 24-diepicastasterone (**16**) and 24-epibrassinolide (**5**) at which the C-24 configuration is R. The (24S)-24-methyl and ethyl substituted brassinosteroids possess higher biological activity. 24-O-β-D-glucopyranosyl-25-methyldolichosterone and 23-O-β-D-glucopyranosyl-2-epi-25-methyldolicho-sterone are two conjugated brassinosteroids.[3] The biological activity of these conjugates most likely depends on the activity of their aglycones.

Figure 1. Structures of naturally occurring brassinosteroids

7-Oxalactonic type



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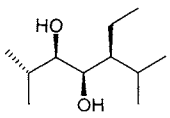

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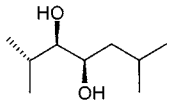

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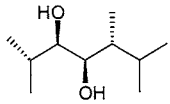

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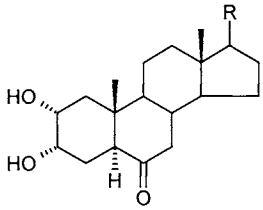

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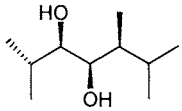

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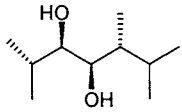

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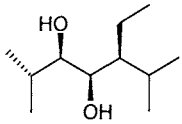

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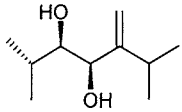

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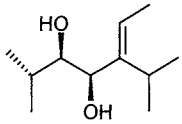

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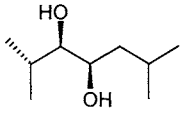

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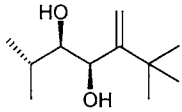

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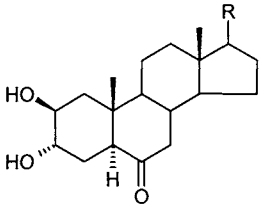

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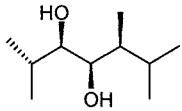

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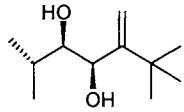

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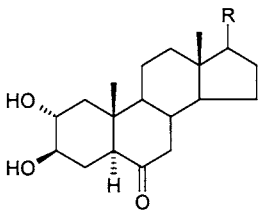

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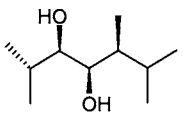

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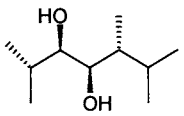

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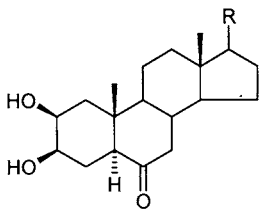

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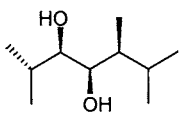

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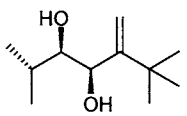

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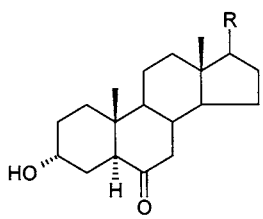

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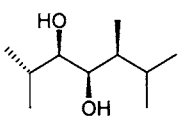

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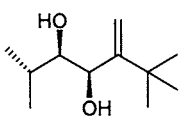

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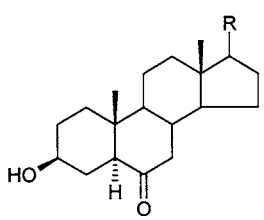

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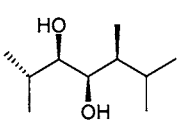

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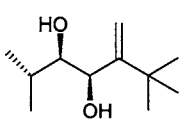

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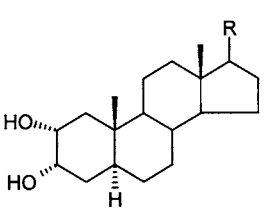
6-Deoxotype



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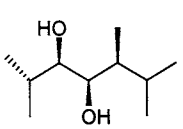

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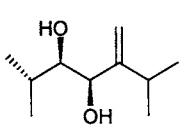

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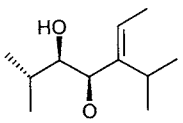

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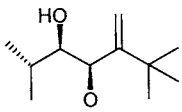

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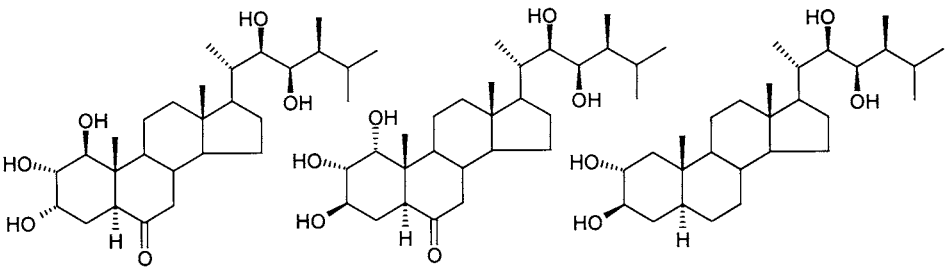
Other type



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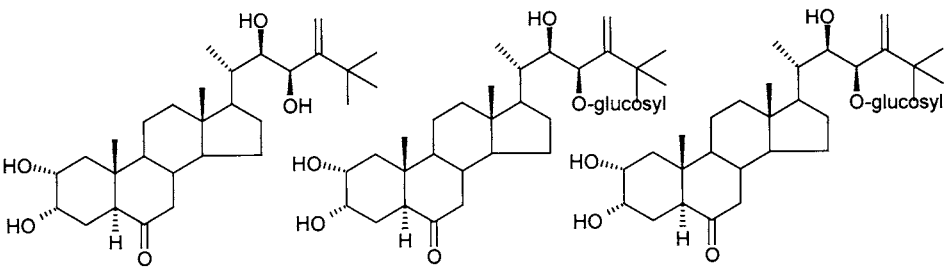

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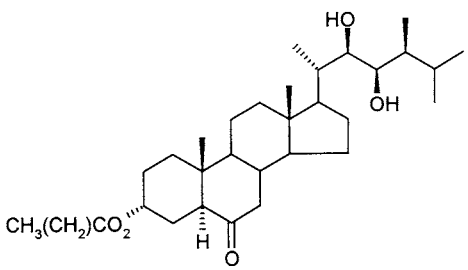

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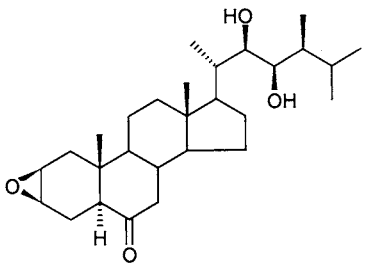
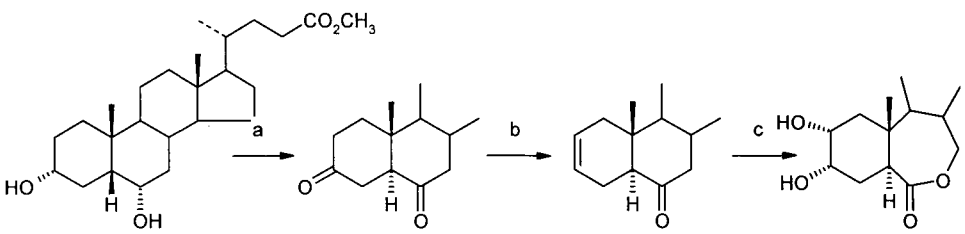


34

4.1. A, B Ring Modification

The synthesis of brassinolide requires a suitable steroid as starting material for the introduction of characteristic structural features in the A, B ring system and stereoselective building of the dihydroxy side chain. Some known methods for the construction of A,B-ring structure units consist of initial construction of the stigmasterol or ergosterol to Δ^2-6-keto steroid which is then converted into the 2,3-dihydroxy-7-oxalactone by hydroxylation with OsO_4-NMMNO and Baeyer-Villiger oxidation.[9] The hyodeoxycholic acid (**1**), which is rich in China, was used as starting materical for the synthesis of brassinolide by us. The Δ^2-6-keto steroid could be more conveniently obtained from hyodeoxycholic acid by Jone's oxidation, followed by selective reduction with Zn(Hg)/TMSCl in THF (**Scheme 1**).[10]



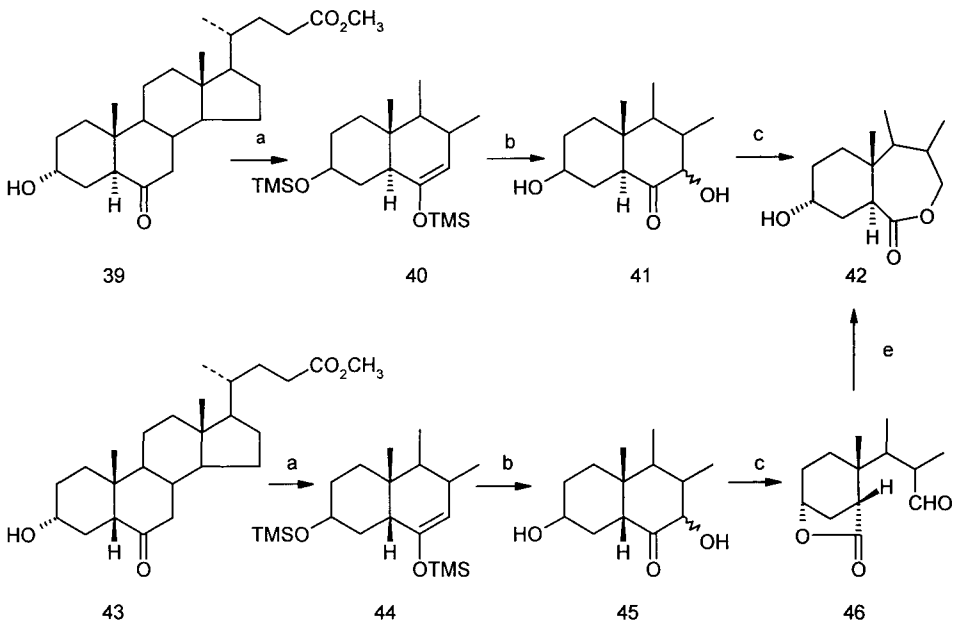
Reagents: a. 1. Jone's Reagents, 100%; 2. 1N HCl/MeOH, 86%; b. Zn(Hg)/TMSCl/THF, 67%; c. 1. OsO_4/NMMNO; b. CF_3O_3H.

Scheme 1

However, in the case of 3-hydroxy-5α(β)-steroid-6-ketone **39**, only a mixture of 6-oxa and 7-oxalactone in the ratio of ca. 1:2 or 3:2 was obtained by Baeyer-Villiger oxidation[11]. An efficient regioselective preparation of the 7-oxalactone was developed by us via ozone oxidation of the 6-enolsilyl ether steroid **40** (**Scheme 2**).[12] The trimethylsilyl enol ether **40** derived from 3-hydroxy-5α-steroid-6-one (**39**) by treatment with LDA and trimethylsilyl chloride was ozonized in CH_2Cl_2 in the presence of a small amount of pyridine to afford the 6-keto-7-hydroxy-steroid (**41**), which was converted into the 7-oxalactone **42** by oxidation of the α-ketol with HIO_4, followed by reduction and acidification.

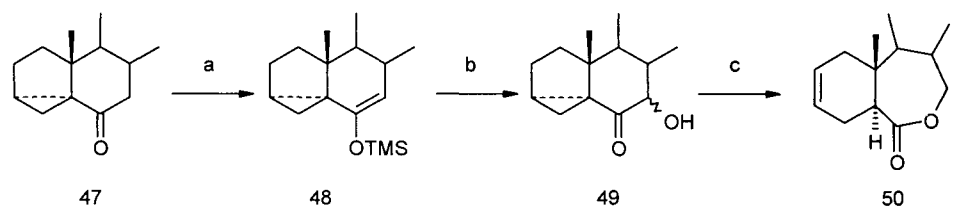
Similarly the trimethyl silyl enol ether **47** obtained from *i*-cholestanone or *i*-ergostanone was transferred into α-ketol **49** by ozone or m-CPBA oxidation. The cyclopropane in **48** was smoothly open to give Δ^2-α-ketol with *p*-TsOH and LiBr in DMF, following oxidation with periodic acid, reduction with sodium borohydride and acidification to gave the Δ^2-7-oxaloctone **50** (**Scheme 3**).[12b] This highly regioselective formation of 7-oxalactone ring by oxidation of 6-enolsilyl ether is a

complement of the Baeyer -Villiger oxidation. We applied this method for construction of the A, B-moiety of 24-epibrassinolide (**5**)[12b] and homobrassinolide(**3**).[51]



Reagents: a. TMSCl/LDA/Et_3N, -78°C; b. O_3/Py/CH_2Cl_2; c. 1. HIO_4/Ether; 2. $NaBH_4$/EtOH; 3. H_3O^+; d. HIO_4/Ether; e. 1. $NaBH_4$ 2. OH^-, 3, H_3O^+; 4. CH_2N_2

Scheme 2



Reagents: a. $CF_3SO_3SiMe_3/Et_3N$, O°C; b. O_3/CH_2Cl_2/Py or mCPBA/CH_2Cl_2; c. 1. *p*-TsOH/LiBr/ DMF; 2. HIO_4/Ether; 3. $NaBH_4$/MeOH, O°C; 4. 6N HCl/THF.

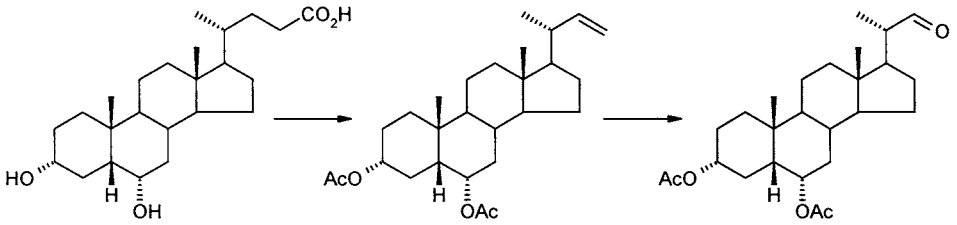
Scheme 3

4.2. Construction of Sidechain of Brassinosteroid

4.2.1. 20-Carbaldehyde Steroid Obtained from Hydeoxycholic Acid as Starting Material

(22R, 23R, 24S)-22, 23-dihydroxy-sidechain of brassinolide is an important characteristic function for its biological activities. An important problem in the construction of sidechain is to

control the stereochemistry at C22, C23, and C24. Many of methods have been reported to build the chiral center of side chain of brassinolide.[9] We have developed various methods for the stereocontrolled construction of sidechain of brassinolide from 20-carbaldehyde steroid **53** obtained from hyodeoxycholic acid (**51**) as starting material (**Scheme 4**).[10a]

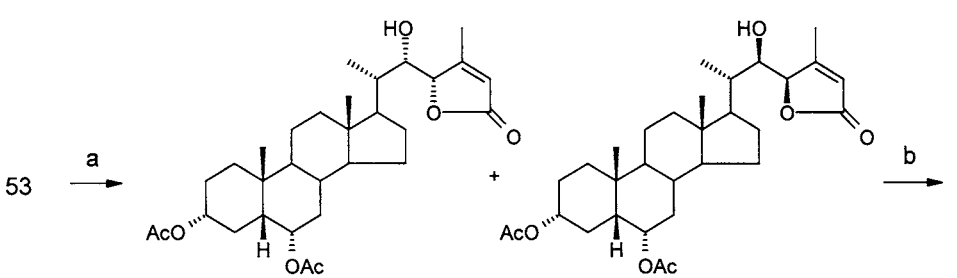


51 52 53

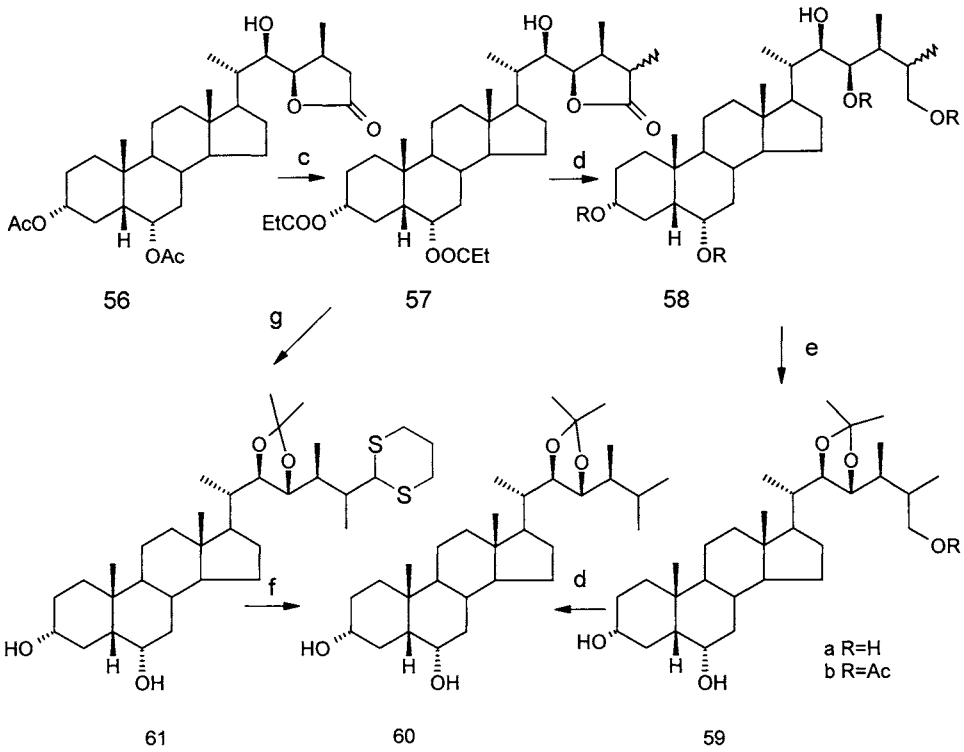
Scheme 4

4.2.1.1. Construction of Sidechain of Brassinosteroid via Aldol Reaction of 20-Carbaldehyde

The aldol reaction of the 20-carbaldehyde **53** with the anion of 3-methylbutenoilde in THF from butenolide and lithium diisopropylamide under kinetic condition yielded a mixture of the Cram **55** and anti-Cram **54** isomers in a ratio of 64:36.The stereochemistry at C23 was determined in the less steric interaction which results in the predominance of the 23R stereochemsitry. Hydrogenation of **55** over PtO_2 yield **56** in 62%yield. Methylation of **56** with CH_3I in the presence of LDA to give **57**, which was reduction with $LiAlH_4$ in THF afforded pentahydroxy compound **58**. Protection of 22,23-dihydyoxyl with 2,2-dimethoxypropane followed by selective tosylation with *p*-toluene-sulfonyl chloride and reduction with $LiAlH_4$ to afford the side chain of brassinolide[13] (**Scheme 5**). An improved route for the synthesis of **60** was achieved from **57** by the sequence of 4 steps in 30% over all yield from **57**, which is much better than that obtained from the mentioned method over all yield from **55** (**Scheme 5**).



54 55

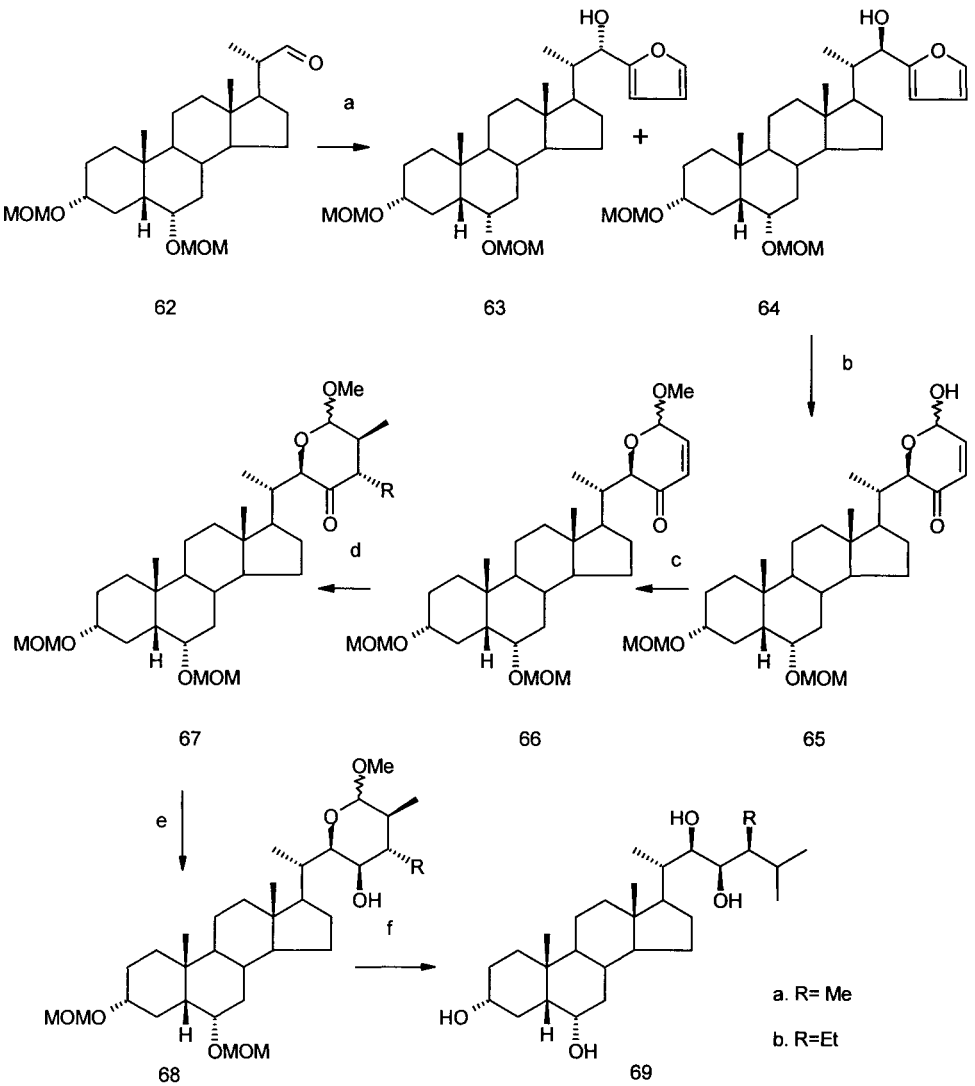


Reagnets: a. 3-methylbutenolide/LDA, -78 °C; b. H_2/PtO_2; c. LDA/MeI; d. $LiAlH_4$; e. 1. $Me_2C(OMe)_2$, 2. *p*-TsOH; e. $LiAlH_4$, f. Raney Ni ; g. 1. DIBAlH, 2. $(CH_2SH)_2$, 3. $Me_2C(OMe)_2$.

Scheme 5

4.2.1.2. Construction of Sidechain of Brassinosteroid via a Tandem Dialkylation of the Pyranone Moiety.

Reaction of the aldehyde **62** with the lithofuran which was generated from furan and n-butyllithium at -78 °C gave a 2.6:1 epimeric mixture of 22S-**63** and 22R-**64** in 90% yield.[14] The 22S-**63** could be converted into the 22R-**64** by oxidation of the 22-hydroxy of **63** followed by reduction with metal hydride. Oxidation of **64** with m-MCPBA gave the cyclic hemiacetal **65**, which was masked as the methyl derivative on treatment with $HC(OMe)_3/BF_3$ etherate, The unsaturated ketone **66** was reacted with the methyl lithium cuprate followed by reaction with CH_3I to give **67**. The tandem reaction offered procedure one step to introduction of both C24 methyl and C25 isopropyl groups, and represented an efficient method to prepare brassinolide (**1**), homobrassinolide (**3**) (**Scheme 6**).



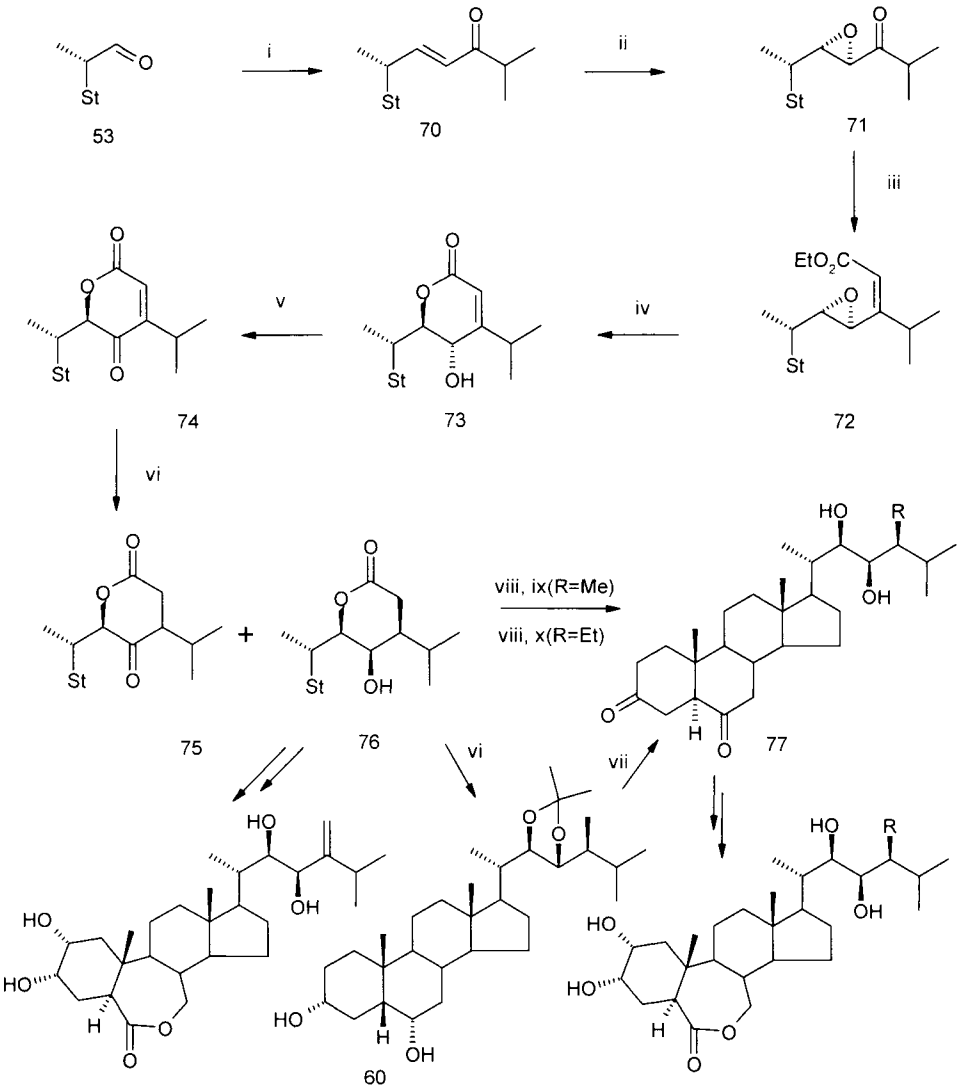
Reagents: a. LDA/Furan; b. m-CPBA; c. $HC(OCH_3)/BF_3$; d. Me_2CuLi/RI; e. $NaBH_4$; f. 1. $(CH_2SH)_2$; 2.Raney Ni

Scheme 6

4.2.1.3. Construction of Sidechain of Brassinosteroid via Reaction of 20 Carbaldehyde with Carbonyl Arsonium Yilde

20-carbaldehyde **53** was treated with isobutylcarbonyl arsonium yilde to form the α,β-unsaturated ketone **70** in 90% yield.[15] Epoxidation of **70** with H_2O_2 in the presence of sodium hydroxide afforded the α, β-epoxyketone **71**. The Wittig-Horner reaction of ethoxycarbonyl methylphosphonic acid dimethylester with **71** furnished a mixture of *Z*- and *E*- α, β-unsaturated-γ-δ-α-expoxy acid ester **72** in a ratio of 10:1 (70%). The key intermediate *Z*-**72** was lactonised under

acidic condition to give an unsturated lactone **73** formed by the carboxylate aided epoxide ring opening of Z-**72** with the inversion of the configuration at C22 in quantitative yield. The 23S-configuration of **73** could be easily converted into the 23R configuration by successive oxidation



Dolicholide (**2**)

R=Me Brassinolide (**1**)
R=Et Homobrassinolide (**3**)

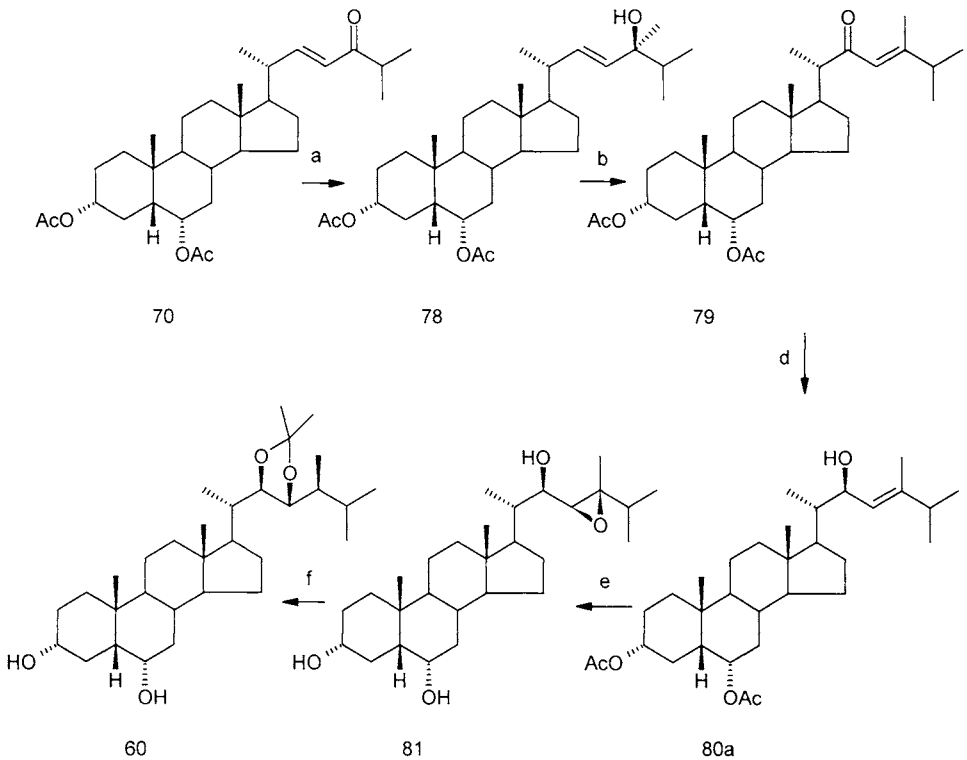
Reagents: i. $Me_2CH_2C(O)CHAsPh_3$; ii. 1. $H_2O_2/NaOH$; 2. Ac_2O/Py; iii. $(MeO)_2P(O)CH_2CO_2Et$; iv. $30\%HClO_4/MeOH$; v. H_2/PtO_2; vi. 1. DIBALH; 2. $p\text{-}TsOH/Me_2C(OMe)_2$; 3. $[(Ph_3P)]_3RhCl$; vii. 1. PDC; 2. 5%HCl/MeOH; vii. 1. $LiAlH_4$, 2. $p\text{-}TsOH/Me_2C(OMe)_2$; viii. 1. CrO_3/Py, 2. $[(Ph_3P)]_3RhCl$; ix. i. CH_3SO_2Cl/Et_3N, 2. $LiAlH_4$, 3. CrO_3/Py, 4. 5%HCl/MeOH.

Scheme 7

and reduction. Oxidation of **73** with pyridinium dichromate followed by hydrogenation over PtO_2 gave a mixture of 22R, 23R-γ-hydroxy δ-lactone **76** and 22R-γ-ketolactone **75** in quantitative yield in a ratio of 88:12. Reduction of the lactone **76** with DIBALH afforded a hemiacetal followed treatment with dimethoxypropane and decarbonylated with tris-(triphenylphosphine) rhodium chloride to give the **60** with 22R, 23R, 24S-side chain of brassonolide (**Scheme 7**).[16] This three step reaction sequence was performed in 76% over yield. The key intermediate **76** could be used for the synthesis of brassinolide (**1**), dolicholide(**2**), homobrassinolide (**3**) and typhasterol (**19**).

4.2.1.4. Construction of Sidechain of Brassinostereoids via the β-Alkylative 1,3-Carbonyl Transposition of the Steroidal 22-en-24-one

In continuation of our efforts to develop a brassinolsteroid sidechain synthesis, we have found a practical route for the stereoselective synthesis of the sidechain of brassinolide which involves β-



Reagents: a. MeLi; b. PCC; d. DIBALH; e. mCPBA; f. $LiBH_4$.

Scheme 8

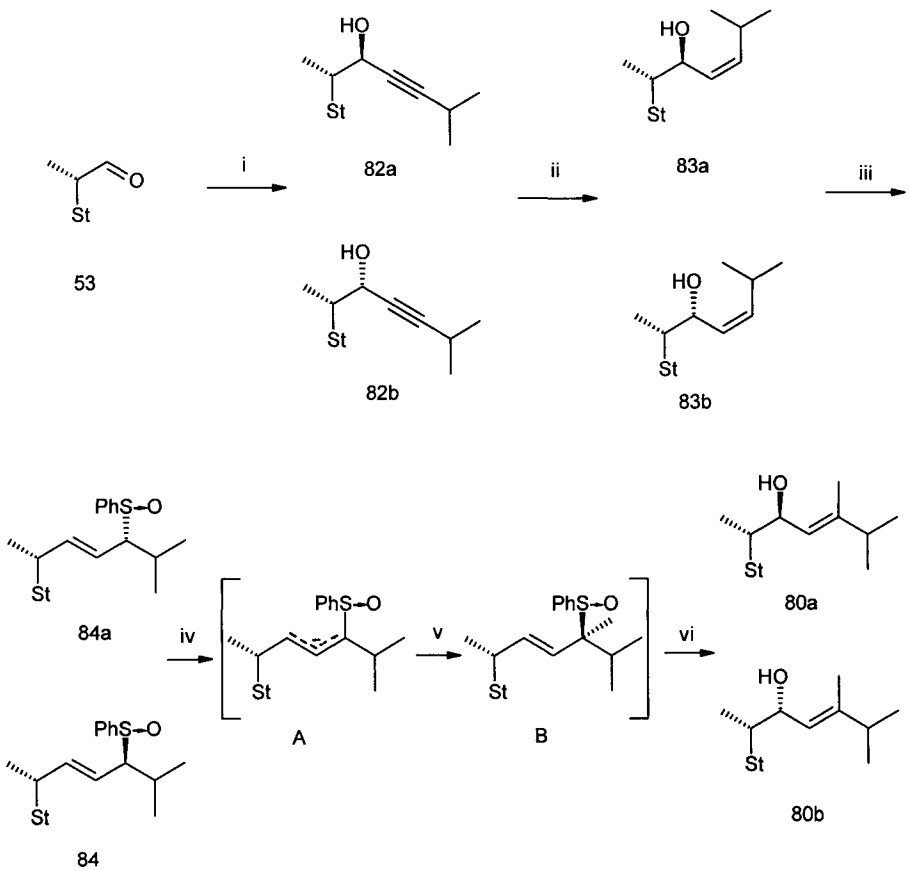
alkylative 1,3-carbonyl transposition of the α,β-unsaturated ketone, 3,6-diacetoxycholest 22-en-24- one.[17] The tertiary allylic alcohol **78** was obtained from the α,β-unsaturated ketone **70** by 1,2-addition of methyllithium which was oxidized with pyridiumchlorochromate to afford the transposed β-alkyl-α,β-unsaturated ketone in 93% yield. The newly introduced methyl group at C-24 was found in only a single configuration, presumed to be the α-orientation due to steric considerations. Stereoselective reduction of the enone **79** with DIBALH gave the (22R, 23E)- 22-hydroxy-24-methyl compound in 95% yield. Hydroxy-directed epoxidation of the enol **80a** with the m-CPBA gave desired the epoxide **81** in 95% yield. The stereo- and regio-selective opening of the epoxide **81** with inversion at C-24, followed by treatment with dimethoxypropane, gave the known compound **60** (**Scheme 8**).

The overall yield from the carbaldehyde **53** to the dioxolane **60** in six steps is 47%. The procedure is simple, flexible to perform, and provides a high overall yield for the construction of the sidechain of brassinolide and related compounds.

4.2.1.5. Construction of Sidechain of Brassinosteroids via Stereoselective 1,3-Sulfoxide Hydroxy Transposition

Alkylation of the C20 carbaldehyde **53** with but-1-ynyl-3-methylstannane[18a] or with 1,1-dibromo-3-methyl-but-1-ene followed by catalytic hydrogenation offerded (22R, Z-)-**83a** and (22S, Z)-**83b** (**Scheme 9**).[18b] Treatment of (22R, Z)-**83a** with benzenesulphenyl chloride and Et_3N at low temperature resulted in [2,3]-sigmastropic rearrangement to afford the (24R, 22E)-24-sulphoixe **84a**, which was treated with lithium diisopropylamide (LDA) and iodomethane at -78 °C to give, via rearrangement, the sulphenate ester of the alcohol **83** via the transition state A. This was cleaved at room temperaure with trimethylphosphite to give a mixture of (22R)-**80a** (47%) and (22S)-**80b** (6%) in the ratio 8.4:1 in 53% overall yield. Thus, conversion of the Z-enol (22R)-**83a** into the E-enol (22R)-**80a** was readily accomplished, although the yield of this reaction has not been optimized. The structure assignment of product **80b** was made based on that of its isomer **80a**. Since the 22-(*p*-nitrobenzoyl) derivative of **80b** exhibited a positive Cotton effect at 262nm, the configuration at C-22 could be assigned as being S. Similarly, when this reaction sequence was carried out on the (22S, Z)-enol **83b**, a mixture of enols **80a** and **80b** was also readily obtained in the ratio 6:1 in 56% overall yield.

Since either enol **83a** or **83b** gave the same compound **80a** in this 1,3-sulphoxide-hydroxy transposition process, this same reaction sequence was carried out on a mixture of substrates **83a** and **83b** to yield a mixture of enols **80a** and **80b** in the ratio of 7:1 in 46% overall yield. The present method for the preparation of enol (22R)-**80a** is both highly stereoselective and highly efficient.

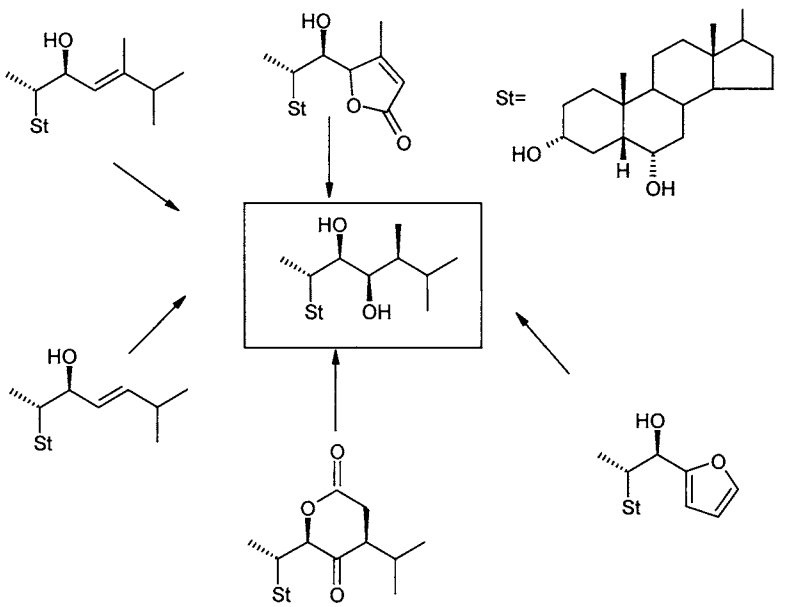


Reagents: i. $TiCl_4/Bu_3SnC{\equiv}CPr^i$ or $BuLi/Pr^iCH{=}CBr_2$; ii. $Pd/BaSO_4$; iii. $PhSCl/Et_3N/THF$; iv. LDA/THF; v. MeI; vi. $P(OMe)_3$

Scheme 9

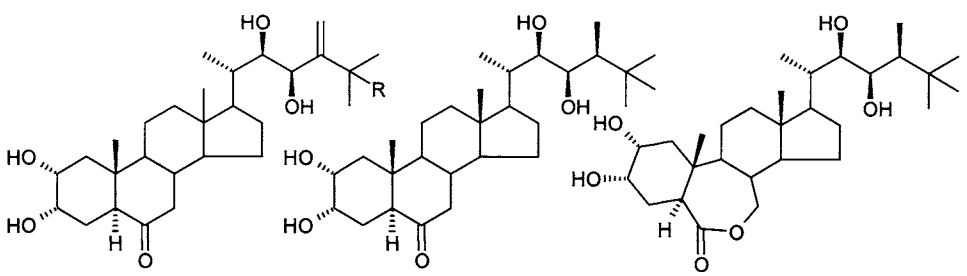
4.2.2. Intact Sidechain of Hyodeoxycholic Acid as the Starting Material

The above section has shown that the construction of brassinolide sidechain is based on C20-carbaldehyde **53**, obtained from hyodeoxycholic acid as the starting material, via various important building blocks (**Scheme 10**). Although the hyodeoxycholic acid is a selective starting material for the construction of the sidechain of brassinosteroids, however, degrading carboxyl sidechain of hyodeoxycholic acid to C20-carbaldehyde is not atom economy for construction of the sidechain of the brassinosteroid. For this reason, we have developed a synthetically useful route to brassinosteroidal sidechains by utilizing directly the intact sidechain of hyodeoxycholic acid, along with the β-alkylative 1,3-carbonyl transposition[19] of α,β-unsaturated ketone and α,β-unsaturated methyl ester as the key step, for construction of various sidechains of brassinosteroids.



Scheme 10

In 1987, Kim and co-workers isolated 25-methyldolichosterone (**12**) from *Phaseolus vulgaris* and found it to be about one order of magnitude more bioacitive than dolichosterone (**9**) itself by the rice lamina inclination test.[20] The introduction of a methyl group at C-25 could enhance the plant growth-promoting activity. This finding prompted us to establish an efficient method for construction of 25-homobrassinolide sidechain. Mori and co-worker first reported the syntheses of 25-homocastasterone (**85**) and 25-homobrassinolide (**86**) (**Figure 2**).[21] Our approach to 25-homo brassinolides from the intact sidechain of hyodeoxycholic acid, by means of β-alkylative 1,3-carbonyl transposition as the key step, is depicted in **Scheme 11**.[22]



9 R = H

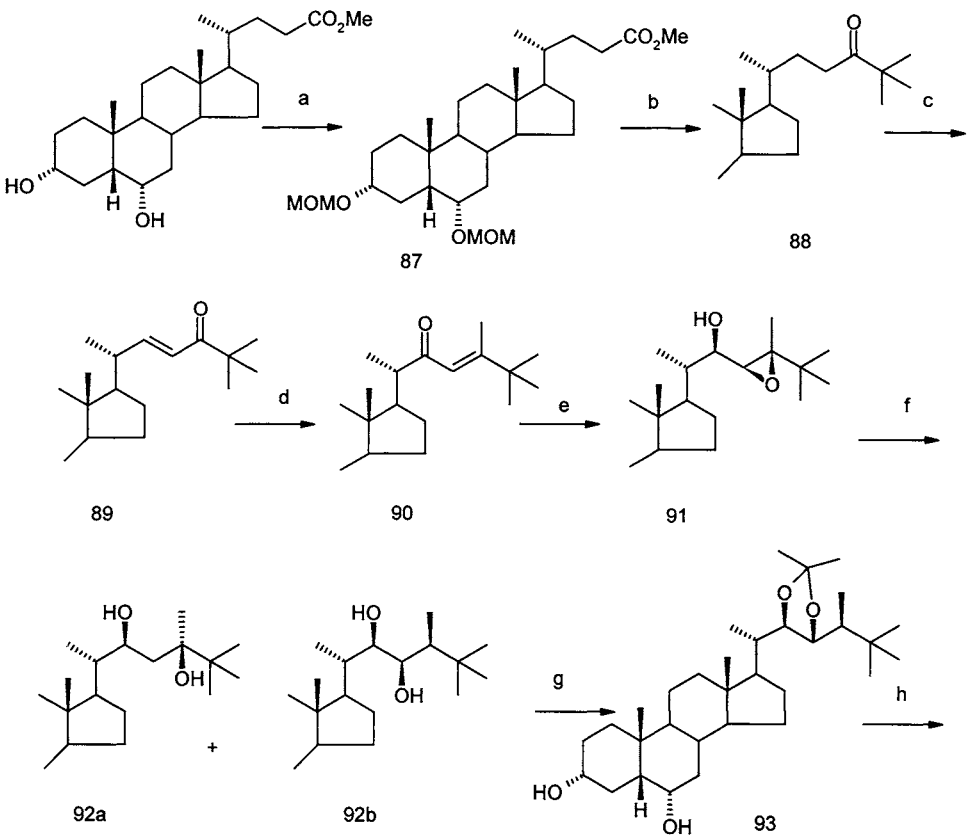
12 R = Me

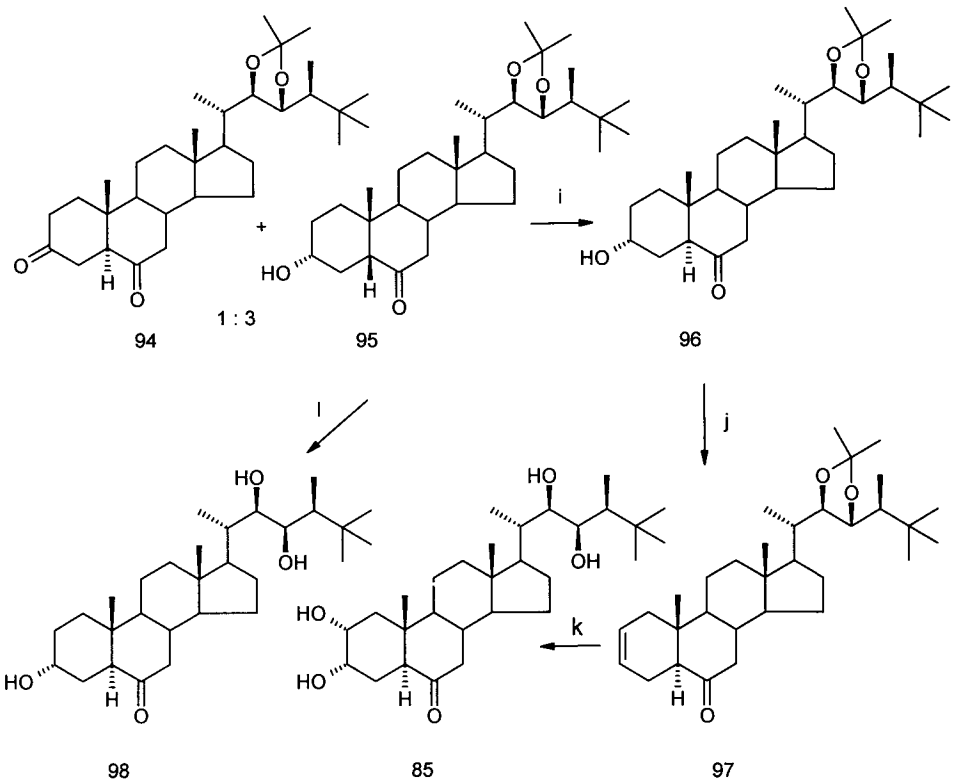
85

86

Figure 2

The first step was aimed to convert the bile acid to the α,β-unsatruated ketone **89**. For the best sequence, the protected **87** derived from methyl hyodeoxycholate was treated with tert-butyllithium at -78^{0}C, to yield the ketone **88** in respectable yield. Dehydrogenation of **88** by PhSeBr resulted in the α,β-unsaturated ketone **89** (92%). Oxidation of the tertiary allylic alcohol generated by the 1,2-addition of methyllithium to the 22-ketonic compound with pyridinium chlorochromate, produced the β-alkylative 1,3-carbonyl transposition product **90**.[17,19] Opening of the epoxide **91**, prepared by epoxidation with m-chloroperbenzoic acid (m-CPBA), with lithium borohydride in the presence of $Ti(OiPr)_4$ in benzene yielded regio and stereroselectively the 1,2-diol **92b** as major product. This procedure provided an improved regioselectivity compared to the $LiBH_4$-BH_3 in THF method used in the similar reduction of the brassinolide sidechain.[9a] 25-Homocastaterone (**85**) was synthesized from **93** through the following sequence of reactions: regioselective oxidation of 6α-



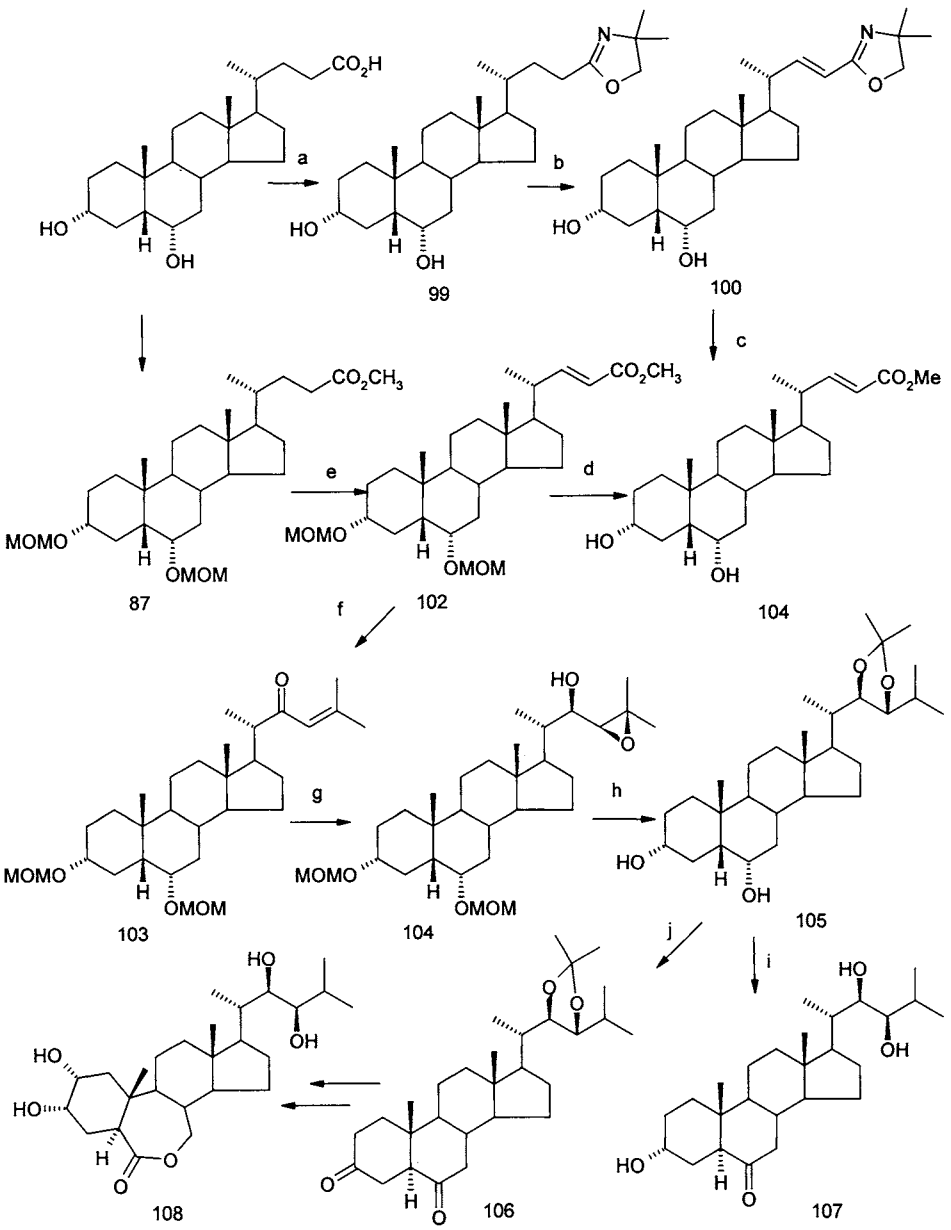


Reagents: a. $CH_2(OMe)_2$, P_2O_5, $CHCl_3$; b. t-BuLi, THF, -78°C; c. 1. LDA, THF, -78 °C; 2. PhSeBr; 3.aq. HOAc, 30% H_2O_2; d. 1. MeLi, THF, -78°C; 2. PCC, CH_2Cl_2; e. 1. DIBALH, THF, -78°C; 2. mCPBA, CH_2Cl_2; f. $LiBH_4$, $Ti(OiPr)_4$, benzene; g. 1. PPTS, t-BuOH; 2. $(MeO)_2CMe_2$, *p*-TsOH, CH_2Cl_2; h. PCC, CH_2Cl_2; i. MeONa, MeOH; j. $CuSO_4$-silica gel; k. OsO_4, $K_3Fe(CN)_6$; 1.25% HCl-MeOH.

Scheme 11

hydroxyl group of **93**; the base treatment for the epimerization of C-5 of **95**; dehydration of the 3-hydroxyl group of **96** by cupric sulfate adsorbed on silica gel and dihydroxylation of 2-ene **97**. Thus, **85** was obtained from **93** in 26% overall yield (four steps). 25-Homocastaterone (**85**) was converted to 25-homobrassinolide (**86**) by the usual Baeyer-Villiger oxidation. In addition, 25-homotyphasterol (**98**) as a new brassinolide analog, was obtained in 40% yield from **93**. The overall yield for the construction of 25-homobrassinosteroidal sidechain (**92b**) from **87** was about 30% in eight steps.

26, 27-bisnorbrassinolide (**108**), a new brassinolide analog, was synthesized first by the Ikekawa group, and it showed almost the same activity as brassinolide.[23] Its simple structure and remarkable activity prompted us to develop practical methods for its synthesis. Several approaches to this compound have been presented from C20-carbaldehyde by us.[24] In **Scheme 12**, we

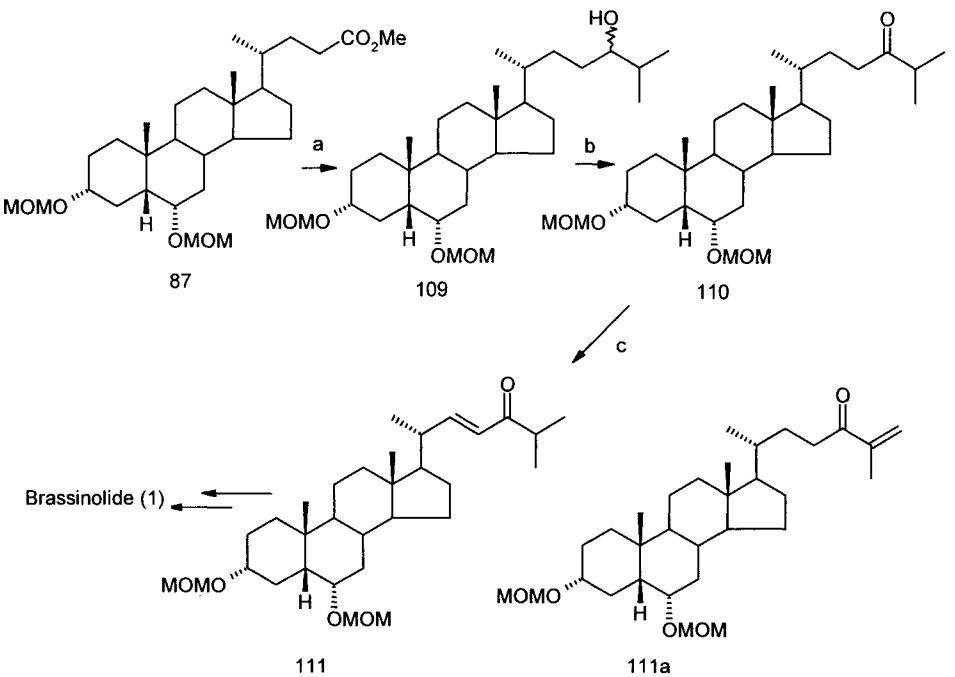


Reagents: a. 2-amino-2-methyl-1-propanol, H_3BO_3, xylene; b. $PhSeO_2H$; c. 5% H_2SO_4 in methanol; d. $CH_2(OMe)_2$, P_2O_5, $CHCl_3$; e. 1. LDA, THF, -78°C; 2. PhSeBr; 3. aq, HOAc, 30% H_2O_2; f.1. MeLi, THF, -78°C; 2. PCC, CH_2Cl_2; g. 1. DIBAL-H, THF, -78°C; 2. m-CPBA, CH_2Cl_2; h. 1. $LiBH_4$, $Ti(OiPr)_4$; 2. PPTS; 3. $(MeO)_2CMe_2$, p-TsOH, CH_2Cl_2; i. 1.PDC, CH_2Cl_2; 2.25% HCl-MeOH; j. 1. PDC, CH_2Cl_2; 2. p-TsOH, acetone.

Scheme 12

describe an improved method for construction of 26, 27-bisnorbrassinosteroidal sidechain from the intact sidechain of hyodeoxycholic acid, employing β-alkylative 1,3-carbonyl transposition as the

key step.[19] For the elaboration of the 26,27-bisnorbrassinosteroidal sidechain, the α, β-unsaturated ester **102** as a key intermediate was prepared from hyodeoxycholic acid via the oxazoline derivative **99**. Another approach to **102** involved the direct dehydrogenation of **87** by PhSeBr in high yield. The 22,23-acetonide **105** was obtained without any intermediate purification from **102** in seven steps via **103** and **104** in the same manner as **90-91** in 39.5% overall yield, involving the β-aklylative 1,3-carbonyl transposition reaction. Oxidation of **105** with PDC and subsequent acid treatment for 5-H epimerzation led to the diketo **106**, which can be readily convert to 26,27-bisnorbrassinolide (**108**) by our previous method.[24] In addition, the conversion of **105** to 26,27-bisnortyphasterol (**107**) was accomplished in 41% yield. The overall yield for the synthesis of 26,27-bisnorbrassinosteroidal sidechain (**105**) from **87** was about 35% in eight steps. To our knowledge, this is one the best method for the construction of the side chain of these two compounds.[23,25]



Reagents: a. iPrMgCl-LiBH$_4$, THF, -25 °C, b. PCC, CH_2Cl_2; c. LDA,THF, -78 °C; PhSeBr, -78 0°C; aq, HOAc, 30% H_2O_2.

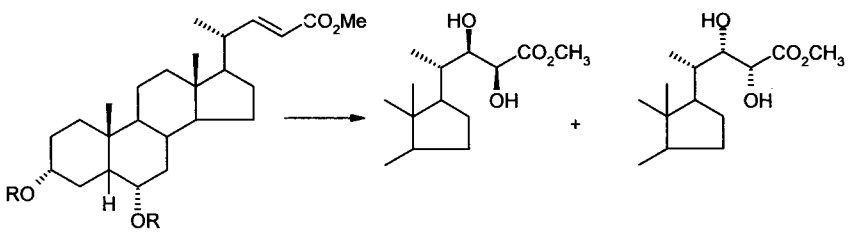
Scheme 13

The synthesis of brassinolide (**1**) from the intact sidechain of hyodeoxycholic acid is shown in **Scheme 13**.[22] Treatment of **87** with iPrMgCl-LiBH$_4$[26] at -25 ^{0}C produced the epimeric mixture of alcohols **109** in 79% yield. Dehydrogenation of the ketone **110** derived from **109** provided 22E-α,β-unsaturated ketone **111** as the major product. Conversion of the pure **111** into brassinolide (**1**) via β-alkylative 1,3-carbonyl transposition was achieved by our previous methods.[17]

We were successful in the construction of the brassinosteroidal sidechain from the intact sidechain of hyodeoxycholic acid. The present methods are simple, flexible and easy to perform and provide a high overall yield, in particular for the construction of the sidechain of 25-homo and 26,27-bisnor-brassinolides.

4.2.3. (22E)-Methyl Hyodeoxycholate as Starting Material

In our continuing effort to prepare brassinolide and its analogues from the intact sidechain of hyodeoxycholic acid, we have found that the dihydroxylation of (22E)-methyl hyodeoxycholate by the asymmetric osmium-catalyzed method[27] afforded the natural configured isomer **112a** as the major product (**Scheme 14**).[28,29] A 4:1 of the enatioselectivity in favor of the natural (22R, 23S)-diol isomer **112a** was obtained by employing dihydroquinidine *p*-chlorobenzoate (DHQD-CLB) as the chiral ligand. In contrast, when the osmylation of **102** was carried out in the absence of chiral ligand, a 8: 1 selectivity in favour of the unnatural (22S,23R)-**112b** was obtained. Our further studies showed that selectivity in favour of **112a** could be greatly improved by means of two new cinchona alkaloids: 9-O-(9'-phenanthryl) dihydroquinidine (DHQD-PHN)[30] and 1,4-bis (dihydroquinidine) phthalazine [(DHQD)$_2$-PHAL][31] respectively (**Scheme 14**).[32-34] The resulting natural (22R,23S)-isomer **112a** could be effectively used as an important building block for syntheses of various brassinosteroids [28,29]



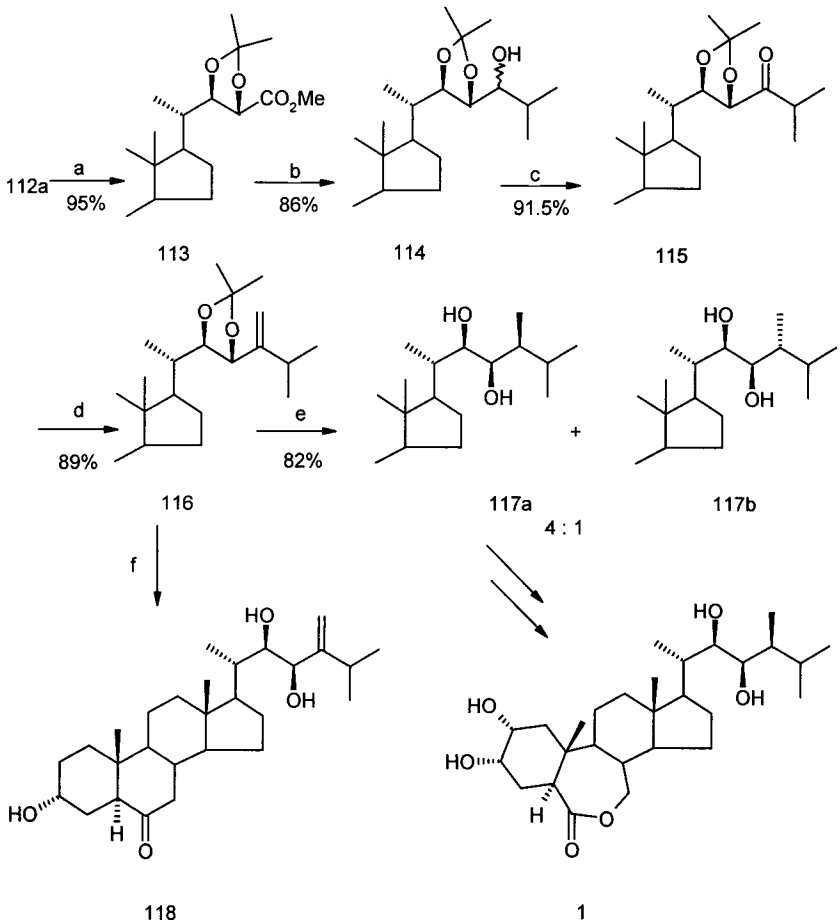
102 R = MOM (22R,23S)-112a (22S,23S)-112b

Scheme 14

Ligand	**112a: 112b**	Yield of **112a** (%)
none	1:8	--
DHQD-CLB	4:1	70
DHQD-PHN	17:1	86
(DHQD)2-PHAL	>100:1	89

Reagents: Cinchona ligand (0.5 equiv.), OsO_4(0.0125 equiv.), $K_3Fe(CN)_6$ (3equiv.), K_2CO_3 (3 equiv.) t-BuOH-H_2O(1:1).

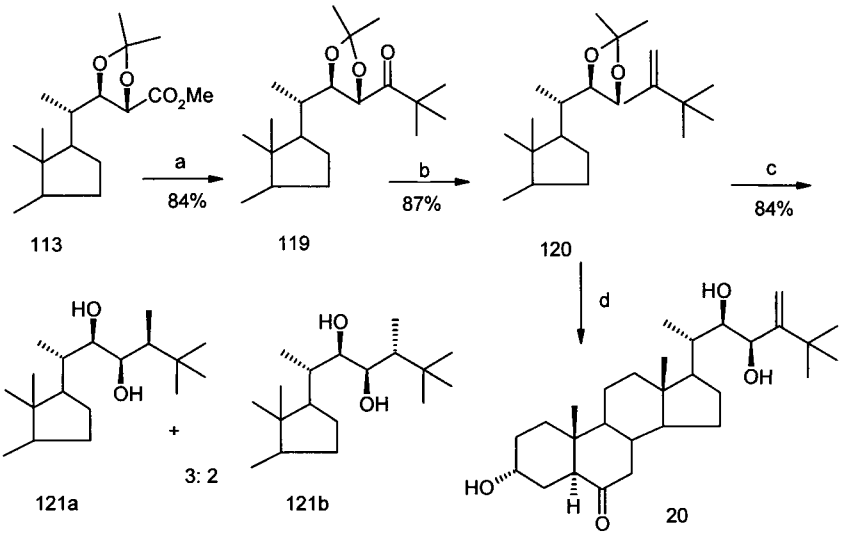
Our new approach to the construction of the sidechain of brassinolide is outlined in **Scheme 15** [28,29] The (22R, 23S)-diol **112a** was first protected as the acetonide **113.** Treatment of **113** with $LiBH_4$-iPrMgCl followed by oxidation of the resulting alcohol **114** with PCC gave rise to the ketone **115** in high yield. The Wittig reaction of the ketone **115** yielded the olefin **116**, which was hydrogenated in the presence of Pd-C, followed by acid treatment to provide the brassinolide sidechain (22R, 23R, 24S)-**117a** as the major product. The six-step overall yield from **102** to **117a** was ca. 36%. Compound **117a** was further converted into brassinolide and typhasterol according to our previous procedure.[22] In addition, starting from **116** through selective deprotection, oxidation and acid treatment enabled us to prepare a new brassinosteroid **118**. This method could be offered a new, stereoselective and high yielding route to the brassinosteroid sidechain.



Reagents: a. $Me_2(OCH_3)_2$/p-TsOH, CH_2Cl_2; b. $LiBH_4$/iPrMgCl, THF; c, PCC/NaOAc, CH_2Cl_2; Ph_3PMeI, t-BuOK, Benzene; e, 1.10%Pd/C, H_2; 2. 2.5%HCl; f. 1.PDC, 2. 2.5%HCl/MeOH

Scheme 15

25-Homobrassinolide (**86**), a more potent brassinosteroid than brassinolide, had been prepared earlier by Mori and co-worker[21] and our group.[22] Here, an attractive synthetic sequence from (22E)-methyl hyodeoxycholate for the synthesis of the target compounds is summarized in **Scheme 16.** In the first step, the acetonide **113** was converted to the ketone **119** by means of t-BuLi. The Wittig reaction of **119** followed by catalytic hydrogenation led to the pivotal intermediate **121a** and its 24-epi isomer **121b** in the ratio of 3:2. **121a** could be further transformed to 25-homobrassinolide and 25-homotyphasterol according to our reported method.[22] Moreover, in the same way as described for the preparation of compound **118**, a natural brassinosteroid **20** (which was first isolated from the kidney bean, *Phaseolus vulgaris*)[35] was prepared from **119** in 38% yield.

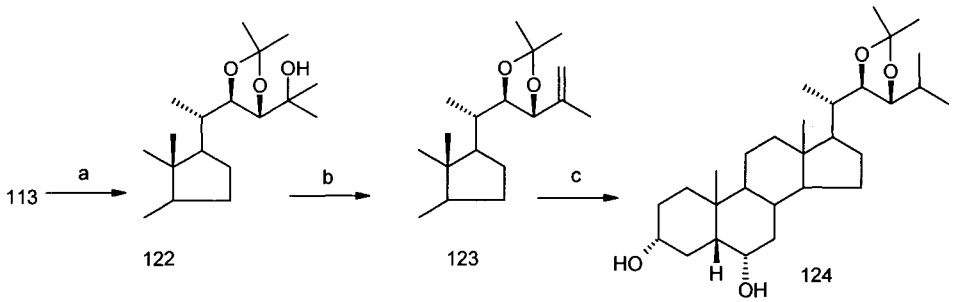


Reagents: a. t-BuLi, THF; b. Ph_3PMeI, t-BuOK, Benzene; c, 1.10%Pd/C, H_2; 2. 2.5%HCl; d 1.PDC, 2. 2.5%HCl/MeOH.

Scheme 16

As mentioned above, the key intermediate **124** for the syntheses of 26, 27-bisnorbrassinolide and 26,27-bisnortyphasterol has been previously prepared by means of β-alkylative 1,3-carbonyl transposition from **102.**[22] An alternative route to **124** was accomplished by reaction of **113** with MeLi and dehydration of corresponding tertiary alcohol **122**, following by catalytic hydrogenation. The overall yield of the four-step synthesis of **124** is ca. 69%. Compound **124** could be converted into the demethylated brassinolide and demethylated typhasterol by a known procedure.[22]

In the search for effective plant-growth promoters among brassinosteroids, a number of brassinolide analogs were synthesized to study the relationships between their structures and biological activities. Cerny and co-workers reported that an analog **125**, with a short acid sidechain

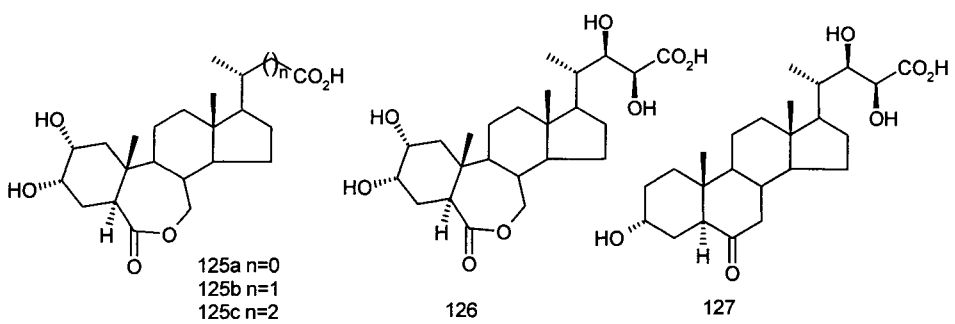


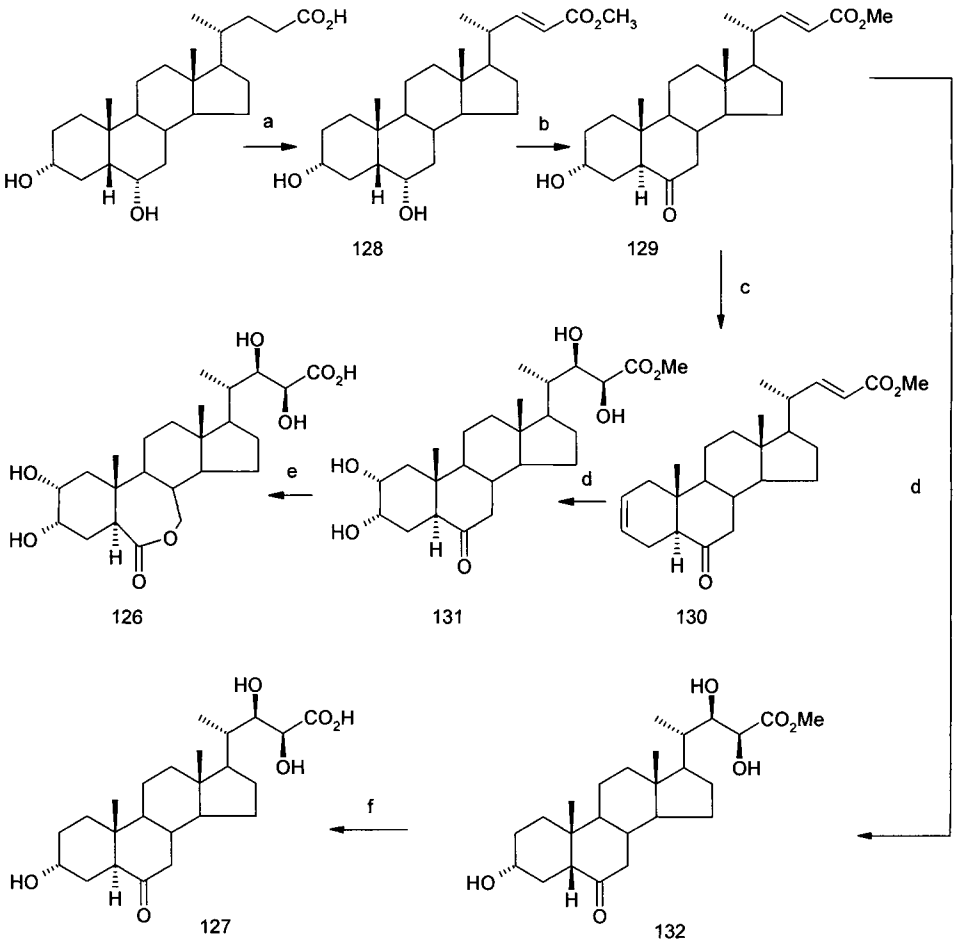
Reagents: a. MeLi, THF; b.$MeSO_2Cl/Et_3N$, DMAP; c. 1. PPTS, t-BuOH; 2.10%Pd/C, H_2.

Scheme 17

and A/B ring unit of brassnolide, showed high activity in both the bean first and second internode assay.[36] We synthesized two analogs **125b** and **125c**[37], and showed that both possessed around 50% activity of 24-epibrassinolide.[38,39] In attempts to prepare more active compounds with acid sidechain, we designed and synthesized two new derivatives **126** and **127** with an active vicinal (22R,23S)- diol moiety at the sidechain of **126**.[40] As shown in **Scheme 18**, the α,β-unsaturated ester **128**, derived from hyodeoxycholic acid was subjected to dehydration with $CuSO_4$ adsorbed on silica gel to yield the diene **130.** Asymmetric dihydroxylation of **130** and **129** by DHQD-PHN as the chiral ligand provided almost the sole natural (22R, 23R)-isomers **131** and **132** respectively. After Baeyer-Villiger oxidation of the ketones **131**, the title compound **126** was obtained. Hydrolysis of **132** gave the acid **127**.

We have developed a new and efficient synthetic approach to brassinosteroidal sidechains from (22E)-methyl hyodeoxycholate via the asymmetric osmylation as the key step. It is obvious that this procedure is quite effective for the construction of $\Delta^{24(28)}$-(22R, 23R)-diol dolicholide and 25-methyldolicholide sidechains, from which various brassinosteroids such as brassinolide, 25-methylbrassinolide and 26, 27-bisnorbrassinolide can be readily formed.





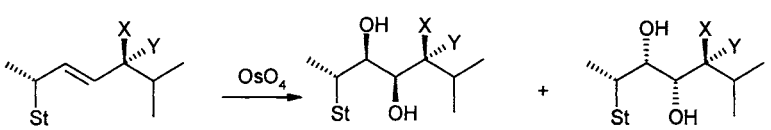
Reagents: a. Ref. 22. b. 1.PDC, CH_2Cl_2; 2. 2.5% HCl-MeOH; c. $CuSO_4$/silica gel, CCl_2=CCl_2; d. OsO_4 (cat.), DHQD-PHN, $K_3Fe(CN)_6$, K_2CO_3, tBuOH-H_2O (1:1); e. 1.Ac_2O, pyridine, DMAP, CH_2Cl_2, 2. CF_3CO_3H, CH_3Cl; 4%KOH-MeOH; 6N HCl-THF; f. 4%KOH-MeOH.

Scheme 18

4.2.4. Osmium Tetroxide Catalyzed Asymmetric Dihydroxylation of the (22E,24R)-and the (24E, 24S)-24-Alkyl Steroidal Unsaturated Sidechains

The direct cis-dihydroxylation of (22E)-unsaturated steroidal sidechain provides a convenient and potential route to the various brassinosteroidal sidechains. In the earlier studies, the osmylation was carried out with a stoichiometric amount of osmium tetraoxide and it was found that the oxidation was accelerated in the presence of pyridine.[41] As a matter of fact, this hydroxylation could be carried out efficiently with a catalytic amount of osmium tetraoxide, and N-methyl morpholine N-oxide (NMMNO) or potassium ferricyanide as cooxidants.[27] The alkyl substituent at C-24 has a significant influence on the dihydroxylation of the 22E-double bond of the steroidal sidechain. As

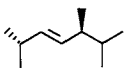
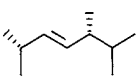
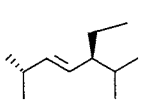
shown in **Table 2**, the steroids with (24S)-24-methyl group or without a methyl substituten at C-24 yielded predominantly the undesired (22S, 23S)-isomers (entry 1),[41-43] while the (24R)-24-methylgroup provided ca. 1:1 mixture of isomers (entry 2).[44-47,12b] Osmylation of (24S)-24-ethyl sustituents led almost exclusively to the unnatural (22S, 23S)-isomers (entry 3).[48-51] Isomers with the unnatural (22S, 23S)-22,23-dihydroxyl groups were inactive or less potent against the plant growth regulator activity.[41,42] Therefore an improved method for obtaining the natural (22R, 23R)-isomers as the major products is highly required. Recently, Sharpless and co-workers reported a very high enantioselectivity for the osmium catalyzed asymmetric dihydroxylation (ADH) of olefins by using cinchona alkaloids as the chiral ligands and potassium ferricyanide as co-oxidant.[27] We have applied this method for the first time to the higher olefin, (22E)-methyl hyodeoxycholate, without a substituent at 24 position, providing the natural (22R, 23S)-isomers as the major product (see 4.2.3). The extension of the scope of this methodology to (22E)-24-alkyl steroidal unsaturated sidechains was found to be also successful. The detailed results of ADH with different cinchona ligands: DHQD-CLB, DHQ-CLB, DHQD-PHN, 9-O-(9'-naphthyl) diphydroquinidine (DHQD-NAP) and $(DHQD)_2$-PHAL are tabulated in **Table 2**. An unexpected ratio of 8:1 of (22R, 23R) to (22S, 23S) was obtained from the (24S)-24-methyl substituted unsaturated sidechain with DHQD-CLB(entry 1),[33,52] in contrast to the old method.[41-43] When the compound with the (24R)-24 methyl unsaturated substituted sidechain was dihydroxylated with (DHQD)2-PHAL, an improved ratio (22R, 23R) to (22S, 23S) from 8:1 (with DHQD-CLB) to 45:1 was observed (entry 2).[34] It was also noted from **Table 2** that dihydroxylation of (22E, 24S)-24-ethyl steroid produced the natural configured isomers as major product, and the ratio of (22R, 23R) to (22S, 23S)-isomers was improved up to a ratio of 8:1 (entry 3)[32,33] Furthermore, we found that the reaction rate and stereoselectivity were decreased when the amount of either chiral ligand or osmium catalyst was diminished (entry 2-3, **Table 2**).Very recently, the same method has been employed by the Brosa,[50,53] McMorris[54,55] and Marino groups[56] for the syntheses of brassinolide, 24-epibrassinolide and homobrassinolide (see entry 1-3, **Table 2**). Marino and co-workers found that for the ADH of 24 (R) and 24 (S)-methyl unsaturated sidechains both gave 100% enantionselectivity in favour of the natural (22R,23R)-diols isomer with $(DHQD)_2$-PHAL.[56]

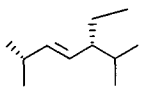


(22R, 23R)-133a (22S, 23S)-133b

X = Me, Et; Y=H
X = H; Y= Me, Et
St = various steroidal nuclei

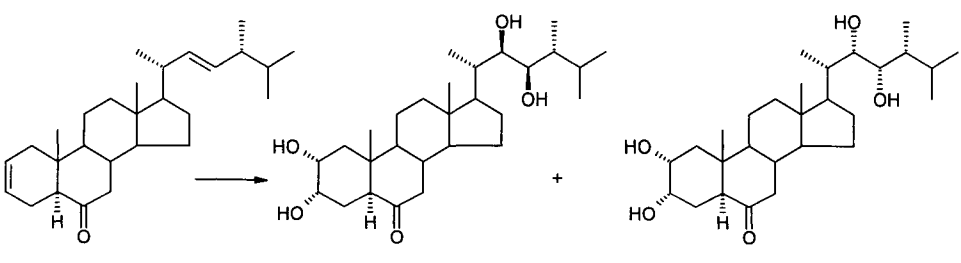
Table 2. Dihydroxylation of (22E)-Steroidal Unsaturated Sidechain with OsO_4

Entry	Sidechain	Chiral Ligand (%)	A[a] (%)	B[c] (eq.)	Co-Oxiddant	C[d]	Ref.
1		--	b	--	--	<1:>4	41,42
		--	b	--	--	1:6.5	43
		DHQD-CLB(100)	10	--	$K_3Fe(CN)_6$	8:1	52,33
		DHQ-CLB(100)	10	--	$K_3Fe(CN)_6$	0:100	52,33
		$(DHQD)_2$-PHAL(100)			$K_3Fe(CN)_6$	100:0	56
2		--	b	--	--	1:1	44,45
		--	4.0	--	NMMNO	1:1	46
		--	1.2	--	NMMNO	3:5	47
		--	6.0	--	NMMNO	3:4	12b
		DHQD-CLB(100)	10	--	$K_3Fe(CN)_6$	8:1	52,33
		DHQ-CLB(100)	10	--	$K_3Fe(CN)_6$	1:9	52,33
		DHQD-CLB(50)	5.0	2.0	$K_3Fe(CN)_6$	14:1	34
		DHQD-CLB(25)	2.5	2.0	$K_3Fe(CN)_6$	10:1	34
		DHQD-CLB(20)	4.0	2.0	$K_3Fe(CN)_6$	9:1	54
		DHQD-PHN(100)	10	--	$K_3Fe(CN)_6$	17:1	32,33
		$(DHQD)_2$-PHAL(100)	10	2.0	$K_3Fe(CN)_6$	14:1	34
		$(DHQD)_2$-PHAL(50)	10	--	$K_3Fe(CN)_6$	45:1	34
		$(DHQD)_2$-PHAL(40)	10	2.0	$K_3Fe(CN)_6$	100:0	56
		$(DHQD)_2$-PHAL(20)	4.0	2.0	$K_3Fe(CN)_6$	10:1	54
3		--	b	--	--	1:16	48
		--	8.3	--	NMMNO	1:24	49
		--	10	--	NMMNO	1:24	50
		--	7.6	--	NMMNO	1:5	51
		DHQD-CLB(100)	10	--	$K_3Fe(CN)_6$	1.5:1	52,33
		DHQ-CLB(100)	10	--	$K_3Fe(CN)_6$	0:100	52,33
		DHQD-CLB(20)	4.0	2.0	$K_3Fe(CN)_6$	1.4:1	55
		DHQD-NAP(100)	10	2.0	$K_3Fe(CN)_6$	4.8:1	34
		DHQD-NAP(100)	10	--	$K_3Fe(CN)_6$	3.9:1	34
		DHQD-NAP(50)	5.0	2.0	$K_3Fe(CN)_6$	2.2:1	34
		DHQD-NAP(25)	2.5	2.0	$K_3Fe(CN)_6$	1.5:1	34
		DHQD-PHN(100)	10	--	$K_3Fe(CN)_6$	8:1	32,33

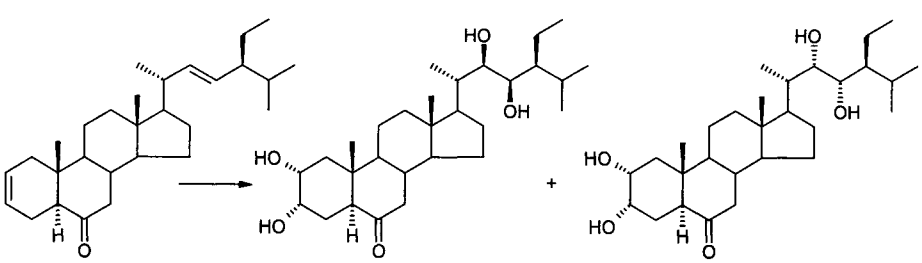
	DHQD-PHN(25)	2.5	2.0	$K_3Fe(CN)_6$	2.0:1	34
	DHQD-PHN(100)	10	--	NMMNO	1:1.5	50
	$(DHQD)_2$-PHAL(100)	10	2.0	$K_3Fe(CN)_6$	1.2:1.	34
	$(DHQD)_2$-PHAL(100)	10	--	$K_3Fe(CN)_6$	1.4:1	34
	$(DHQD)_2$-PHAL(20)	4.0	2.0	$K_3Fe(CN)_6$	2.1:1	55
	$(DHQD)_2$-PHAL(40)	10	2.0	$K_3Fe(CN)_6$	4:1	56
4		--	3.6	--	NMMNO	1:2	57

a. A= OsO_4; b. Stoichiometric amount of OsO_4; c. B=$MeSO_2NH_2$; d. C=Ratio of **133a:133b**

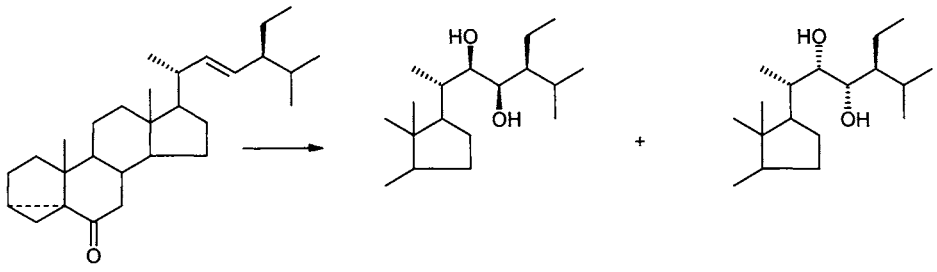
On the other hand, methylsulfonamide played an important role in the ADH of 22E-unsaturated sidechains.[27,34] The results are incorporated in **Table 3.** The reaction rate was increased substantially in the presence of $MeSO_2NH_2$ in case of dienes **134** and **136** which contained one more double bond in ring A (entry 1-4, **Table 3**).[34] Simultaneously, with regard to **138**, only slight effects of the reaction rate were observed (entry 5, 6 **Table 3**). However , there is no significant influence on the enantioselectivity in all cases with methylsulfonamide (entry 2,3, **Table 2**).



134 135a 135b



136 8 137



138 139a 139b

Tabab 3. Effects of methanesulfonamide on the ADH of (22E)-24-alkyl unsaturated sidechain[a]

Entry	Oleifin	Chiral Ligand	$MeSO_2NH_2$(eq.)	React. Time(day)
1	**134**	DHQD-CLB	2.0	1
2	**134**	DHQD-CLB	--	5
3	**136**	DHQD-NAP	2.0	1
4	**136**	DHQD-NAP	--	4
5	**138**	DHQD-PHN	2.0	5
6	**138**	DHQD-PHN	--	6

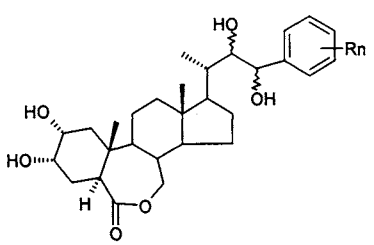
a. 1.0 equiv. ligand and 0.1equiv. osmium tetroxide was used.

This highly effective new dihydroxylation method was proved to be useful for the preparation of the (22R,23R)-configured bioactive isomer in spite of the existence of (24S) or (24R)-methyl in the unsaturated sidechain. It is equally effective for (22E,24S)-24-ethyl substituted sidechain.

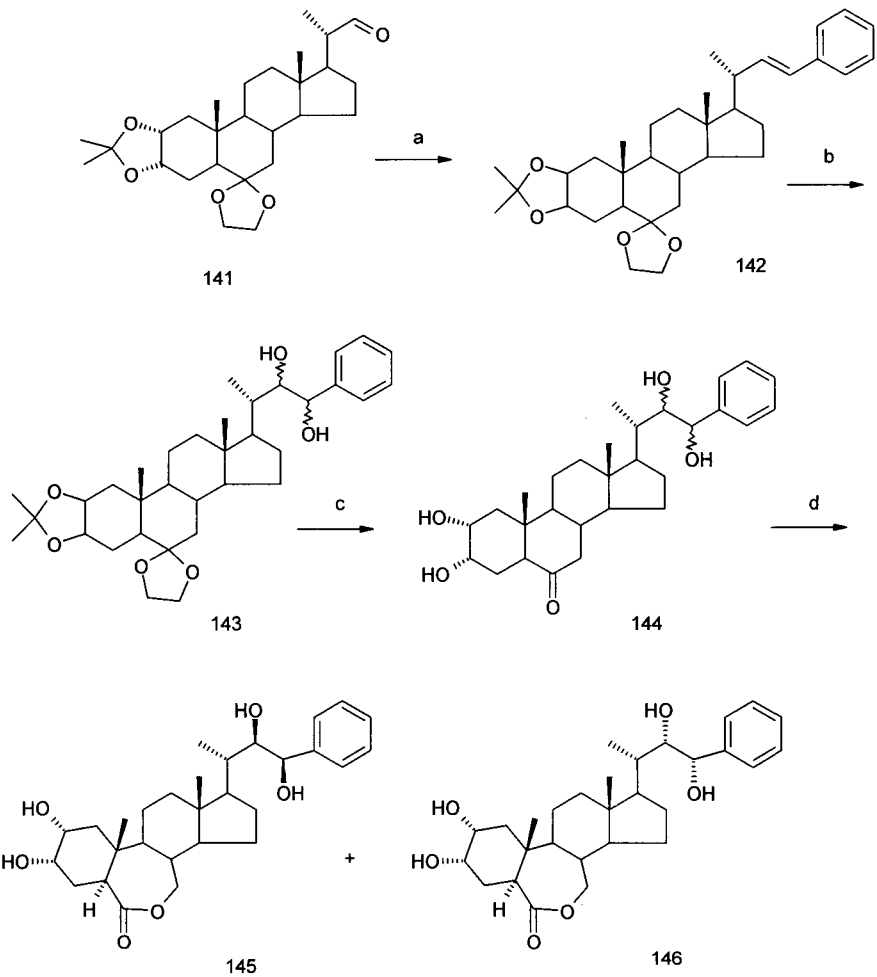
4.2.5. 22-Olefin Steroid Obtained from Hyodeoxycholic Acid as Starting Material

4.2.5.1. Synthesis of 23-Arylbrassinolides via Heck Arylyation and Asymmetric Dihydroxylation

As a part of our efforts toward the synthesis of brassinosteroids, we have reported a practical method for the stereoselective construction of the sidechain, which involved direct osmium catalyzed asymmetric dihydroxylation [27] from the readily available (22E)-unsaturated steroidal sidechains, to provide the naturally configured (22R, 23R)-diol isomer as the major product.[58] This



140 (22R,23R) and (22S,23S); R=H, Halo,etc; n=1,3

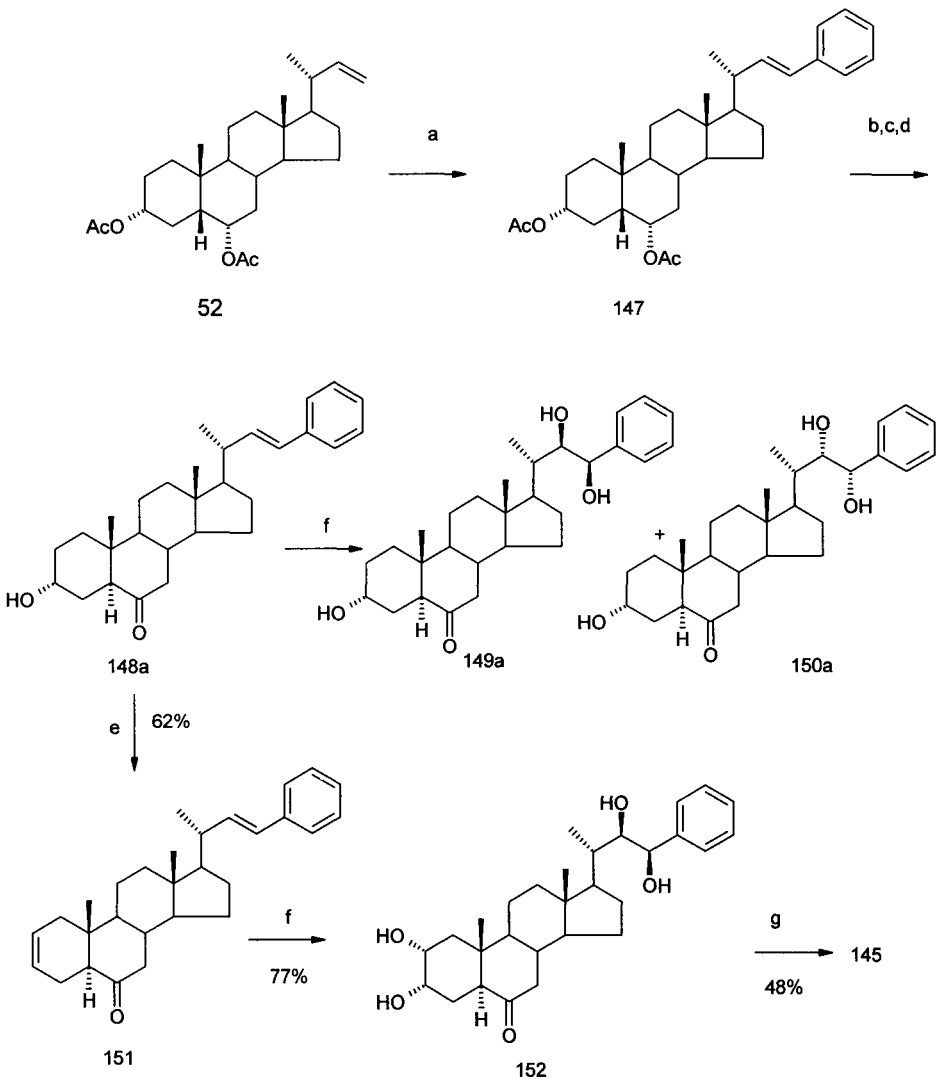


Reagents: a. n-BuLi/$Ph_3P^+CH_2PhCl^-$; b. cat. OsO_4, NMMNO; c. 80% HOAc; d. 1. Ac_2O, Py, cat. DMAP; 2. CF_3CO_3H, Na_2HPO_4; 3. aq. NaOH; 6NHCl

Scheme 19

approach shows the promise of considerable practical value due to its efficiency and simplicity. On the other hand, in our laboratory numerous new analogues have been synthesized for the investigation of structure-activity relationships. Ikekawa and coworkers have disclosed that the 23-phenylbrassinosteroids **140** are potential plant growth promotors and they are promising candidates for application in agriculture.[59] However, as shown in **Scheme 19** for the synthesis of the above compounds **140**, the procedure was suffered from a large amount of BuLi in the Wittig reaction of the 20-carbaldehyde**141**. Furthermore, osmylation of the 22E compound **142** with NMMNO as co-oxidant yielded mainly the unnatural (22S, 23S)-diol isomer. Therefore, an efficient synthesis of 23-

phenylbrassinosteroids **140**, particularly of the natural (22R,23R)-configuration, should be of great significance based on its simple structure and high activity. Our synthetic strategy toward (22R, 23R)-**140** involves both Heck coupling [60] and asymmetric osmylation as key steps (**Scheme 20**).



Reagents: a. PhI, 2% $Pd(OAc)_2$, 4%Ph_3P, Et_3N; b. 4%KOH-MeOH; c. PDC,CH_2Cl_2; d. 2.5%HCl-MeOH; e. $CuSO_4$-silica gel, THF; f. DHQD-CLB, cat. OsO_4, $K_3Fe(CN)_6$, K_2CO_3 aq. t-BuOH(1:1, v/v); g. 1. Ac_2O, Py, cat. DMAP, CH_2Cl_2; 2. CF_3CO_3H, CH_2Cl_2; 3. 4%KOH-MeOH; 4. aq. HCl, THF.

Scheme 20

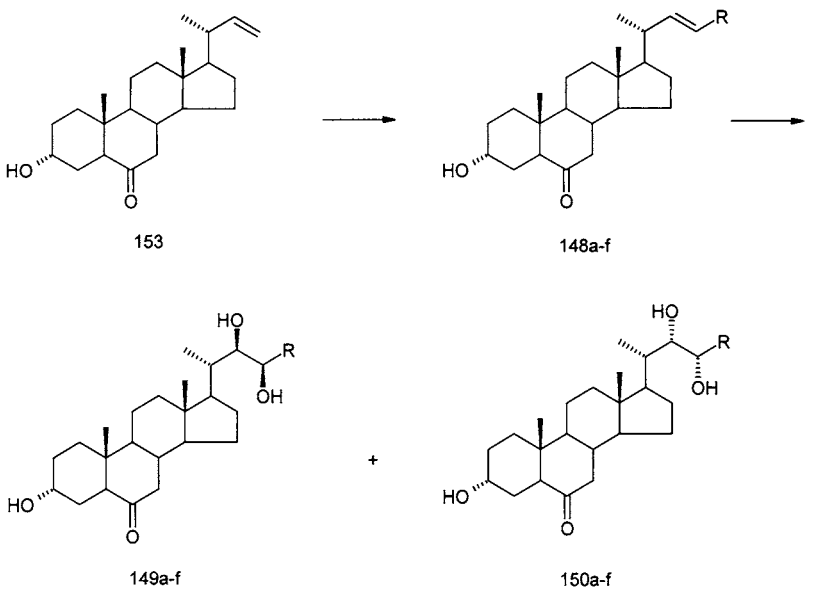
Specifically, in **Scheme 20** summarized the preparation of 23-phenylbrassinosteroid **145** and **149a**. Olefin **52**[61], on Heck coupling with iodobenzene, produced the (22E)-23-phenyl olefin **147** (81%). The advantages of Heck arylation are its simplicity and high yield. After functionalization of the A/B ring of compound **147**, osmylation of the resulting styrene **148a** was studied under different conditions (**Table 4**). With the NMMNO method, there was mainly obtained the unnatural (22S, 23S)-diol isomer **150a** (entry 1). On the other hand, when the asymmetric dihydroxylation method was employed, the major component was the natural isomer **149a** obtained with high enantioselectivity (entries 2-4). Similarly, the 2,22-diene **151** was subjected to the dihydroxylation with DHQD-CLB as chiral ligand, producing the (22R,23R)-2α,3α22,23-tetrahydroxy compound **152** in high yield. Baeyer- Villiger oxidation of ketone **152** afforded the target compound **145.** Thus, we have presented an efficient and practical procedure with high overall yield to prepare 23-phenylbrassinosteroid **145a** (10%, seven steps) and **149a** (37%, five steps) from the olefin **147**. It is noteworthy that the synthetic **149a** showed almost the same activity as 24-epibrassinolide on the rice lamina inclination test.[62] Since the introduction of a C-23 phenyl moiety has a significant effect on the bioactivity, it gives us considerable incentive to prepare various substituted 23-arylbrasinosteroids for further structure-activity studies.

Table 4. Osmylation of olefin 140a to glycols 149a and 150a

Entry	Ligang	Co-oxidant	Ratio of **149a : 150a**	Isolated yield(%)
1	--	NMMNO	1:2	88
2	DHQD-CLB	$K_3Fe(CN)_6$	8.8:1	90
3	DHQD-NAP	$K_3Fe(CN)_6$	8.9:1	82
4	DHQD-PHN	$K_3Fe(CN)_6$	9.3:1	89

In order to synthesize substituted 23-arylbrassinosteroids, we explored the Heck arylation of the olefin **153** with a variety of aryl iodides (**Scheme 21**, **Table 5**). We found that the coupling of compound **153** with an electron-donating aryl iodide proceeds very well under the usual Heck reaction conditions (entries 1-3, Method A), but the electron-withdrawing *p*-nitrophenyl iodide refuses to react with olefin **153** (entry 4, Method A). After several trials, it was found that this coupling reaction of the electron-withdrawing aryliodide could be realized under phase-transfer conditions [63] (entry 4, Method B) and was effective in the case of the electron-donating aryl iodide (entry 3,5, Method B). However, the coupling of aryl bromide with olefin **153** is not satisfactory with either method A or B. For example, there was little or no reaction of the olefin **153** with *p*-tolyl bromide to give product **148f**, and we were unable to obtain any diol **149f/150f**. The resulting

22E-23-aryl compounds **148b-e**, when subjected to asymmetric dihydroxylation, afforded the natural (22R, 23R)-isomers **149b-c** as the major products with 8:1-10:1 enantioselectivity.



Scheme 21

Table 5. Aryation of olefin 16 with an aryl halide and subsequent asymmetric dihydroxylation of styrene 148 to 22,23-diols 149 and 150

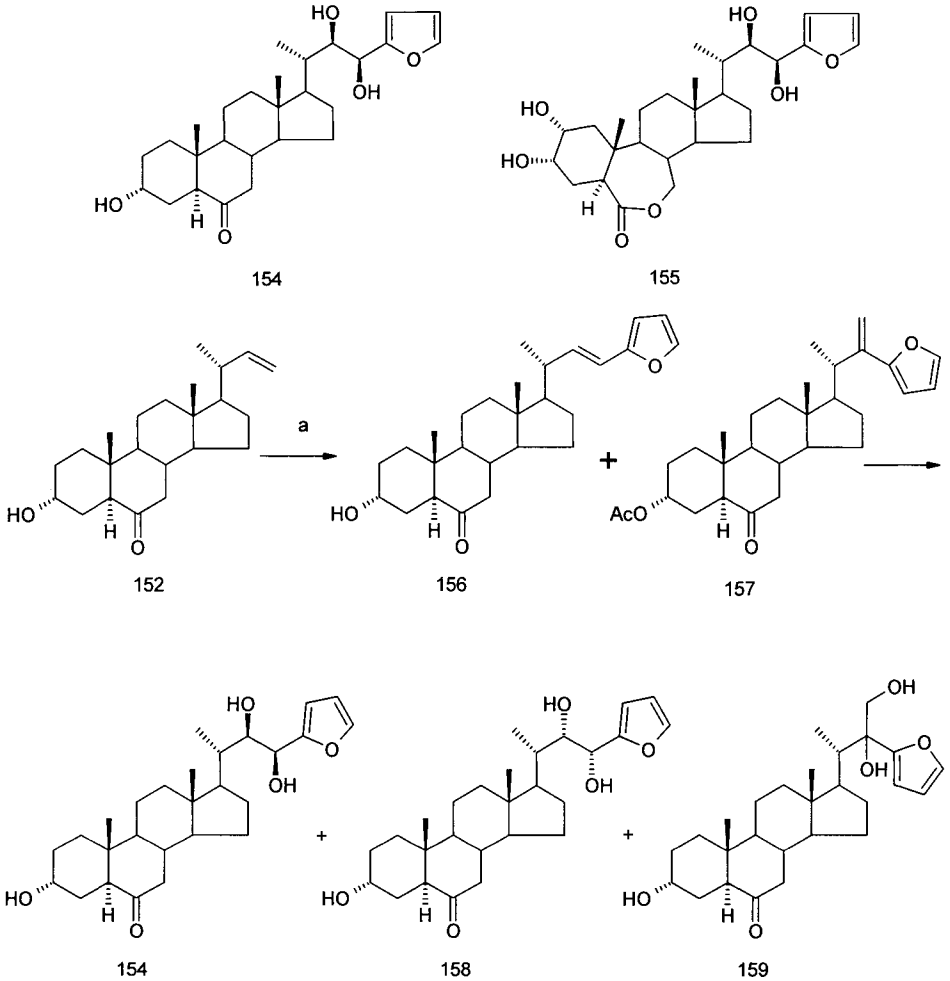
Entry	ArX	R		148 Yield(%)[a] Yield(%)[b]		22,23-diol Yield%	**149:150**
1	PhI	Ph	**a**	79(88)		90	8.8:1
2	*p*-ClC_6H_6I	*p*-ClC_6H_6	**b**	80(90)		87	9.3:1
3	*p*-BrC_6H_6I	*p*-BrC_6H_6	**c**	74(84)	50(66)	88	10:1
4	*p*-$NO_2C_6H_6I$	*p*-$NO_2C_6H_6$	**d**	0	52(63)	83	8.6:1
5	1-Naphthyl Iodide	1-Naphthyl	**e**	74(84)	55(66)	88	10:1
6	*p*-MeC_6H_6Br	*p*-MeC_6H_6	**f**	0	trace		

a. Method A: $Pd(OAc)_2$(2%), Ph_3P(4%), Et_3N; b, Method B: $Pd(OAc)_2$(8%), Bu_4NCl, $NaHCO_3$ DMF.

4.2.5.2 Synthesis of 23-Furylbrassinolide via Heck reaction and Asymmetric Dihydroxylation

Biological evaluation of 23-phenylbrassinosteriods indicated that the synthetic **149a** has almost the same activity as that of 24-epibrassinolide, while **145** possesses much stronger activity than 24-epibrassinolide.[62] In comparison with the phenyl group, furan has greater aromatic character, and it also widely occurs in the natural products as a bioactive moiety. We therefore decided to prepare

two new brassinosteroids **154** and **155**, with a furan moiety at C-23, in the hope of increasing the biological activity[64]



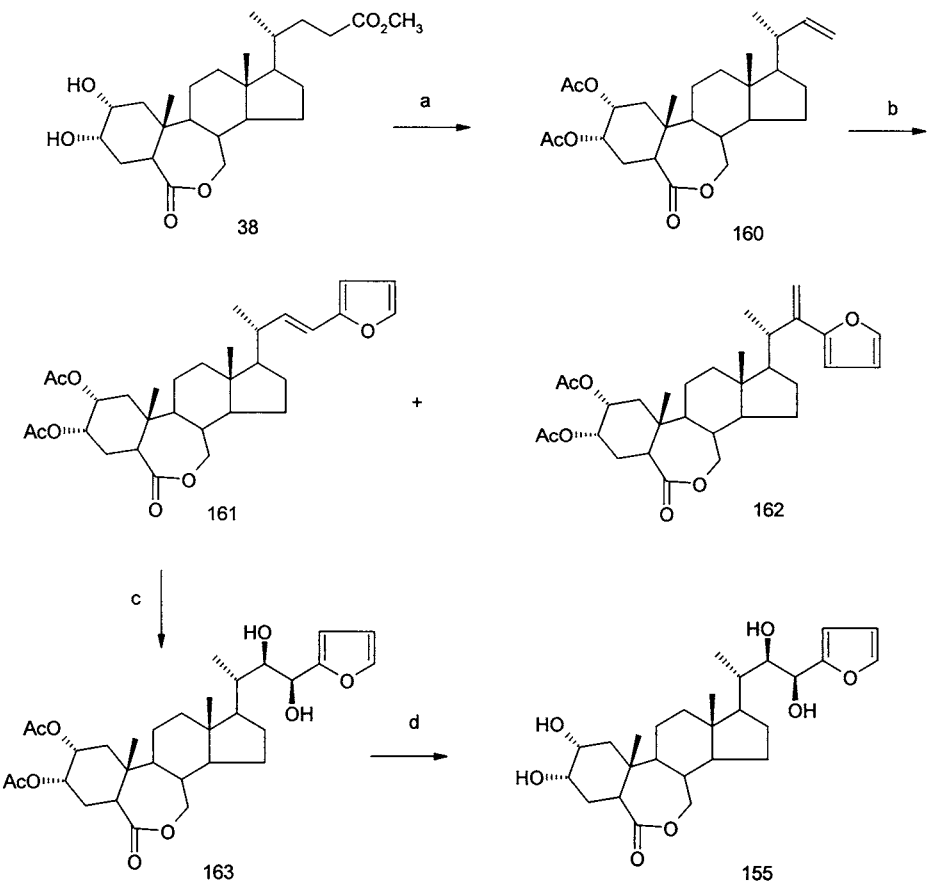
Reagents: a. 2-bromofuran, $Pd(OAc)_2$(8%), $NaHCO_3$, n-Bu_4NCl, DMF; b. DHQD-CLB, cat. OsO_4, $Ke_3Fe(CN)_6$, K_2CO_3, aq. tBuOH(1:1)

Scheme 22

For the synthesis of **154**, initially, the key step to introduce the 22E-23-furan sidechain was accomplished by Heck reaction of the olefin **152** with 2-bromofuran under phase transfer conditions [cat. $Pd(OAc)_2$ $NaHCO_3$, n-Bu_4NCl, DMF] (**Scheme 22**).[63] An inseparatable mixture of the Heck α-β-adducts **156** and **157** in a ratio of 2.6:1 (62%) was obtained, which is quite different from the results for the phenyl group (where only the α-adduct was observed). In the meantime, we also tried this reaction under the usual Heck condition [cat. $Pd(OAc)_2$, Ph_3P, Et_3N),[60] which was unsuccessful. After dihydroxylation of the above mixture, the title compound **154** was obtained

in 53% yield as the major product (**154:158**=8.9:1) with a trace amount of **159**. The biological activity testing revealed the synthetic **154** is less active than its phenyl derivative **148a**.[62]

Next, our synthetic effort was focused on the 23-furylbrassinolide **155**. It is well-known that furan in the presence of an α-hydroxyl group, is quite sensitive to some oxidants such as CF_3CO_3H and mCPBA. So, for the preparation of **155**, we planned to functionalize the A/B rings first and then build the sidechain (**Scheme 23**). The 2,3-dihydroxy-7-oxolactone **38**[10] was converted into 22-olefin **160** by decarboxylation. Using a very similar strategy as that described for the preparation of **154**, the protected 23-furylbrassinosteroid **163** could be synthesized from olefin **160**. Hydrolysis of **163** gave **155**.



Reagents: a. 1. 4%KOH, 2. Ac_2O/Py, 3. $PhI(OAc)_2$, $Cu(OAc)_2$, Py; b. 2-bromofuran, $Pd(OAc)_2$(8%), $NaHCO_3$, n-Bu_4NCl, DMF; c. DHQD-CLB, cat. OsO_4, $Ke_3Fe(CN)_6$, K_2CO_3, aq. tBuOH(1:1); d. KHCO3.

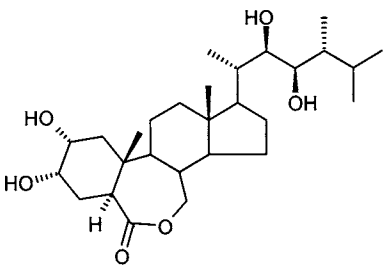
Scheme 23

5. Structure-Activity Relationship[62]

The study of the structure activity relationship of brassinolide (**1**) is an active research area. Using hyodeoxycholic acid as the starting material the authors synthesized natural and unnatural steroids as mentioned above, as well as a number of steroidal compounds with different long side chains of bile acid.[37] However, their plant growth promoting activity is only 50%-70% of 24-epibrassinolide (**5**). In this review we report the biological activity of brassinosteroids with 26,27-bisnor, 25-methyl, 24-ethyl, 24-methylene, 23-phenyl, 23-furyl and 23-carboxylic acid.

A rice bioassay has been reported as highly specific and sensitive to brassinosteroids.[48] The following steroidal compounds, such as 26,27-bisnorbrassinolide (**108**) and 26, 27-bisnortyphasterol (**107**) lacking a hydroxy group at C2, 25-homocasterone (**85**) and 25-homotyphesterol (**98**), 23-phenylbrassinosteroids (**149a**), (**152**), (**164**), (**150b**), (**145**), homobrassinosterone (**8**), 23-furyl (**154**) as well as the 23-carboxyl compounds (**132**) and (**131**),have been synthesized from hyodeoxycholic acid. Among them,**107, 98, 149a, 152, 164, 150b, 119, 20, 132, 131** and **154** were new ones which were first synthesized by our lab. **Table 6** shows the effects of various concentrations of the new compounds in rice lamina inclination test. Among them 23-phenylbrassinolide (**145**) and 23-phenylbrassinosterone (**152**) had the strongest biological activity. When the concentration of **145** was 0.00001 ppm, their inclination angles increased by 20%. When the concentration of **145** was 0.00001 to 1 ppm, and its activity was also much higher than that of 24-epibrassinolide (**5**) (as standard). They possess the similar activity as 26, 27-bisnorbrassinolide (**108**). The activities of compounds **149a** and **164** lacking hydroxy group at C2 decrease with the decreasing concentrations. The (22S, 23S)-**150b** is an isomer of (22R,23R)-**149a**, in which the 22, 23-vicinal hydroxy groups possess an unnatural configuration. Therefore its activity is only 33% of that of (22R, 23R)-**149a**. The activity of the 3-α-hydroxy-6-one steroid **154** with a 23-furyl group is similar to that of the 23-phenyl substituetn group only at higher concentrations. In addition, the homobrassinosterone (**8**) is obviously more active than the 24-epibrassinolide (**5**). 25-Homocasterone (**85**) is less active than the corresponding phenylbrassinosterone (**152**) and its activity is similar to that of the 24-epibrassinolide (**5**). The activity of homobrassinosterone (**8**) increased by 30% as compared to that of 24-epibrassinolide (**5**) at a concentration of 0.001ppm.

Brassinosteroids with 6-oxo group such as the 24-methylene **119** and the 24-methylene-25-methyl **20** showed a weaker effect than 24-epibrassinolide (**5**), **20** being less active than 24(R) 24, 25-dimethyl steroid **98**. The 6-oxo compounds with carboxylic esters at C23, **132** and **131,** were much less active than the 24-epibrassinolide (**5**). Thus it can be seen that the biological activities of 6-oxo-brassinosteroids were less than those of the brassinosteroids with a 7-oxalactone group.



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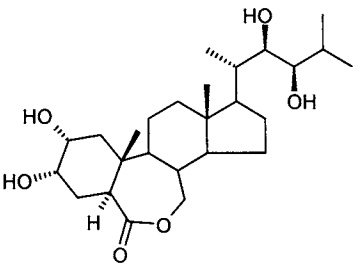

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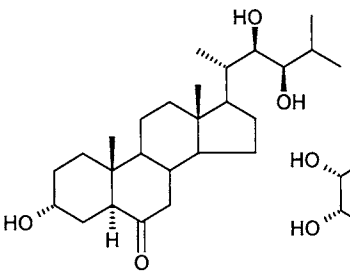

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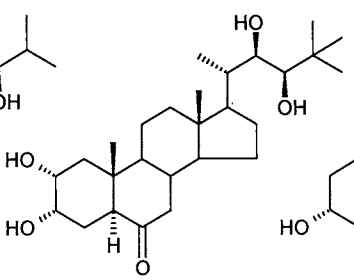

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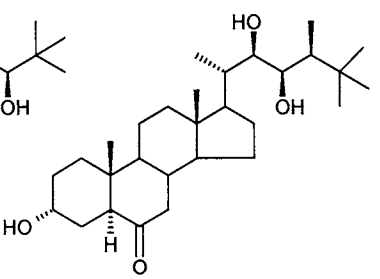

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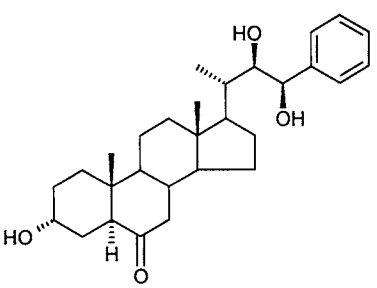
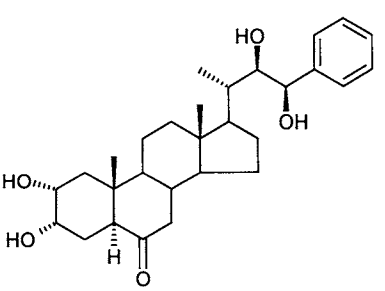


149a



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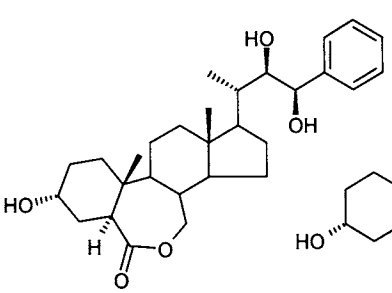

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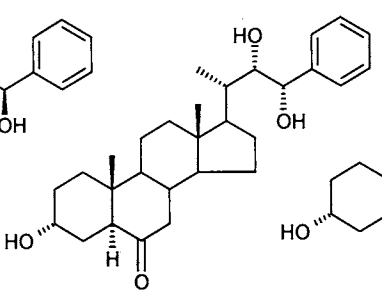
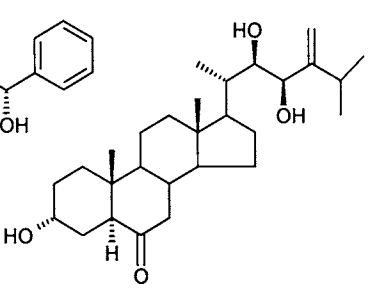


150b



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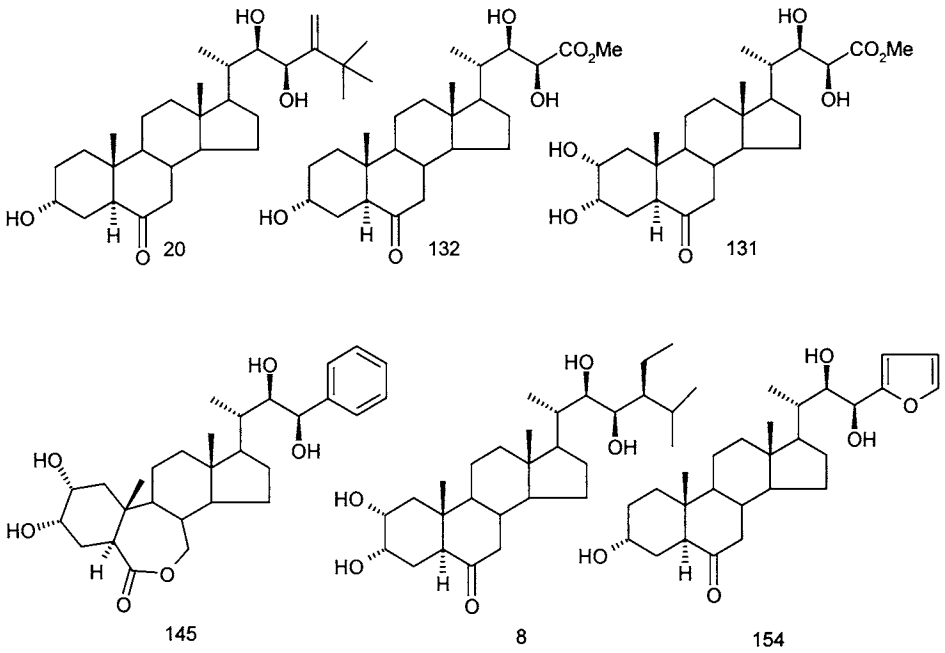

Figure 3. Structure of brassinolide analogoues

Table 6. Effect of brassinosteroids on the lamina inclination of rice seedlings

	Concentration ($\times 10^{-6}$)						
Compound	Angle degrees between laminate and sheaths (±se)						
	1	0.1	0.01	0.001	0.0001	0.00001	control
5	159±9	148±16	141±13	110±16	102±11	89±8	89±10
108	—	162±9	148±16	138±11	107±13	—	
107	136±18	125±16	97±14	96±10	98±19	—	
85	153±17	142±9	113±15	107±14	95±9	—	
98	149±15	124±15	98±9	87±9	—	—	
149a	157±15	143±17	137±15	108±9	—	—	
152	—	159±11	149±14	142±13	115±13	—	
164	159±19	142±11	119±14	94±15	—	98±12	
150b	151±7	128±18	103±13	—	—	—	
119	145±13	100±12	—	—	—	—	
20	152±11	107±16	—	—	—	—	
132	98±12	87±10	85±12	—	—	—	
131	88±10	88±8	84±14	—	—	—	
145	—	161±9	154±16	148±13	126±18	102±11	
8	—	157±15	153±18	140±14	100±14		
154	136±17	135±13	102±10	89±8	—		

The clarification of the structure-activity relationship of brassinosteroids is theoretically important. It contributes not only to the chemical synthesis and the improvement of the related compounds, but also to the illumination of the mechanism and biosynthesis of brassinosteroids.

6. Conclusions

We have employed hyodeoxycholic acid as a starting material for the synthesis of brassinosteroids. For the A, B ring modification, we have developed a highly regioselective synthesis of the 7-oxalactone ring by ozone oxidation of the enolsilyl ether, which could be regarded as a complement of the Baeyer-Villiger oxidation. For the construction of the sidechain the hyodeoxycholic acid must be first degraded to the 20-carbaldehyde as ergosterol or stigmasterol. We then used various stereocontrolled methods for building of the dihydroxy sidechain with 22R, 23R, 24S-configuration. For economy, we used directly the intact sidechain of hyodeoxycholic acid and (22E)-methylhyodeoxycholate as the staring material. For the synthesis of brassinosteroids from the intact sidechain of hyodeoxycholic acid we employed the β-alkylative 1,3-carbonyl transposition methods as the key step.

We have applied the very high enantioselectivity of the osmium catalyzed asymmetric dihydroxylation (ADH) of the olefin, developed by Sharpless for the first time, to (22E)-methyl hyodeoxycholate, providing the natural (22R, 23S)-isomer as the major products. The extension of the scope of this methodology to the (22E)-24-alkyl steroidal unsaturated sidechain was also found to be successful. Finally, we have employed the 22-olefin steroid from hyodeoxycholic acid as the staring material for the synthesis of 23-aryl and 23-furyl brassinosteroids via the Heck reaction and asymmetric dihydroxylation. The activity of the known 23-phenyl brassinolide (**145**) is much higher than that of 24-epibrassinolide (**5**) whereas the activity of the new 3-α-hydroxy-6-one brassinosteroid (**154**) bearing a 23-furyl group is similar to that of 23-phenyl group only at high concentration.

Acknowledgment

This investigation was supported by the National Science Foundation of China. We wish to thank Professor Y. J. Zhao, Professor Y. Q. Wang, Miss W. H. Luo and Miss R. J. Xu for the rice-lamina inclination test for brassinosteroids which was carried out at the Shanghai Institute of Plant Physiology, Chinese Academy of Sciences.

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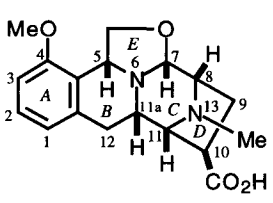
Total Synthesis of Quinocarcin and its related Compounds

Tadashi Katoh and Shiro Terashima

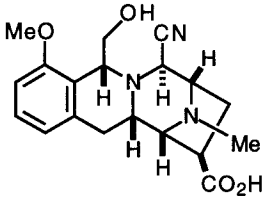
1. Introduction

(–)-Quinocarcin (**1**)[2] isolated from the culture broth of *Streptomyces melanovinaceus* at Kyowa Hakko Co. in Japan in 1983,[3] displays weak antimicrobial activity against Gram-negative organisms as well as exhibits potent antitumor activity against several types of solid mammalian carcinomas including St-4 gastric carcinoma, Co-3 human colon carcinoma, M5076 sarcoma, B16 melanoma, and MX-1 human mammary carcinoma.[4] DX-52-1 (**2**), the more stable semi-synthetic derivative of **1**, also retains significant antitumor activities similarly to **1**.[5]

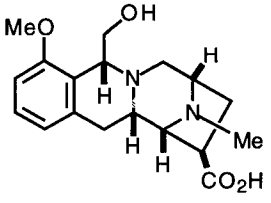
Figure 1. Structures of quinocarcin (**1**), DX-52-1 (**2**), quinocarcinol (**3**), quinocarcinamide (**4**), and tetrazomine (**5**)



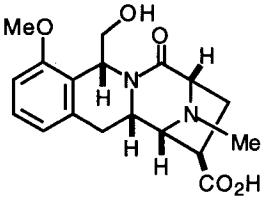
1 : quinocarcin



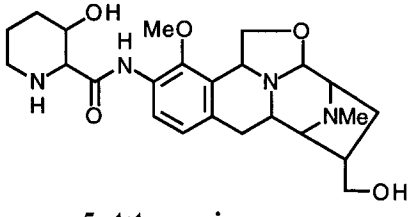
2 : DX-52-1



3 : quinocarcinol



4 : quinocarcinamide



5 : tetrazomine

Quinocarcinol (**3**), the pharmacologically inactive dihydroderivative of **1**, was also isolated from the same culture broth.[3] It is reported that **1** spontaneously disproportionates anaerobically into **3** and an oxidation product, quinocarcinamide (**4**), which is also biologically inactive.[6]

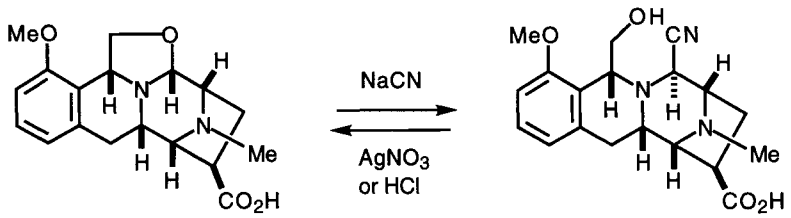
The stereostructure of **1** except absolute configuration was revealed by spectroscopic analyses and chemical correlation with **3** to have a novel 8,11-iminoazepino[1,2-*b*]isoquinoline skeleton with six asymmetric centers.[7] The relative stereochemistry of **3** was established by X-ray diffraction. The absolute configuration of **1** pictured in **Figure 1** was suggested by Remers *et al.* in 1988 on the basis of computer simulation of binding of **1** to DNA.[8] In 1992, Garner *et al.* completed the asymmetric total synthesis of (–)-**1**, leading to the confirmation of its absolute configuration.[9]

Tetrazomine (**5**), a new member of this class of compounds, was isolated from the culture broth of *Saccharothrix mutabilis* subsp. *chichijimaensis* subsp. nov. at Yamanouchi Pharmaceutical Co. in Japan in 1991.[10] Tetrazomine displays broad antimicrobial activity against both Gram-negative and Gram-positive bacteria *in vitro*.[11] It also exhibits *in vitro* cytotoxicity and *in vivo* antitumor activity against P388 murine leukemia cells.[11] The relative and absolute stereochemistries of **5** have not been established to date.[10]

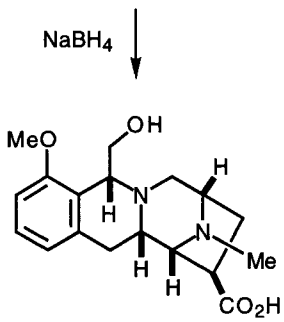
Chemical modification of quinocarcin (**1**) is severely limited owing to its chemical instability inherent in the oxazolidine (E) ring. Therefore, scientists at Kyowa Hakko Laboratories have developed a method for masking the oxazolidine ring and its reproduction to prepare useful quinocarcin derivatives (see, section ***4.1.***).[5] Thus, as shown in **Scheme 1**, cyanation of **1** with sodium cyanide readily provides the amino nitrile derivative, DX-52-1 (**2**), which not only has sufficient chemical stability but also significant antitumor activity. Upon treatment with silver nitrate or hydrochloric acid, **2** can cleanly regenerate **1**. On the other hand, reduction of **1** with sodium borohydride in methanol effectively produces quinocarcinol (**3**).

It was reported that quinocarcin (**1**) inhibits DNA synthesis in preference to RNA and protein synthesis in *Bacillus subtilis*.[4a] Two plausible modes of antitumor actions have hitherto been proposed for **1** as shown in **Scheme 2**. Thus, one is the alkylation of the 2-amino group of guanine in a minor grove of DNA by the

Scheme 1. Chemical transformation of quinocarcin (**1**) to DX-52-1 (**2**) and quinocarcinol (**3**)



1 : quinocarcin **2 : DX-52-1**

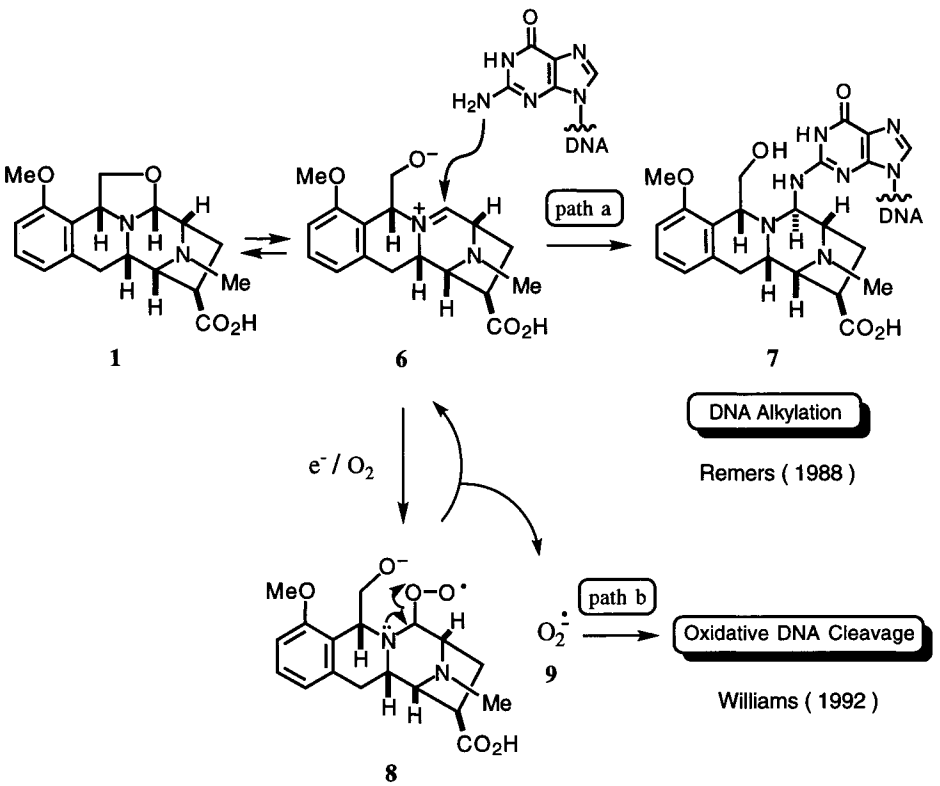


3 : quinocarcinol

iminium ion **6** generated by opening of the oxazolidine ring in **1** (path a).[8] The other one is the oxidative cleavage of DNA by a superoxide radical anion **9** produced by auto-redox disproportionation of the oxazolidine ring of **1** *via* intermediate **8** under aerobic conditions (path b).[6] Superoxide production by **1** was first reported by Tomita *et al.* in 1984,[4a] and its mechanistic rationale described above was presented by Williams *et al.* in 1991.[6] Although it is not yet clear which mode of action is responsible for the antitumor properties, these suggestions indicate that the oxazolidine ring or its equivalent such as an α-cyanoamino functionality plays an important role in exhibiting significant antitumor activity. The fact that quinocarcinol (**3**) and quinocarcinamide (**4**) lacking the oxazolidine ring exhibit no antitumor activity, supports this hypothesis.

Due to the unique structural features as well as the remarkable antitumor activity, considerable attention has been focused on the total synthesis of this class of

Scheme 2. Proposed mode of action of quinocarcin (**1**)



natural products and the synthesis of biologically active structural congeners. In 1986, we embarked on a project directed at the total synthesis of optically active quinocarcin (**1**) with the aim of determining the unknown absolute configuration as well as disclosing the structure-activity relationships. In 1994, our earnest endeavors culminated in completing the total synthesis of an enantiomeric pair of **1**[12] and in exploring unnatural 10-decarboxyquinocarcin and its 7-cyano congeners which are more highly cytotoxic than the corresponding natural **1** and **2**.[13] The total synthesis of racemic quinocarcinol [(±)-**3**] and the first total synthesis of racemic quinocarcin [(±)-**1**] were accomplished by Danishefsky *et al.*[14] in 1985 and by Fukuyama *et al.*[15] in 1988, respectively. Garner *et al.*[9] successfully achieved the first asymmetric total synthesis of (–)-**1** in 1992, and quite recently, the total synthesis of racemic quinocarcinamide [(±)-**4**] was reported by Williams *et al.*[16]

In this article, synthetic studies on quinocarcin (**1**) and its related compounds including three independent total syntheses of **1** (and one formal total synthesis of **1**) are reviewed with particular focus on their strategies. Furthermore, the structure-activity relationships of **1** are presented, disclosing their novel aspects which might be useful for both elucidating the mode of action and developing more effective anticancer agents.

2. Synthesis of Racemic Quinocarcin and Its Related Compounds

2.1. Total Synthesis of Racemic Quinocarcinol [Danishefsky et al., 1985]

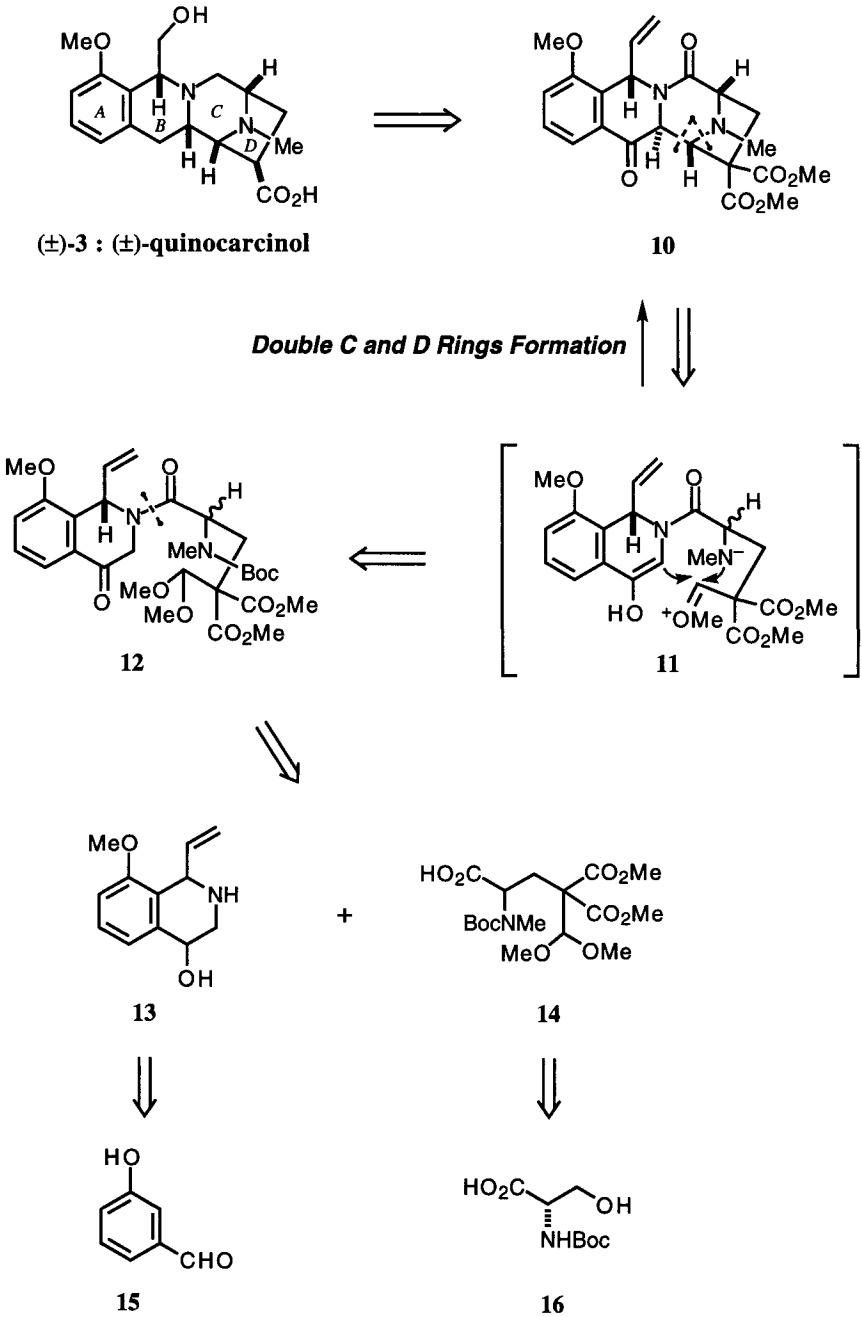
2.1.1. Synthetic Strategy

The total synthesis of racemic quinocarcinol [(±)-**3**] was achieved by Danishefsky *et al.* in 1985.[14] The retrosynthetic analysis for (±)-**3** is outlined in **Scheme 3**. The key step in this approach is envisaged to be a Lewis acid-promoted double cyclization of the tetrahydroisoquinoline **12** through the intermediate oxonium ion **11** to construct the requisite tetracyclic ring system **10** in a single operation (**12**→**11**→**10**). The cyclization product **10** would be converted into the target compound (±)-**3** by sequential functional group manipulations. The key cyclization precursor **12**, in turn, is anticipated to be elaborated by the coupling of the aromatic and the racemic aliphatic fragments **13** and **14** accessible from the benzaldehyde **15** and *N*-(*tert*-butoxycarbonyl) (Boc)-L-serine (**16**), respectively.

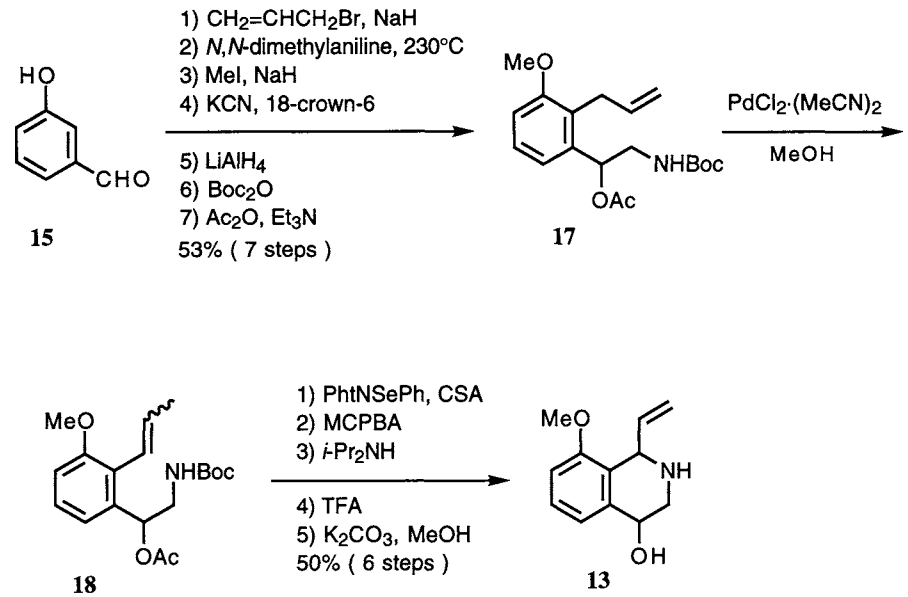
2.1.2. Total Synthesis

At first, the synthesis of the aromatic fragment **13** was investigated as shown in **Scheme 4**. Thus, commercially available 3-hydroxybenzaldehyde (**15**) was converted to the *N,O*-protected amino alcohol **17** *via* a seven-step sequence in 53% overall yield. Isomerization of the terminal olefin in **17** was effected by treatment with palladium(II) bis(acetonitrile)dichloride in methanol[17] to provide olefin **18** as a 3.5 : 1 mixture of E/Z isomers. Transformation of **18** to the aromatic fragment **13** was carried out in 50% overall yield by a sequential five step operation involving cyclization with the Nicolaou reagent (*N*-phenylselenophthalimide)[18] and subsequent oxidative elimination of the resulting selenide.[19] Compound **13** was obtained as a single diastereomer of unassigned relative stereochemistry.

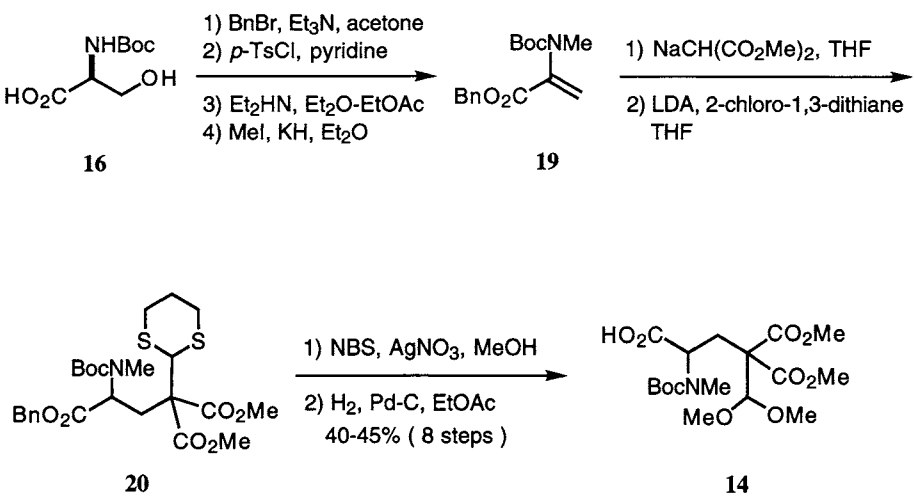
Scheme 3. Retrosynthetic analysis of (±)-quinocarcinol [(±)-**3**] according to Danishefsky *et al.*



Scheme 4. Synthesis of the intermediate **13**



Scheme 5. Synthesis of the intermediate **14**



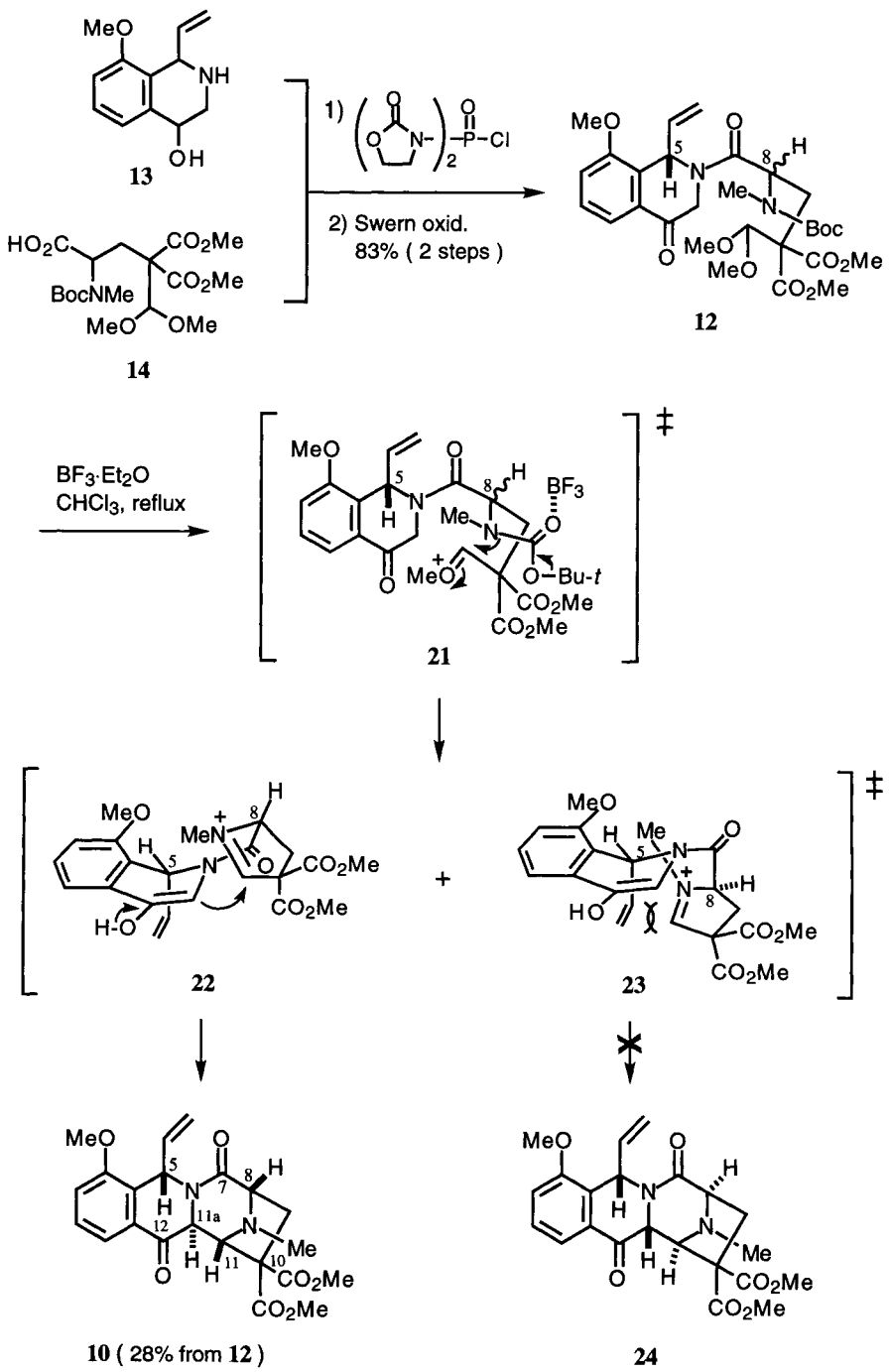
Next, as shown in **Scheme 5**, the racemic aliphatic fragment **14** was prepared from commercially available *N*-Boc-L-serine (**16**) in ca. 40 - 45% overall yield *via* the dehydroalanine **19** and the dithioacetal **20**.[20]

The coupling reaction of the aromatic and aliphatic fragments **13** and **14** under the conditions of Palomo-Coll[21] proceeded cleanly to give the adduct as a mixture of diastereomers in a quantitative crude yield, which was further subjected to Swern oxidation to provide the key intermediate **12** in 83 % yield for the two steps (**Scheme 6**). Now, the stage is set for the crucial intramolecular double cyclization to construct the tetracyclic ring system **10**. Towards this end, treatment of **12** with borontrifluoride etherate in chloroform under reflux resulted in the formation of the desired **10** in 28% yield as a sole product. The structure of **10** including stereochemistry was revealed by its ^{1}H-NMR spectral analysis.

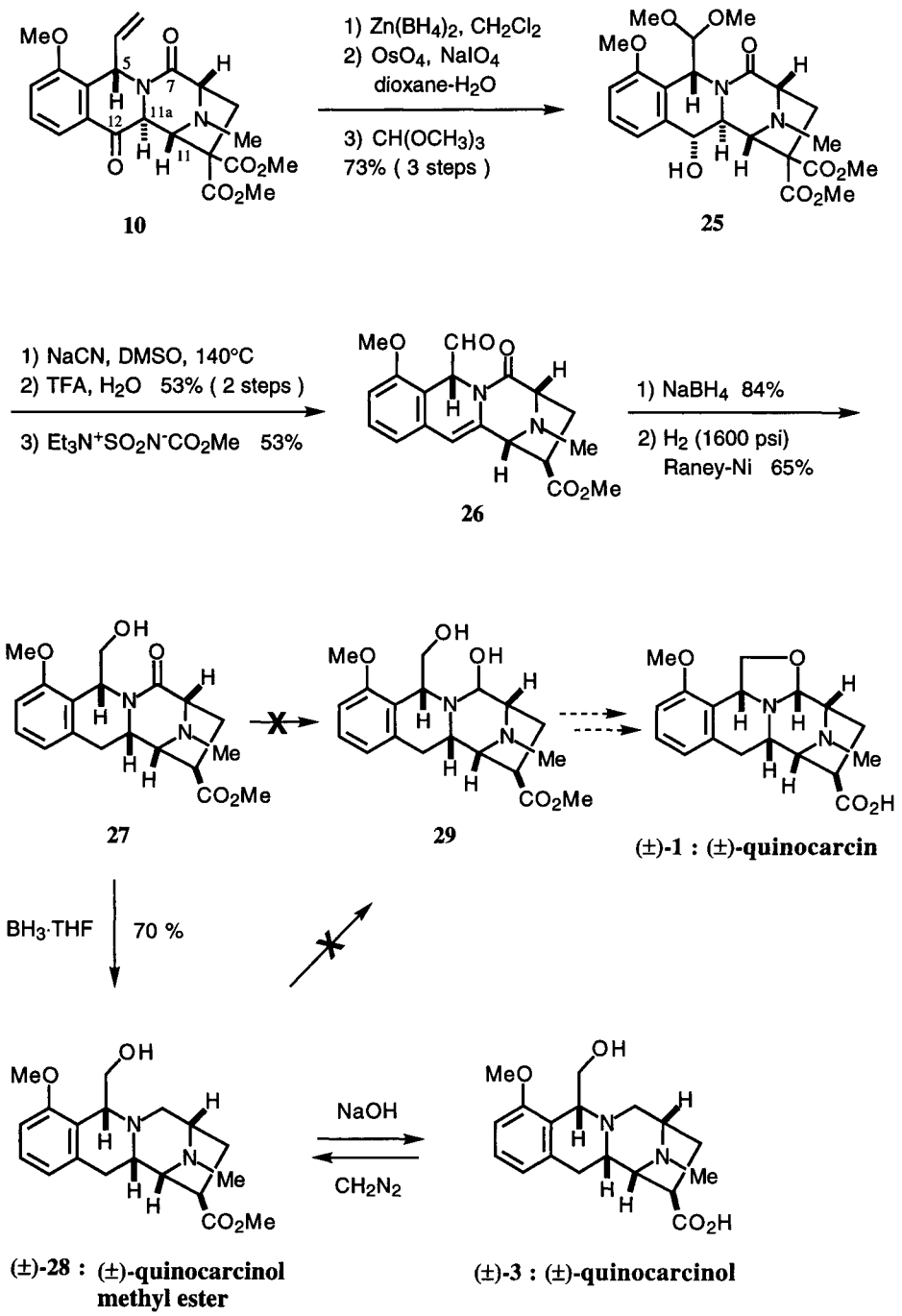
This remarkable double cyclization reaction presumably involves the formation of the iminium ion **22** through the pre-formed oxonium ion **21** and subsequent intramolecular nucleophilic reaction of the enol with the iminium ion moieties. In this cyclization, the iminium ion **23** produced from one of the two possible diastereomers **21** will not cyclize to the corresponding tetracyclic compound **24** because **23** suffers from a very serious steric interaction between the C-5 vinyl group and the pyrrolidinium ring approaching the enol moiety from its α-face.[22]

With compound **10** in hand, efforts are devoted to the completion of the total synthesis (**Scheme 7**). There remain the requirements of conversion of the vinyl group at the C-5 position to a hydroxymethyl group, inversion of configuration at the C-11a position, removal of the endo-methoxycarbonyl group at the C-10 position, and reduction of the carbonyl functionality at the C-7 position. For achieving these adjustments, sequential reduction of **10** with zinc borohydride, oxidative cleavage of the terminal olefin, and acetal formation provided alcohol **25** in 73% overall yield. Compound **25** was further converted to the aldehyde **26** *via* a three-step sequence of reactions involving stereoselective demethoxy-carbonylation,[23] deprotection of the dimethylacetal functionality, and dehydration of the benzylic alcohol with Burgess reagent.[24] Reduction of **26** with sodium borohydride followed by stereoselective hydrogenation of the double bond resulted in the formation of 7-oxoquinocarcinol methyl ester (**27**) in 55% yield for the two

Scheme 6. Synthesis of the intermediate **10**



Scheme 7. Completion of the total synthesis of (±)-quinocarcinol [(±)-**3**]



steps. Chemoselective reduction of the lactam moiety in **27** with borane-tetrahydrofuran complex afforded racemic quinocarcinol methyl ester (**28**) in 70% yield, which was identical with an authentic sample prepared by esterification of natural quinocarcinol (**3**) with diazomethane. Finally, saponification of the synthetic (±)-**28** furnished racemic quinocarcinol [(±)-**3**].

For conversion of **27** to racemic quinocarcin [(±)-**1**], attempts to achieve the partial reduction of lactam **27** to the corresponding hemiaminal **29**, a potential precursor of (±)-**1**, resulted in failure. Furthermore, oxidation of **28** to **29** was also unsuccessful.

2.2. Total Synthesis of Racemic Quinocarcin [Fukuyama et al., 1988]

2.2.1. Synthetic Strategy

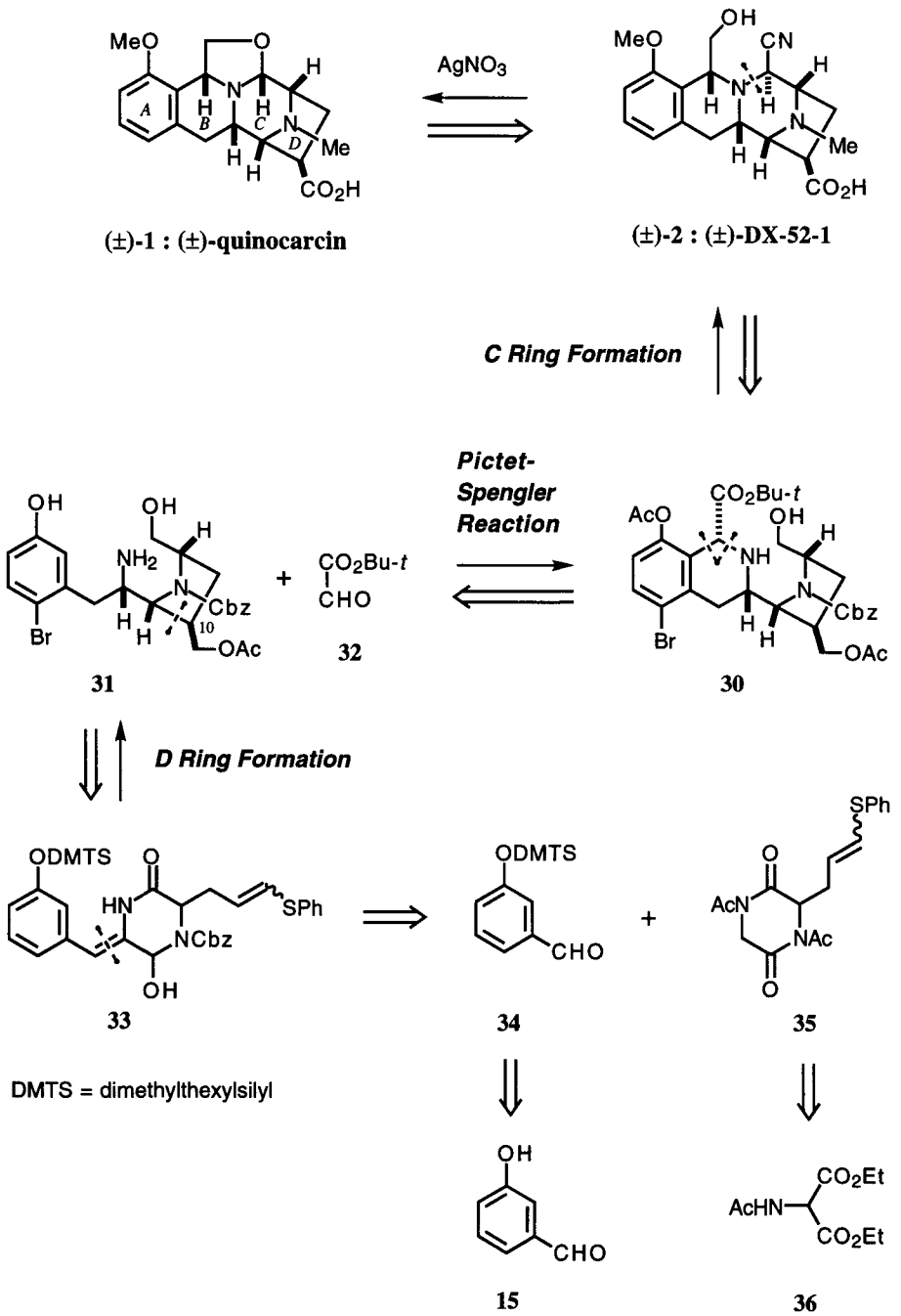
Fukuyama *et al.* accomplished the first total synthesis of racemic quinocarcin [(±)-**1**] in 1988.[15] Their retrosynthetic analysis for (±)-**1** is illustrated in **Scheme 8**. The first crucial step in this contemplated scheme is envisaged to be the stereoselective Pictet-Spengler reaction of the phenethylamine **31** with *tert*-butyl glyoxylate (**32**) to construct the requisite tetrahydroisoquinoline **30** (**31** + **32**→**30**). The cyclization product **30** would be transformed to racemic DX-52-1 [(±)-**2**] by sequential formation of the C ring, functional group manipulation, and deprotection or *vice versa*. The second critical step is envisioned to be the intramolecular cyclization of the hemiaminal **33** to construct the D ring bearing the requisite functional group with correct stereochemistry at the C-10 position (**33**→**31**). The cyclization precursor **33**, in turn, can be elaborated by condensation of the protected benzaldehyde **34** with the piperizinedione **35**, both of which are obtainable from the benzaldehyde **15** and the malonate **36**, respectively.

2.2.2. Total Synthesis

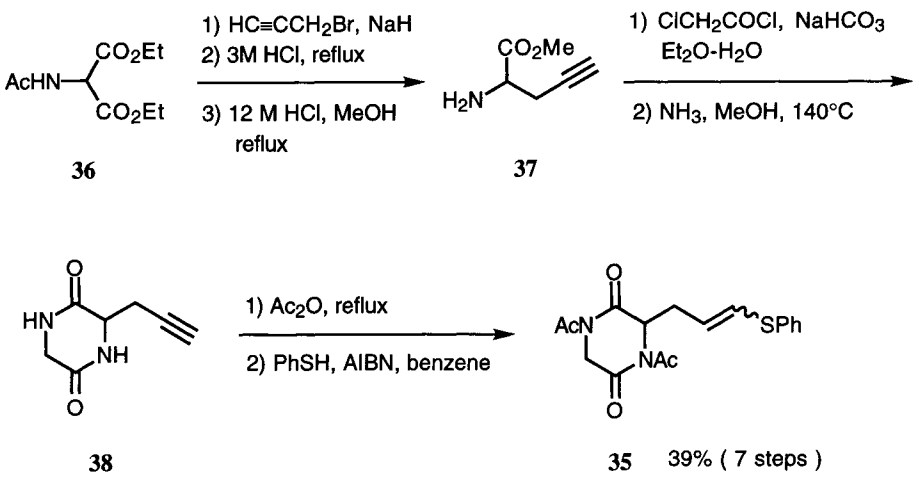
At first, the preparation of the aliphatic fragment **35** was carried out starting with commercially available diethyl acetamidomalonate (**36**) in 39% overall yield in seven steps *via* methyl ester **37** and piperizinedione **38** as shown in **Scheme 9**.

Condensation of the protected benzaldehyde derivative prepared from commercially available 3-hydroxybenzaldehyde (**15**), with the aliphatic fragment **35** provided the desired coupling product, which was further converted to

Scheme 8. Retrosynthetic analysis of (±)-quinocarcin [(±)-**1**] according to Fukuyama *et al.*



Scheme 9. Synthesis of the intermediate **35**

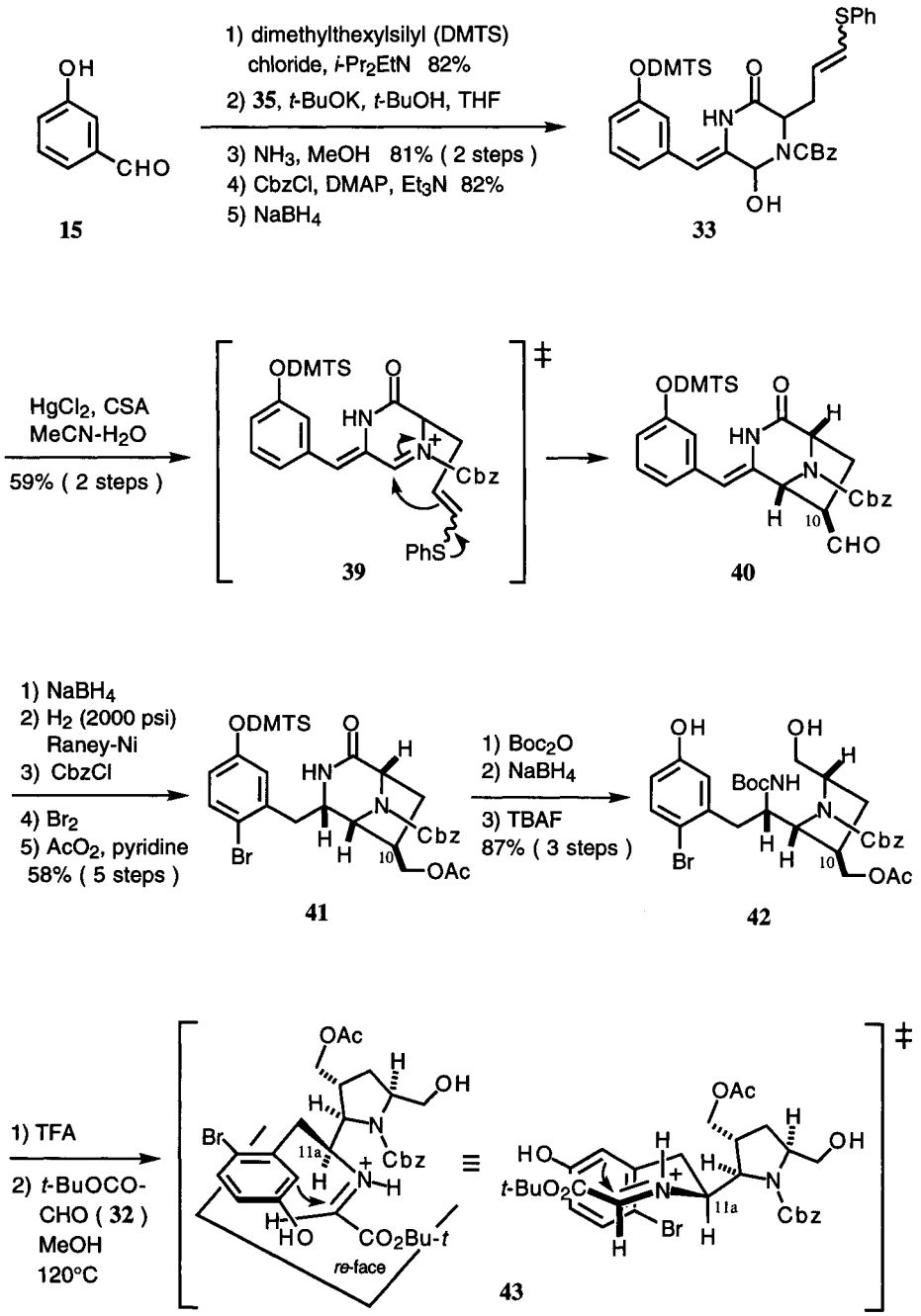


36 **37** **38** **35** 39% (7 steps)

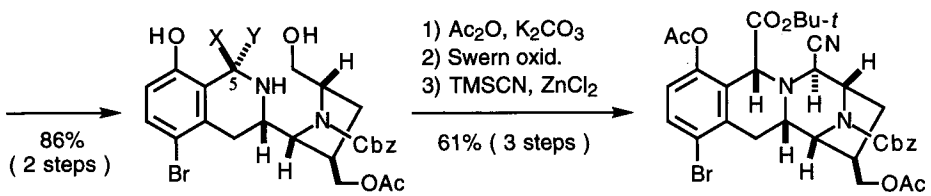
the hemiainal **33** by sequential ammonolysis, selective activation of the amide nitrogen, and partial reduction of the activated amide (**Scheme 10**). The first crucial acyliminium-mediated cyclization was achieved by treating **33** with mercury (II) chloride in the presence of camphorsulfonic acid in acetonitrile-water, giving rise to the aldehyde **40** possessing a formyl group with correct stereochemistry at the C-10 position. Aldehyde **40** was further transformed to the bromobenzene **41** in 58% overall yield *via* a five-step sequence of reactions involving reduction, stereoselective hydrogenation, reprotection of the amino group, blocking of the *para* position to the siloxy group and acetylation. Further activation of the lactam moiety in **41** followed by cleavage of the bicyclic system and deprotection of the silyl group led to the formation of the phenol **42** in 87% overall yield. After deprotection of the Boc group in **42** with trifluoroacetic acid (TFA), the most crucial Pictet-Spengler cyclization was carried out by subjecting the resultant amine salt to 10 equiv of *tert*-butyl glyoxylate (**32**) in methanol for 20 min at 120°C, giving rise to a separable mixture of the desired tetrahydroisoquinolines **44** and its C-5 epimer **45** in a ratio of 8 : 1 in 86% overall yield from **42**.

The stereoselectivity observed for this cyclization can be rationalized with a transition state such as the iminium salt **43**, wherein the nucleophilic attack of the

Scheme 10. Synthesis of the intermediate **46**



Scheme 10. Synthesis of the intermediate **46** (continued)

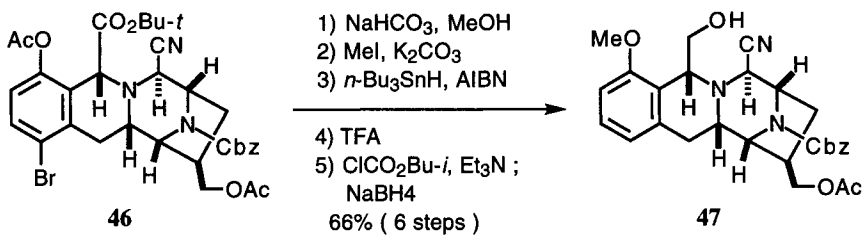


44 : X = H, Y = CO_2Bu-*t*

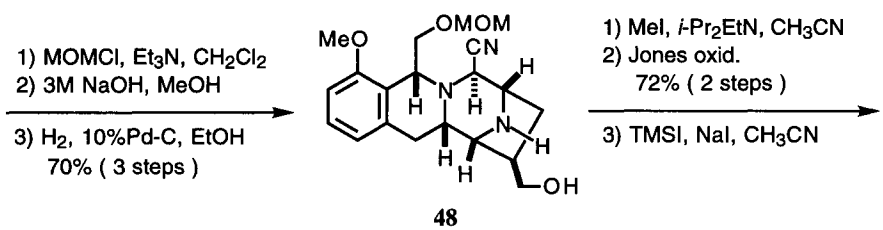
[**45** : X = CO_2Bu-*t*, Y =H (**44** : **45** = 8 : 1)]

46

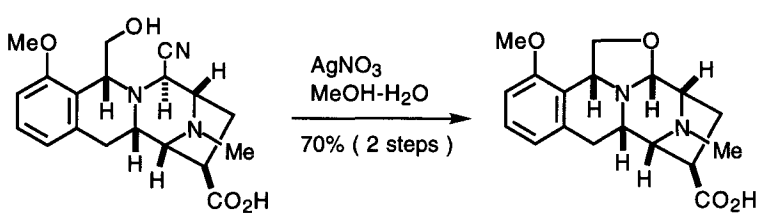
Scheme 11. Completion of the total synthesis of (±)-quinocarcin [(±)-**1**]



46 **47**



48



(±)-**2** : (±)-**DX-52-1** (±)-**1** : (±)-**quinocarcin**

phenolic ring to the iminium function predominantly occurs from its *re*-face under an influence of the C-11a stereocenter. To continue the synthesis, chemoselective protection of the phenol **44** followed by Swern oxidation led to the formation of the unstable tetracyclic hemiaminal, which was immediately treated with trimethylsilyl cyanide in the presence of zinc chloride to provide the amino nitrile **46** in 61% overall yield for the three steps.

The tetracyclic compound **46** was converted to the alcohol **47** in 66% overall yield, as shown in **Scheme 11**, *via* a five-step sequence involving selective deprotection of the phenolic acetate, methyl ether formation, debromination, cleavage of the *tert*-butyl ester, and final reduction of the liberated carboxylic acid. Sequential protection of the primary hydroxy group in **47**, saponification of the acetyl group, and hydrogenolysis of the *N*-Cbz group furnished the MOM ether **48** in 70% overall yield. The conversion of **48** to racemic DX-52-1 [(±)-**2**] was performed by methylation of the amino group followed by Jones oxidation and removal of the MOM group. Finally, (±)-**2** was treated with silver nitrate under the same conditions as reported,[5] affording racemic quinocarcin [(±)-**1**].

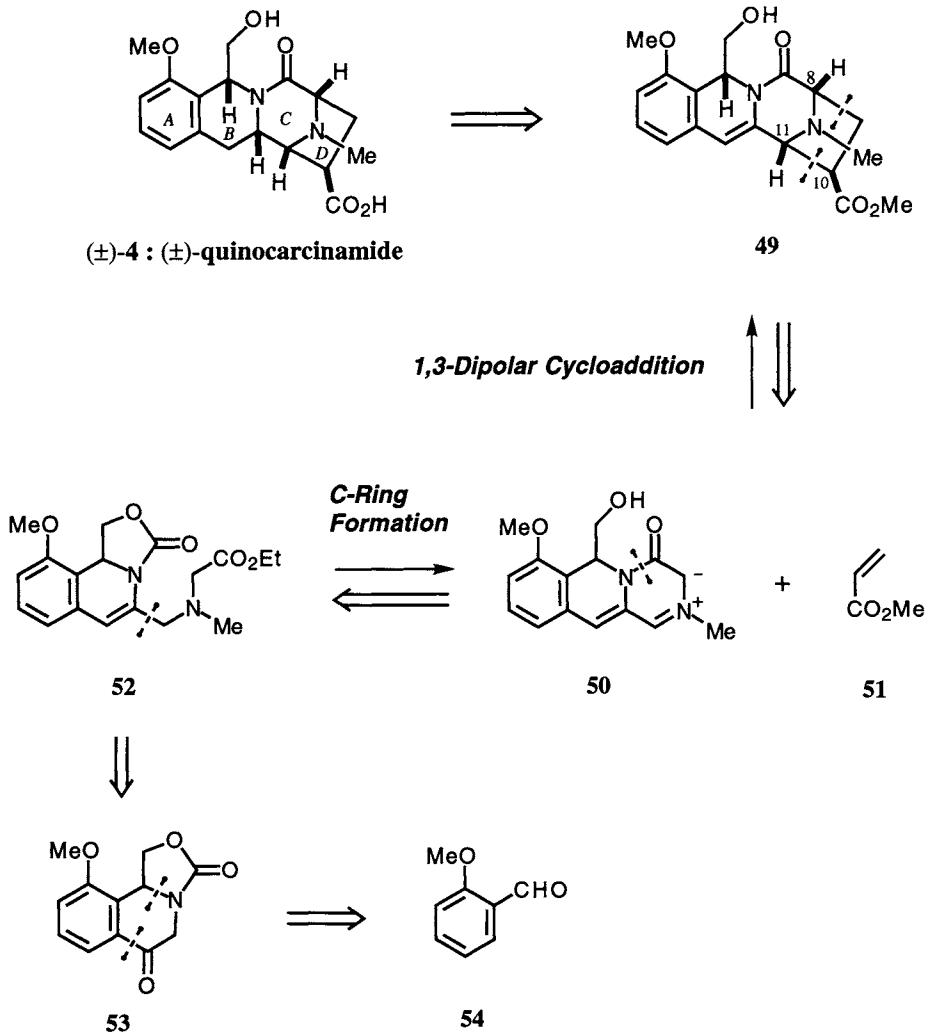
2.3. Total Synthesis of Racemic Quinocarcinamide [Williams et al., 1995]

2.3.1. Synthetic Strategy

The total synthesis of racemic quinocarcinamide [(±)-**4**] was reported by Williams *et al.* in 1995.[16] The retrosynthetic analysis of (±)-**4** is shown in **Scheme 12**. The most crucial step in this scheme is envisaged to be the intermolecular 1,3-dipolar cycloaddition of the azomethine ylide **50** with methyl acrylate (**51**) to construct the tetracyclic ring system **49** possessing the complete carbon framework and the requisite functional groups with correct relative stereochemistries at the C-8, C-10, and C-11 positions (**50** + **51**→**49**). The key substrate **50** would be elaborated from the benzaldehyde **54** *via* the isoquinoline **53** and amine **52**.

Similar strategies in which the key 3,8-diazabicyclo[3,2,1]octane nucleus (the CD ring system) is constructed *via* an intermolecular 1,3-dipolar cycloaddtion, have been already employed by Joule *et al.*[25] and Garner *et al.*,[9,26] respectively, while the methods for generating the azomethine ylides are quite different (see, sections ***2.4.*** and ***3.1.***).

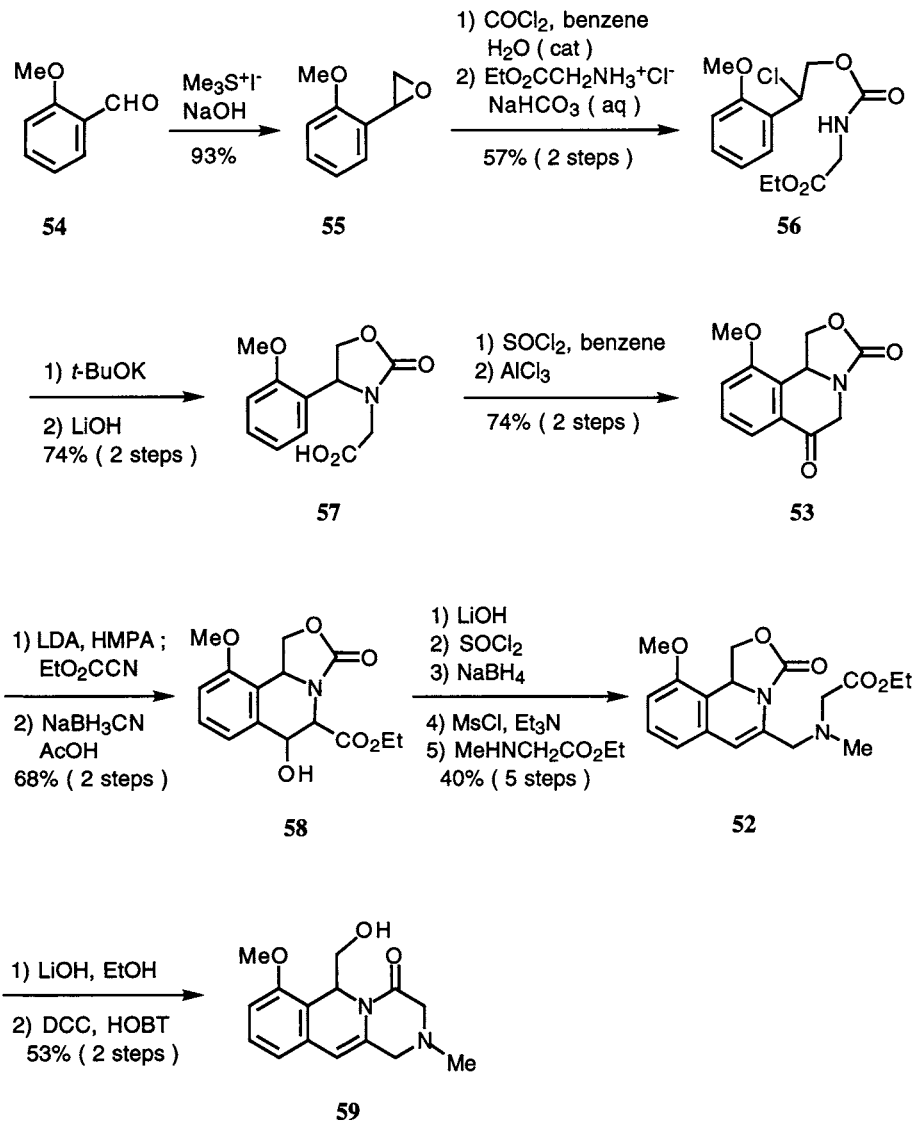
Scheme 12. Retrosynthetic analysis of (±)-quinocarcinamide [(±)-**4**] according to Williams *et al.*



2.3.2. Total Synthesis

The substrate **50** for the key 1,3-dipolar cycloaddition was prepared starting from commercially available 2-methoxybenzaldehyde (**54**) as shown in **Scheme 13**. Thus, epoxide **55** derived from **54** was converted to the urethane **56** by opening the epoxide ring followed by the reaction with glycine ethyl ester.[6b,27]

Scheme 13. Synthesis of the intermediate **59**



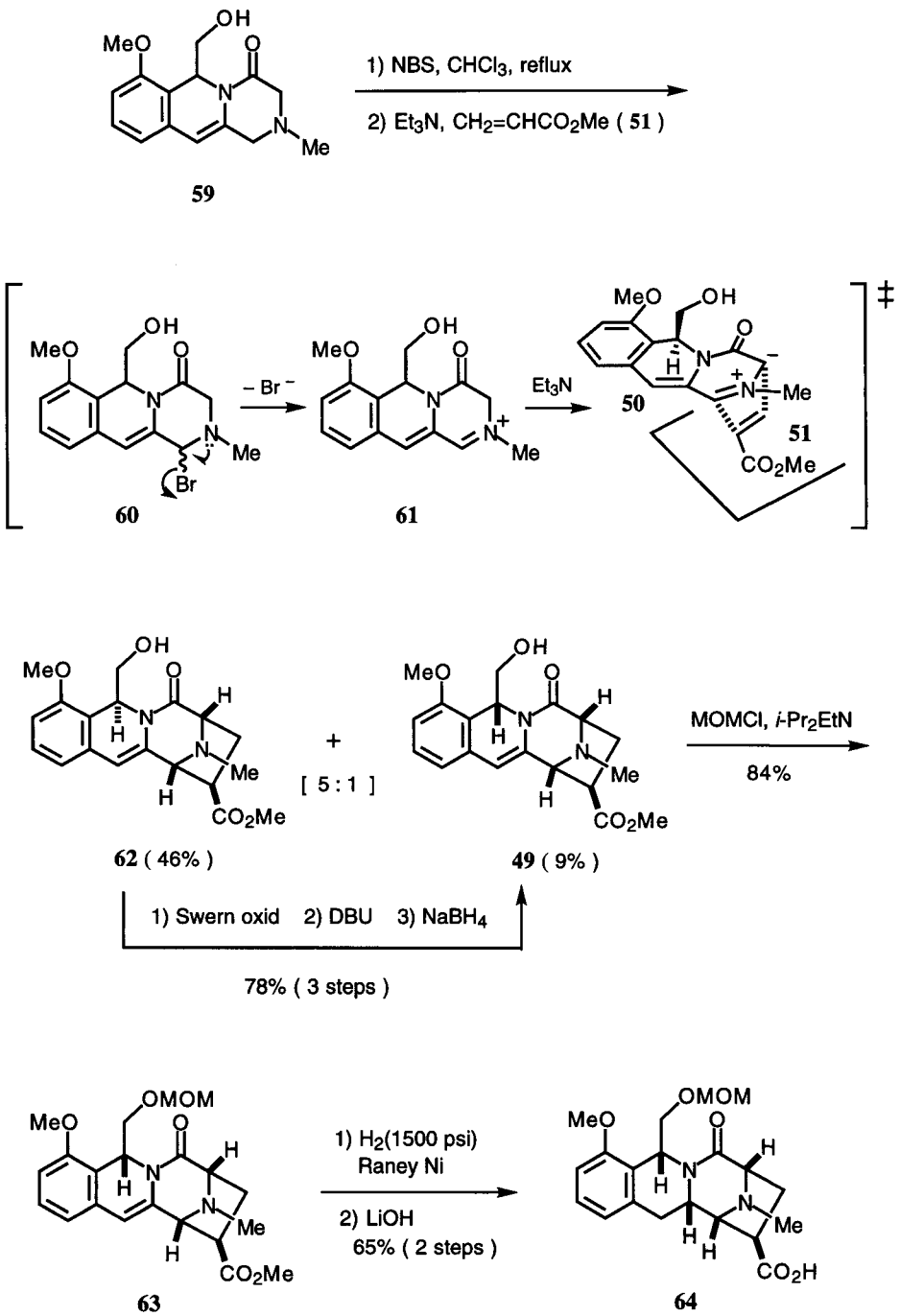
Treatment of **56** with potassium *tert*-butoxide resulted in the cyclization to the corresponding oxazolidinone, which was then saponified to the carboxylic acid **57**. Acid chloride formation of **57** and subsequent intramolecular Friedel-Crafts reaction furnished the isoquinolinone **53**. Ethoxycarbonylation of **53** followed by

reduction gave the alcohol **58** as a single product in 68% overall yield for the two steps. Conversion of **58** into the amine **52** was achieved *via* a five-step sequence of reactions involving saponification, simultaneous dehydration and acid chloride formation, reduction, mesylation, and substitution with sarcosine ethyl ester. Synchronous saponification of the ethyl ester group and deprotection of the oxazolidinone moiety in **52** provided the corresponding amino alcohol which was further converted to the tricyclic compound **59** (53%, 2 steps) by cyclization with 1,3-dicyclohexyl-carbodiimide (DCC) and *N*-hydroxybenzotriazole (HOBT).

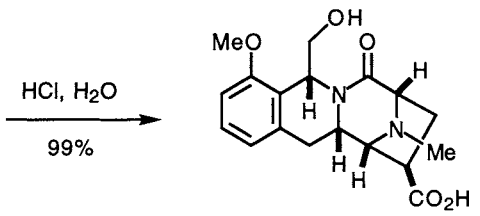
With the key substrate **59** in hand, the crucial 1,3-dipolar cycloaddition was next investigated as shown in **Scheme 14**. Thus, reaction of **59** with 1 equiv of *N*-bromosuccinimide (NBS) in refluxing chloroform followed by treatment of this solution with triethylamine in the presence of methyl acrylate (**51**), resulted in the formation of cycloadducts **62** and **49** in a ratio of 5 : 1 in 55% yield for the two operations. No other cycloadducts were isolated from the reaction mixture. In the sequence of reactions, the azomethine ylide **50** is presumably produced *in situ* through the bromide **60** and the iminium ion **61**. The approach of the dipolarphile **51** is probably governed by the conformation of the ylide **50** which positions the hydroxymethyl group in a pseudoaxial orientation effectively shielding the top face of the ylide and favoring formation of the observed major isomer **62**. Since the desired product **49** is the minor diastereomer from the cycloaddition reaction, **62** was subsequently converted to **49** in 78% overall yield by sequential three step operations involving Swern oxidation, epimerization with 1 equiv of 1,8-diaza-bicyclo[5,4,0]undec-7-ene(DBU) (α-CHO : β-CHO = *ca.* 4 : 1), and reduction with sodium borohydride.

The compound **49** was further transformed to the carboxylic acid **64** *via* a three-step sequence involving protection of the primary hydroxy group, hydrogenation of the double bond of the resulting MOM ether **63**, and saponification of the methyl ester functionality. Finally, treatment of **64** with hydrochloric acid in water provided the racemic quinocarcinamide [(±)-**4**] in essentially quantitative yield, which was identical by ^{1}H-NMR and mobility on TLC to an authentic sample of **4** obtained by the autoredox disproportionation of quinocarcin (**1**). Optically active **64** has been previously converted to **1** *via* DX-52-1 (**2**) by Garner *et al.*[9] through a three-step sequence involving partial

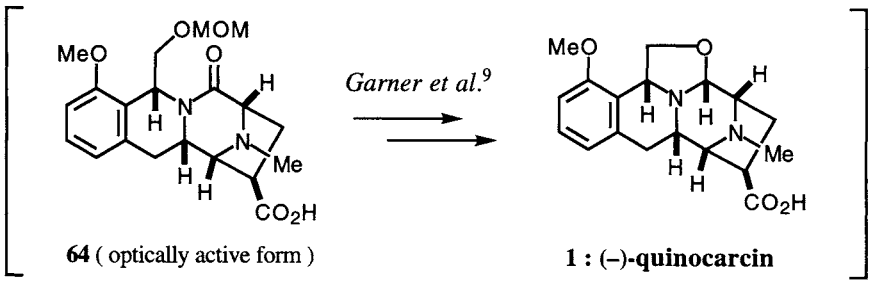
Scheme 14. Completion of the total synthesis of (±)-quinocarcinamide [(±)-**4**]



Scheme 14. Completion of the total synthesis of (±)-quinocarcinamide [(±)-**4**] (continued)



(±)-4 : (±)-quinocarcinamide



64 (optically active form) **1 : (–)-quinocarcin**

reduction of the amide function, treatment with sodium cyanide, and subjection of the resulting amino nitrile to silver nitrate (see, section ***3.1.***). Thus, the formal total synthesis of racemic quinocarcin [(±)-**1**] is now completed.

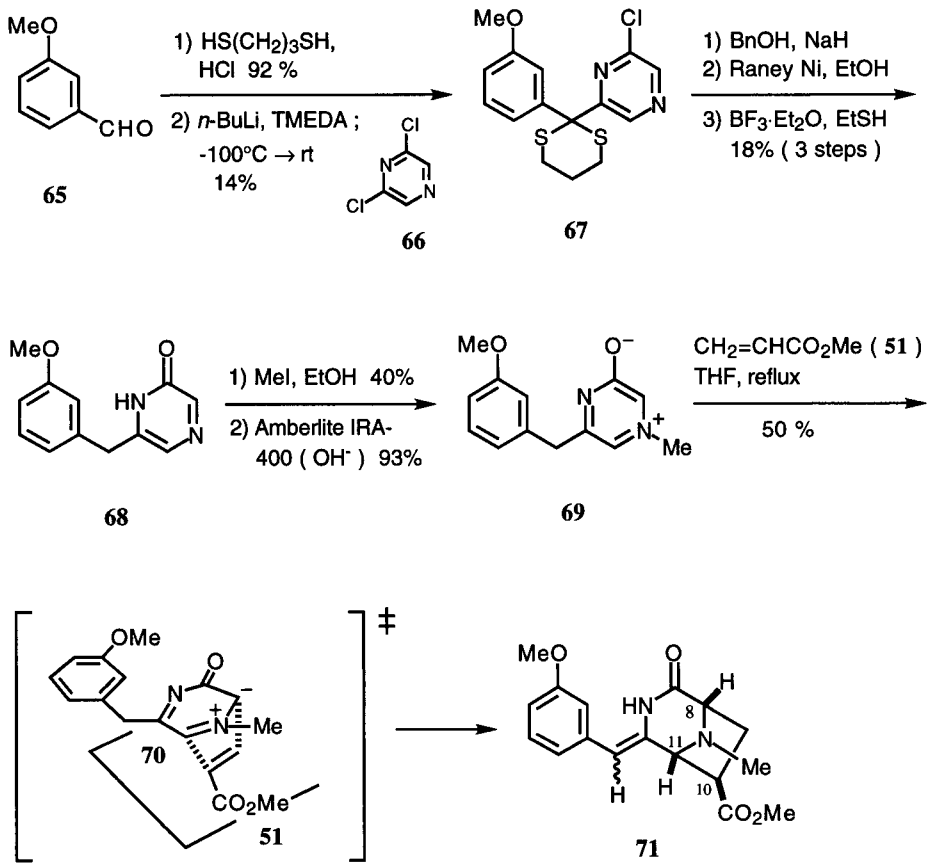
2.4. Synthesis of the Racemic ACD Ring System of Quinocarcin

[Joule et al., 1990]

In 1990, Joule *et al.* presented a novel method for the preparation of the racemic ACD ring system **71** (**Scheme 15**).[25b] The key feature in this synthesis involves the intermolecular 1,3-dipolar cycloaddition of methyl acrylate (**51**) to the azomethine ylide **70** generated *in situ* from the oxidopyrazinium **69** to construct the critical 3,8-diazabicyclo[3,2,1]octane (the CD ring) system possessing the requisite functional groups with correct stereochemistries at the C-8, C-10, and C-11 positions in a single step (**69** + **51**→**71**).

The synthesis commences with displacement of one chlorine for 2,6-dichloropyrazine (**66**) by the anion of dithiane prepared from 3-methoxy-

Scheme 15. Synthesis of the racemic ACD ring system [(±)-**71**]



benzaldehyde (**65**), providing the coupling product **67** in 13% yield for the two steps. Further displacement of the remaining chlorine atom in **67** with a benzyloxy group followed by desulphurization and debenzylation[28] afforded the requisite pyrazinone **68** in 18% overall yield from **67**. Quarternization of **68** with iodomethane and subsequent deprotonation of the resulting methiodide led to the formation of the zwitterion **69**. The crucial 1,3-dipolar cycloaddition of **69** with methyl acrylate (**51**) was carried out in refluxing tetrahydrofuran, giving rise to the bicyclic compound **71** in 50% yield as a single geometrical isomer. The stereochemistry of the olefinic moiety in **71** has not been determined.

3. *Synthesis of Optically Active Quinocarcin and Its Related Compounds*

3.1. Total Synthesis of Natural (–)-Quinocarcin [Garner et al., 1992]

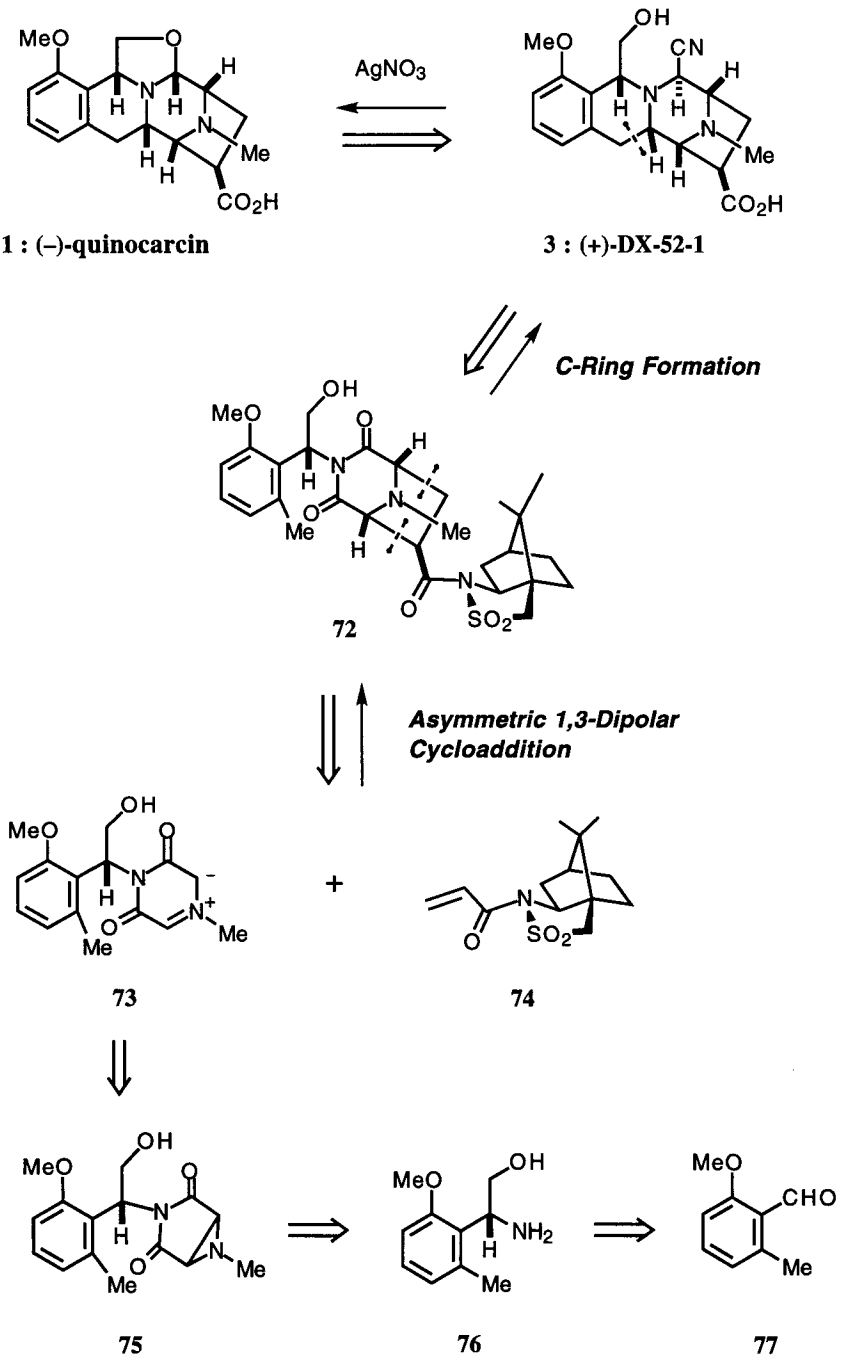
3.1.1. Synthetic Strategy

In 1992, Garner *et al.* accomplished the first asymmetric total synthesis of (–)-quinocarcin (**1**).[9] It must be emphasized that this synthesis verified the unsettled absolute stereochemistry of **1**. The retrosynthetic plan for **1** is outlined in **Scheme 16**. The most crucial step is envisaged to be the intermolecular 1,3-dipolar cycloaddition of the azomethine ylide **73** and Oppolzer's chiral acryloyl sultam **74** to construct the key 3,8-diazabicyclo[3,2,1]octane (the CD ring) system **72** in a diastereo- and regiocontrolled manner in one step (**73+74→72**). The cycloadduct **72** possesses four of the six stereogenic centers present in **1** and also provides a suitable template for introducing the remaining functionality and chirality as well. Generation of the cyclic azomethine ylide **73** can be accomplished by means of a photochemically initiated electrocyclic ring opening of the aziridine **75** accessible from the optically pure phenylglycinol derivative **76**. The compound **76** is envisioned to be prepared from the known 2-methoxy-6-methylbenzaldehyde (**77**)[29] *via* an asymmetric azidation reaction.

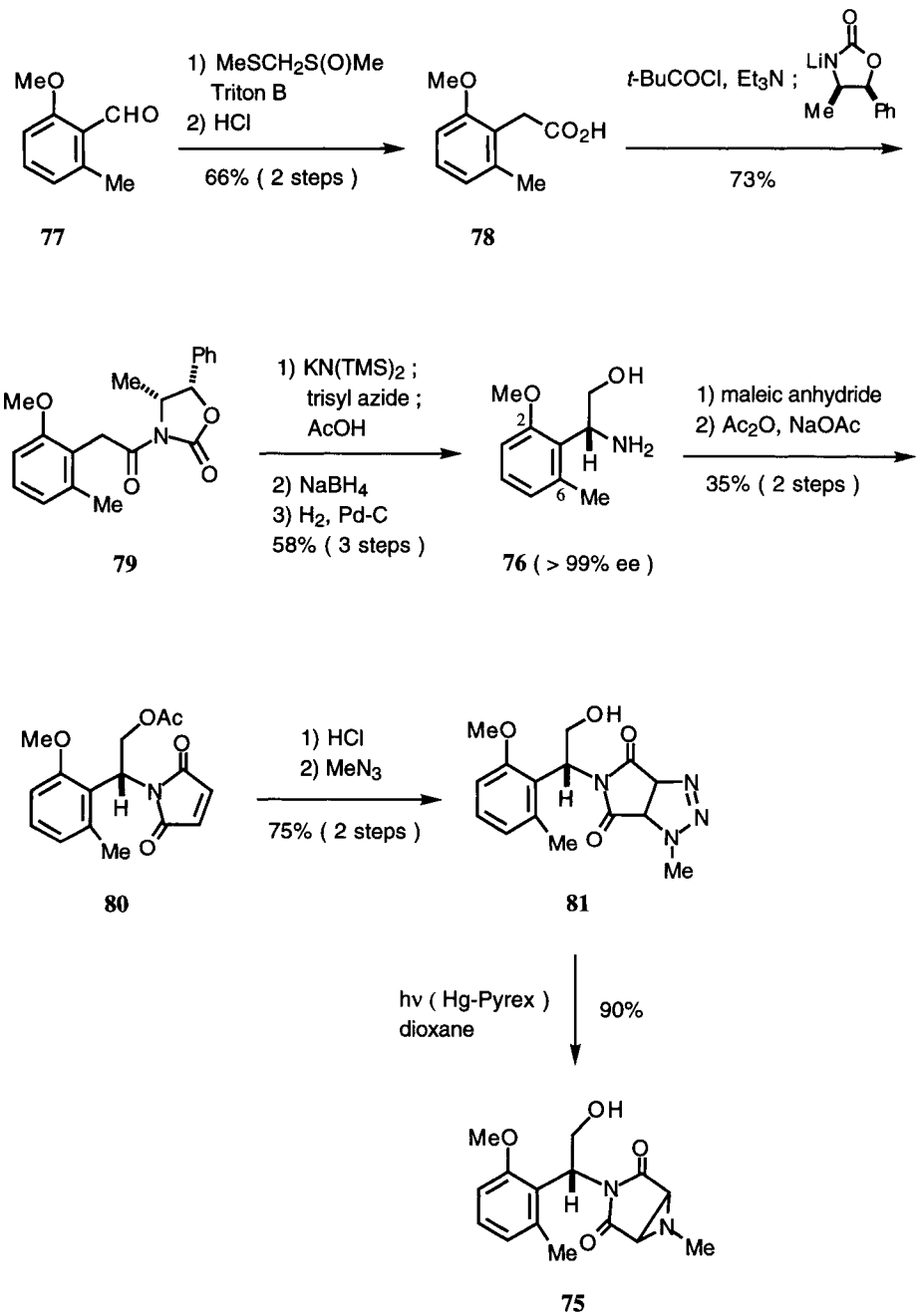
3.1.2. Total Synthesis

The preparation of the key precursor aziridine **75** was first investigated as shown in **Scheme 17**. Thus, the base-catalyzed condensation of the known 2-methoxy-6-methylbenzaldehyde (**77**)[29] with methylsulfinylmethyl sulfide followed by acidic hydrolysis provided 2-methoxy-6-methylphenylacetic acid (**78**).[30] The phenylacetic acid **78** was then converted to the phenylglycinol **76** (>99% *ee*) in 42% overall yield through the oxazolidinone **79** by employing Evans' asymmetric azidation protocol.[31] Treatment of **76** with maleic anhydride followed by acetic anhydride-mediated maleimide formation gave the acetate **80**. Removal of the acetyl group in **80** with hydrochloric acid and subsequent treatment with methyl azide led to the triazoline **81** in 75% yield for the two steps. Irradiation of **81** with a high-pressure Hg lamp through a Pyrex filter resulted in the formation of the desired aziridine **75**.[32] The crucial 1,3-dipolar cycloaddition was carried out by

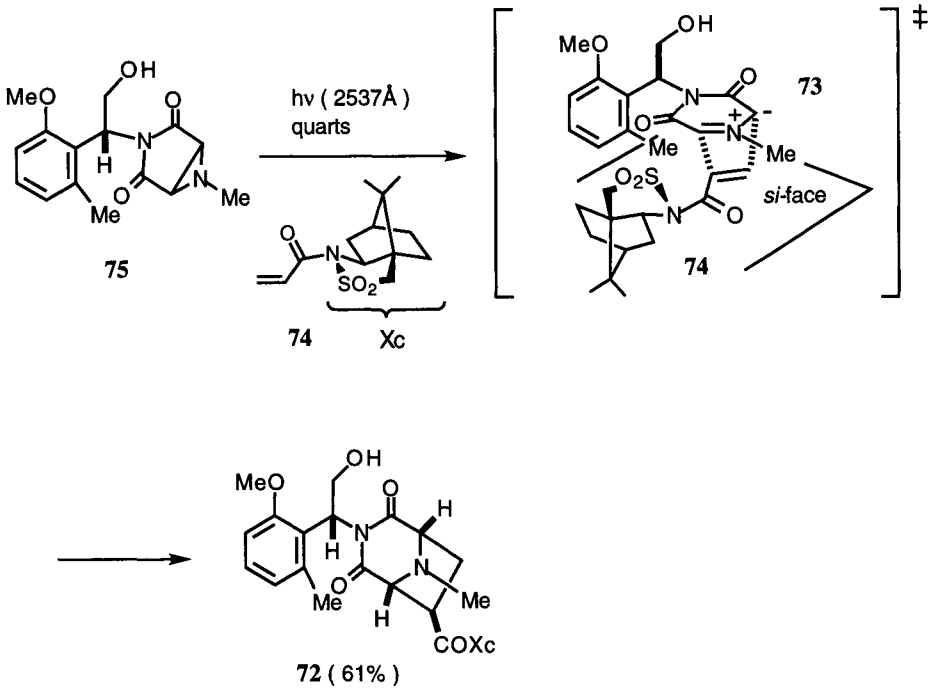
Scheme 16. Retrosynthetic analysis of (–)-quinocarcin (**1**) according to Garner *et al.*



Scheme 17. Synthesis of the intermediate **75**



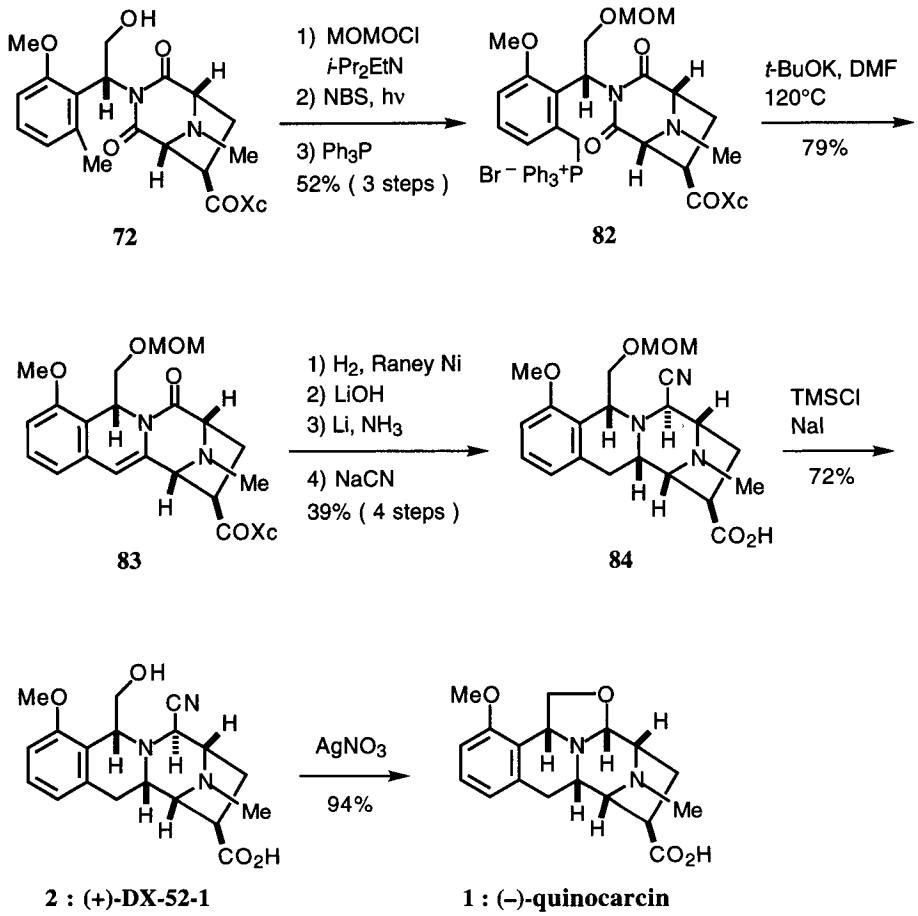
Scheme 18. Synthesis of the intermediate **72**



addition of Oppolzer's chiral acryloyl sultam **74**[33] to an irradiated (2537 Å, quartz) solution of **75** in 1,4-dioxane, providing the exo-*si* adduct **72** in 61% isolated yield (corrected for 14% recovery of **75**) after flash chromatography (**Scheme 18**). The absence of any other detectable stereoisomers in the crude reaction mixture was indicative of the high level of stereocontrol generally achieved for addition reactions with the chiral sultam **74**.[34]

The completion of the total synthesis of (–)-quinocarcin (**1**) is outlined in **Scheme 19**. Thus, the cycloadduct **72** was converted to the phosphonim salt **82** in 52% overall yield *via* a three-step sequence of reactions involving protection of the primary hydroxy group, chemoselective benzylic bromination with NBS, and substitution with triphenyl phosphine. Treatment of **82** with potassium *tert*-butoxide in *N,N*-dimethylformamide resulted in the formation of a phosphonium ylide, which, upon heating to 120°C, cyclized to give the required dihydroisoquinoline **83** as a single regioisomer in 79% yield. Sequential hydrogenation of

Scheme 19. Completion of the total synthesis of (–)-quinocarcin (**1**)



the double bond in **83**, saponification of the imide moiety, partial reduction of the amide functinality, and immediate treatment with sodium cyanide afforded the amino nitrile **84** in 39% yield for the four steps. Finally, deprotection of **84** furnished (+)-DX-52-1 (**2**), which was then treated with silver nitrate to provide (–)-quinocarcin (**1**). The ^{1}H-NMR and ^{13}C-NMR data obtained for synthetic **1** matched well with those reported in the literature. Comparison of the optical rotation of synthetic **1** with that of natural **1** led to the confirmation of the absolute configuration of natural quinocarcin.

3.2. Total Synthesis of an Enantiomeric Pair of Quinocarcin

[Katoh-Terashima et al., 1994]

3.2.1. Synthetic Strategy

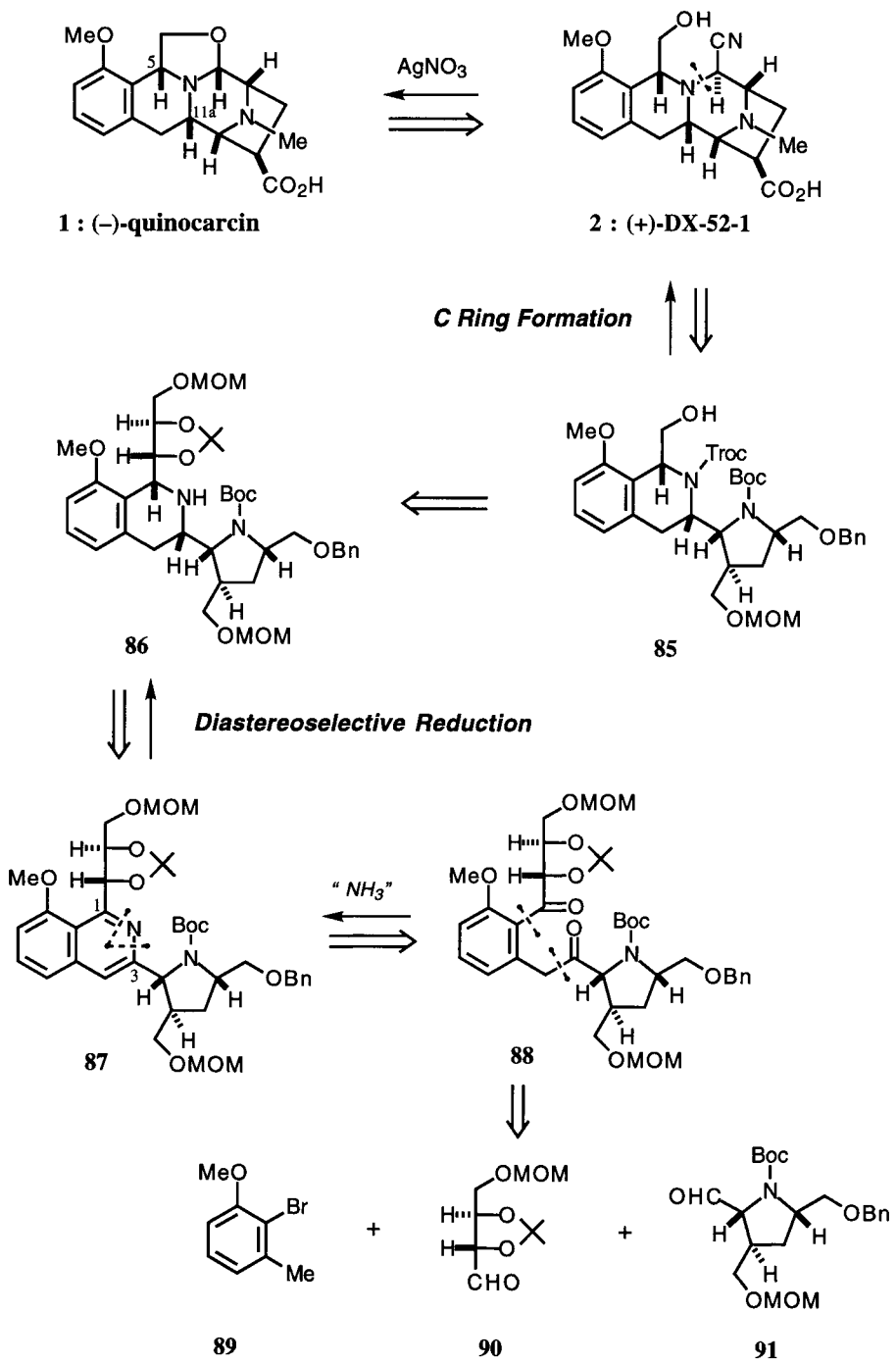
At the time when we initiated this project, the absolute configuration of quinocarcin (**1**) was unknown. Therefore, prior to embarking on the total synthesis of optically active **1**,[12] the syntheses of both enantiomers of the ABE,[35] ABC,[36] ABCD,[37] and ABCDE[37] ring systems of **1** were first carried out to establish an efficient and reliable synthetic method for constructing asymmetric centers at the C-5 and C-11a positions of **1** with defined absolute configurations (see, section *4.2.*).

On the basis of the results accumulated in these model studies, a novel synthetic strategy for **1** was designed as shown in **Scheme 20**. The most crucial step in this scheme is envisaged to be the diasteroselective reduction of the 1,3-disubstituted isoquinoline **87** to control the stereochemistries of asymmetric centers at the C-5 and C-11a positions in **1** simultaneously in a single step (**87**→**86**). The reduction product **86** would be converted to the tetrahydroisoquinoline **85** possessing the requisite carbon frameworks and functional groups with correct absolute stereochemistries, by appropriate functional group manipulations. The substrate **87** for the key diastereoselective reduction can, in turn, be elaborated from the anisole **89**, the threose **90**, and the pyrrolidine **91** by way of the diketone **88**.

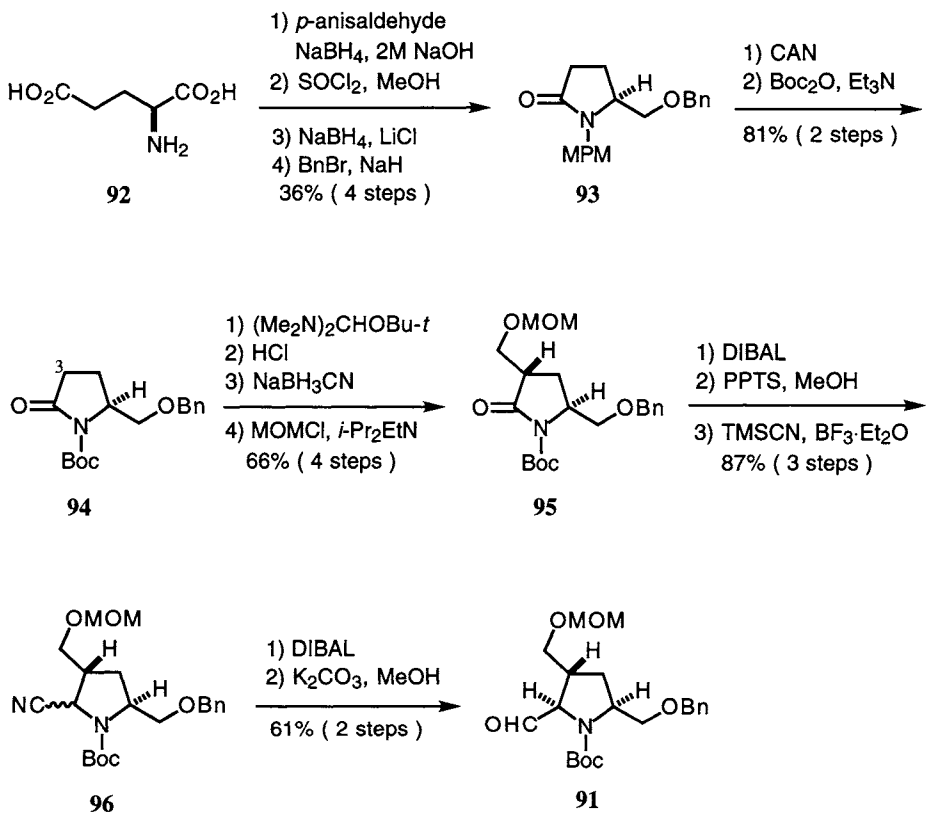
3.2.2. Total Synthesis

We commenced the preparation of optically pure 3,5-disubstituted-2-formylpyrrolidine **91** representing the D ring fragment of **1**, starting from (*S*)-glutamic acid (**92**) as shown in **Scheme 21**.[38] Thus, sequential protection of the amino group[39] in **92**, simultaneous lactam and methyl ester formation,[40] lithium borohydride reduction,[41] and protection of the resulting primary hydroxy group furnished the benzyl ether **93** in 36% overall yield from **92**. Exchange of the *N*-protecting group afforded *N*-Boc lactam **94**[42] in 81% yield for the two steps. Stereocontrolled introduction of a methoxymethyl (MOM) protected hydroxymethyl group into the C-3 position in **94** was achieved by sequential treatment with Bredereck reagent,[43] acidic hydrolysis of the resulting enamide, reduction, and final protection. The MOM ether **95** was then transformed to the

Scheme 20. Retrosynthetic Analysis of (–)-quinocarcin (**1**) according to Katoh-Terashima *et al.*



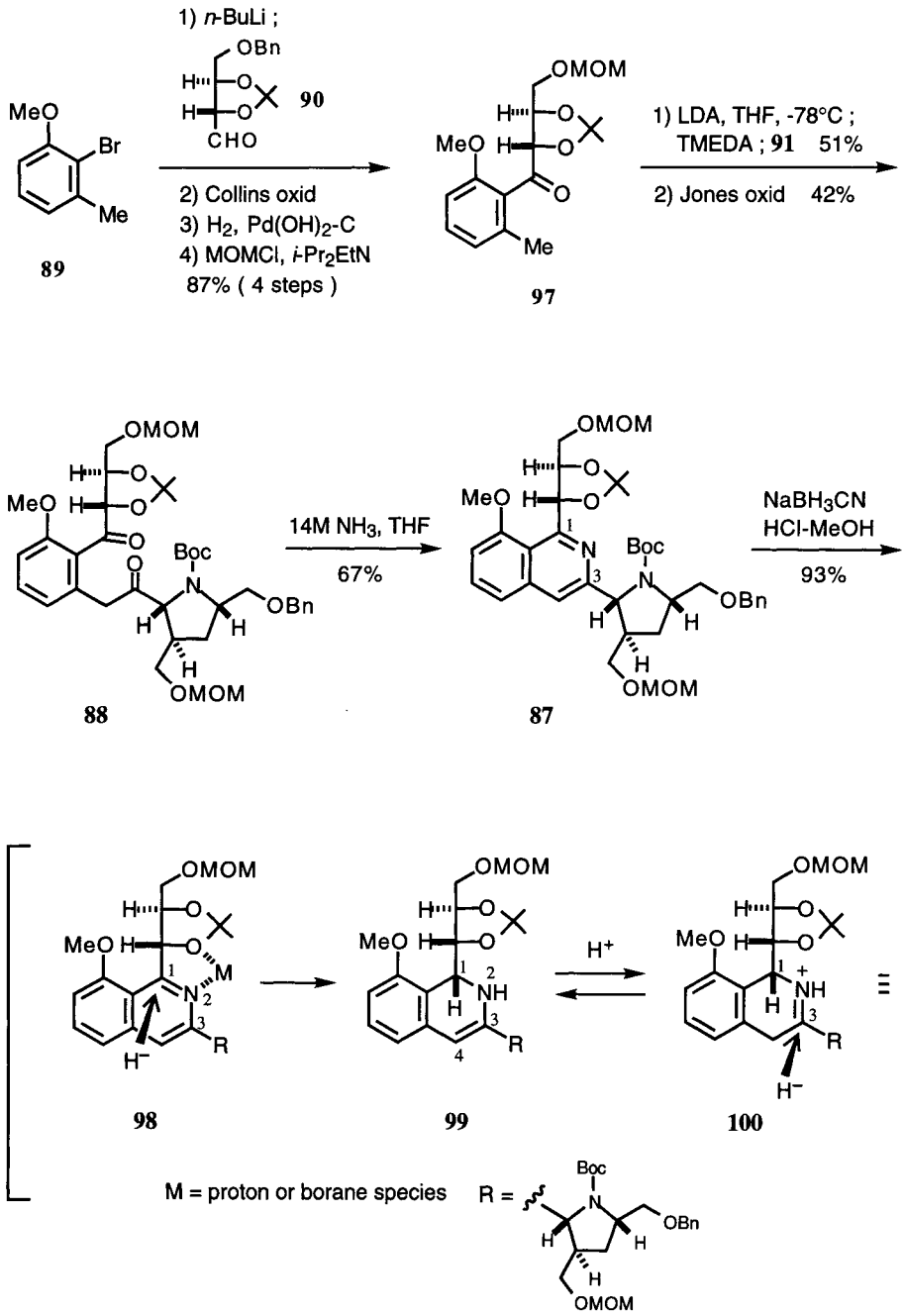
Scheme 21. Synthesis of the intermediate **91**



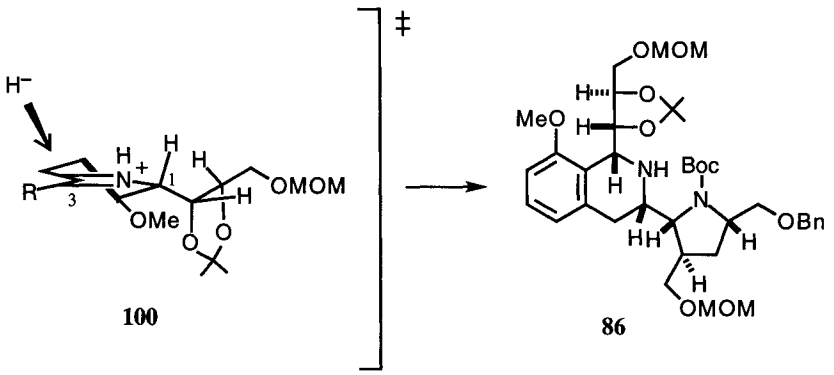
corresponding nitrile **96** *via* a three-step sequence involving diisobutyl aluaminium hydride (DIBAL) reduction, acid-catalyzed aminal formation, and nucleophilic addition of a cyanide anion to the reactive *N*-acyliminium ion generated *in situ* from the intermediate 2-methoxypyrrolidine. Finally, DIBAL reduction of **96** followed by base-catalyzed epimerization of the resulting formyl group provided **91**.[44,45].

With the D ring fragment **91** in hand, we next addressed ourselves to the synthesis of the key intermediate **86** as shown in **Scheme 22**. Thus, the known 2-bromo-3-methylanisole (**89**)[46] prepared from commercially available 2-methyl-6-nitroaniline was transformed to the ketone **97** having a chiral auxiliary by a

Scheme 22. Synthesis of the intermediate **86**



Scheme 22. Synthesis of the intermediate **86** (continued)

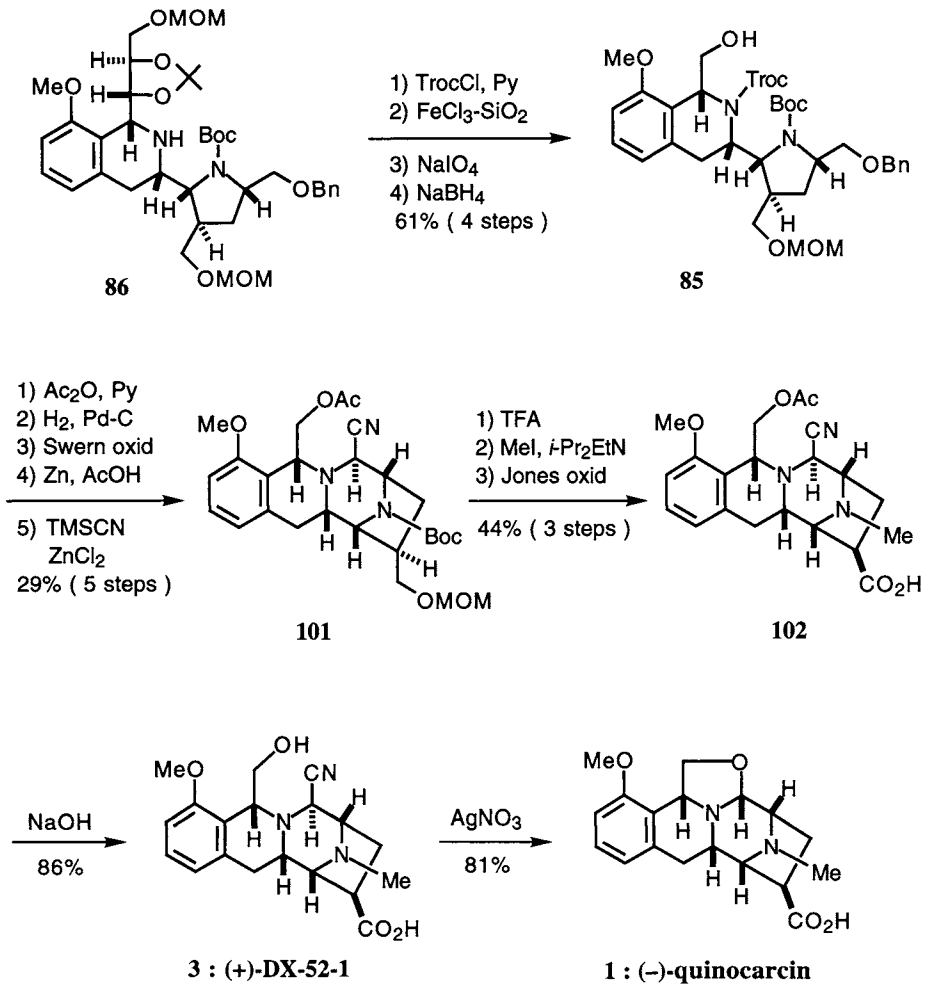


sequential four step operation including coupling reaction with the D-threose **90**,[47] Collins oxidation, and subsequent exchange of the *O*-protecting group. Additional coupling reaction of **97** with **91**[48] followed by Jones oxidation provided the diketone **88**. Further treatment of **88** with aqueous ammonia resulted in the formation of the desired isoquinoline **87**.

The crucial reduction of **87** with sodium cyanoborohydride[49] in an acidic medium proceeded in a completely diastereoselective manner, providing the tetrahydroisoquinoline **86** as the sole product in 93% yield. The diastereoselectivity observed for this reduction can be rationalized by a sequential two-step asymmetric induction. Thus, the first hydride attack may proceed through the usual Cram's chelation model[50] (see, **98**) which interacts with the alkoxy group adjacent to the C1-N2 double bond, exclusively affording the 1,2-dihydroisoquinoline **99**. Subsequent rapid and reversible protonation at the C-4 position of **99** in an acidic medium would generate the readily reducible iminium salt **100**,[49,51] and then the second hydride attack presumably occurs under an influence of the so-called stereoelectronic effect[52] (see, **100**).

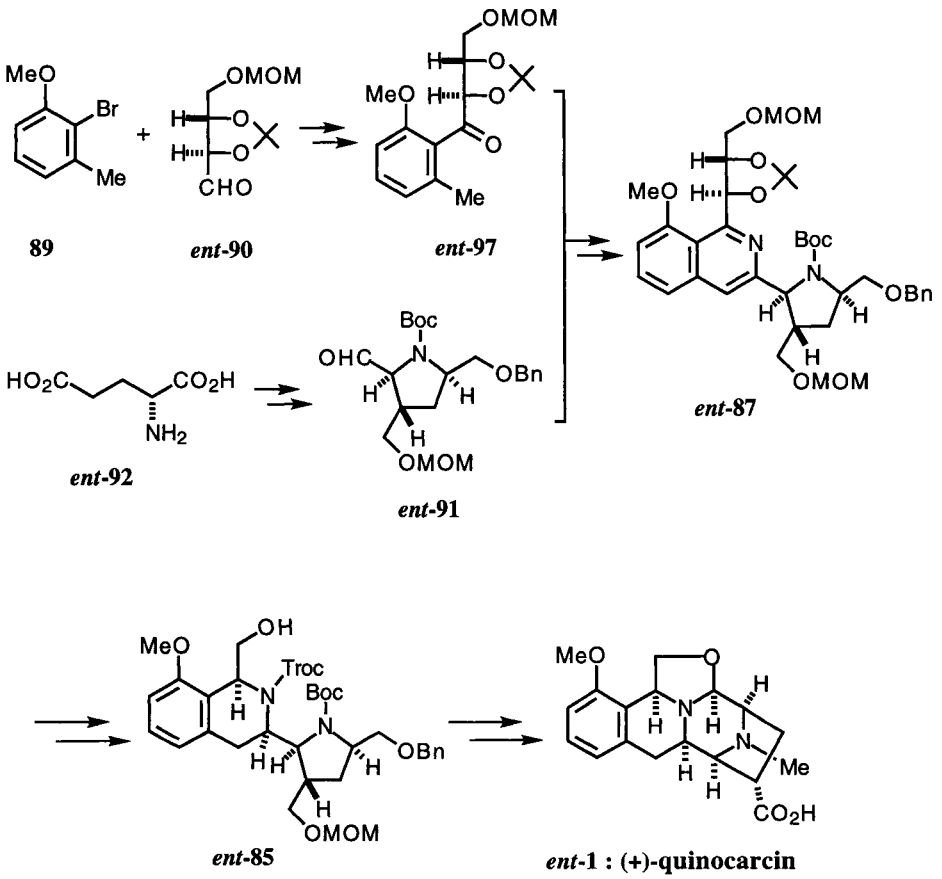
Completion of the synthesis of (–)-**1** is outlined in **Scheme 23**. Thus, conversion of the tetrahydroisoquinoline **86** to the corresponding alcohol **85** was achieved by sequential protection of the amino functionality with 2,2,2-trichloroethoxycarbonyl (Troc)[53] group, chemoselective acid hydrolysis of the acetonide

Scheme 23. Completion of the total synthesis of (–)-quinocarcin (**1**)



moiety with a combination of ferric chloride and silica gel,[54] oxidative cleavage of the intermediate diol, and final reduction of the resulting aldehyde. After acetylation of **85** followed by debenzylation and Swern oxidation, the *N*-Troc group in the resulting aldehyde was removed[53] to yield the unstable tetracyclic hemiaminal, which was immediately treated with trimethylsilyl cyanide in the presence of zinc chloride to afford the requisite amino nitrile **101**. Removal of the Boc group in **101** followed by chemoselective *N*-methylation and Jones oxidation

Scheme 24. The total synthesis of *ent*-quinocarcin [(+)-**1**]



gave the carboxylic acid **102**. Finally, saponification of **102** furnished (+)-DX-52-1 (**2**), which was then treated with silver nitrate to afford (–)-quinocarcin (**1**).

Unnatural *ent*-quinocarcin [(+)-**1**] was synthesized in the same manner as described above by employing *ent*-**97** and *ent*-**91** instead of **97** and **91**, respectively, by way of *ent*-**87** and *ent*-**85** (**Scheme 24**). The ketone *ent*-**97** was prepared from the anisole **89** and the L-threose *ent*-**90** similarly to the previous case. Preparation of the aldehyde *ent*-**91** was also achieved starting from (*R*)-glutamic acid (*ent*-**92**).

3.3. Synthetic Studies on Optically Active Quinocarcin

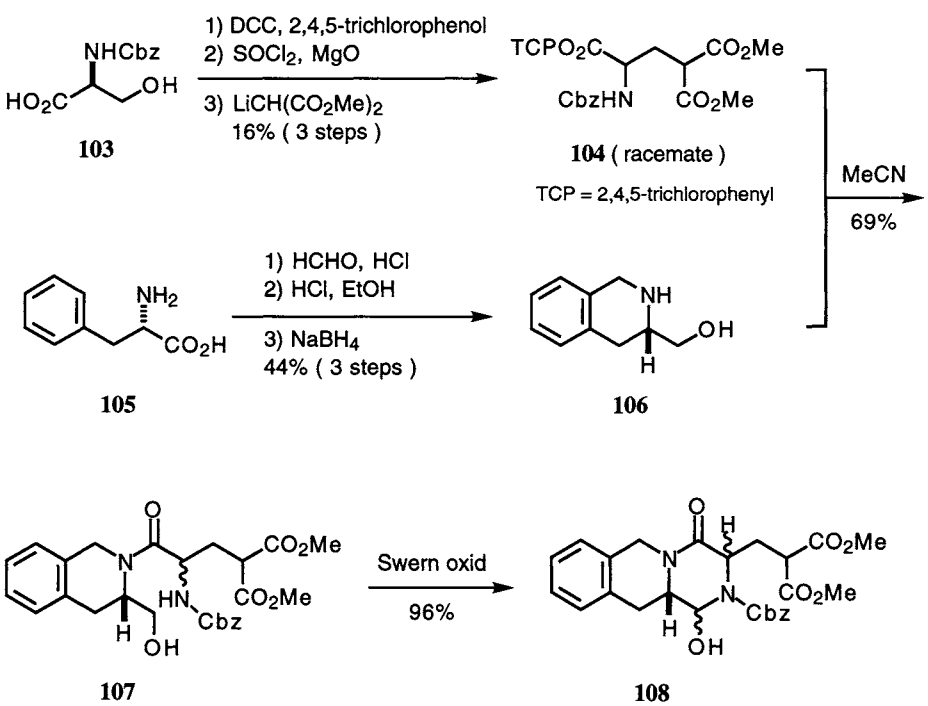
3.3.1. Synthesis of the Optically Active ABCD Ring System of Quinocarcin [Kyowa Hakko, 1987]

In 1987, scientists at Kyowa Hakko Co. reported the synthesis of the basic ABCD ring systems **113** and **114** in optically active forms (**Schemes 25** and **26**).[5a] The key feature in the synthesis involves the intramolecular acyliminium ion mediated cyclization of the aminal **108** to construct the 3,8-diazabicyclo[3,2,1]-octane system (the CD ring) **110** (**108**→**109**→**110**).[5]

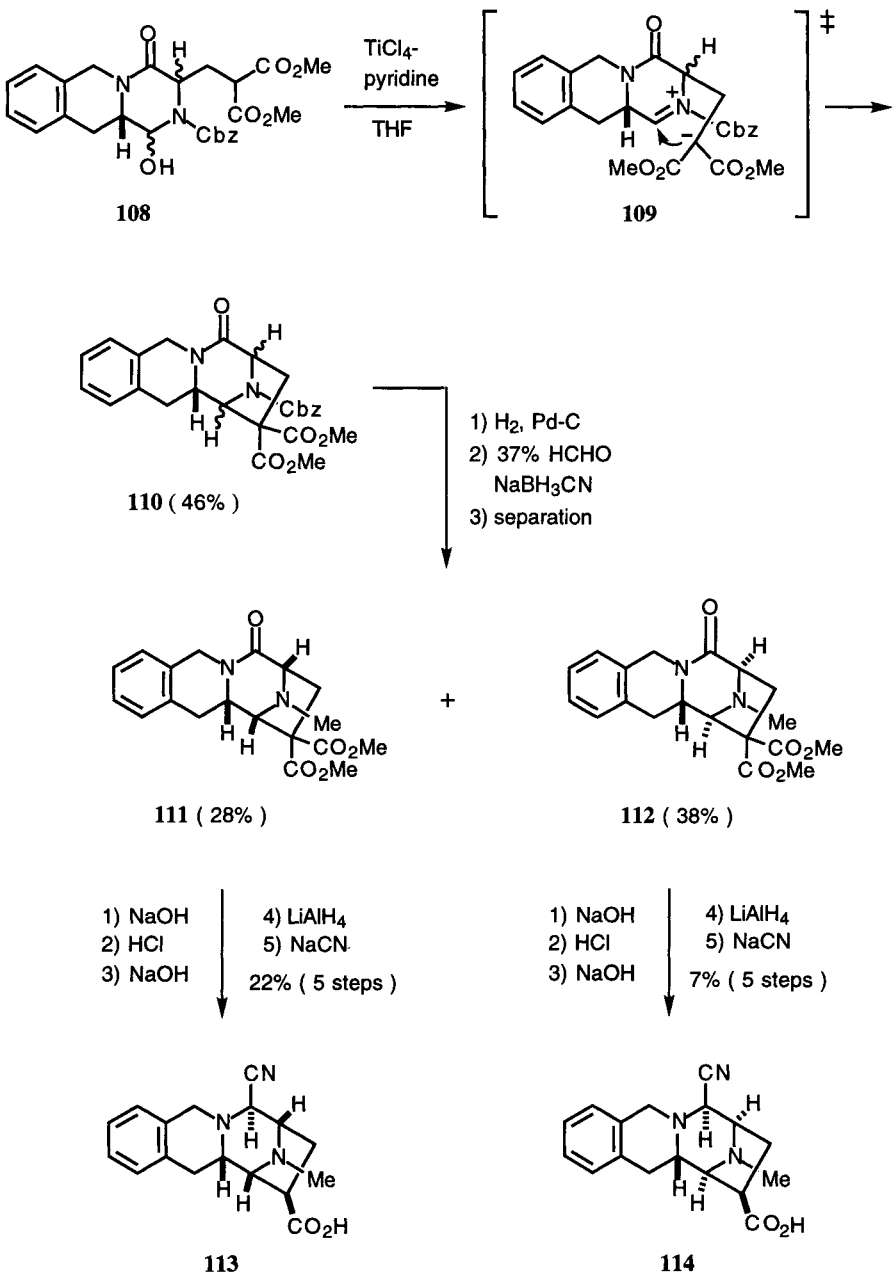
The aliphatic fragment **104** was prepared in a racemic form starting with *N*-Cbz-L-serine (**103**) *via* a three-step sequence of reactions involving esterification, dehydration, and Michael addition of dimethyl malonate.[55]

On the other hand, the preparation of the optically active aromatic fragment **106** was achieved starting from L-phenylalanine (**105**) by employing Pictet-

Scheme 25. Synyhesis of the intermediate **108**



Scheme 26. Synthesis of the optically active ABCD ring systems (**113** and **114**)



Spengler isoquinoline synthesis followed by esterification and reduction.[56]

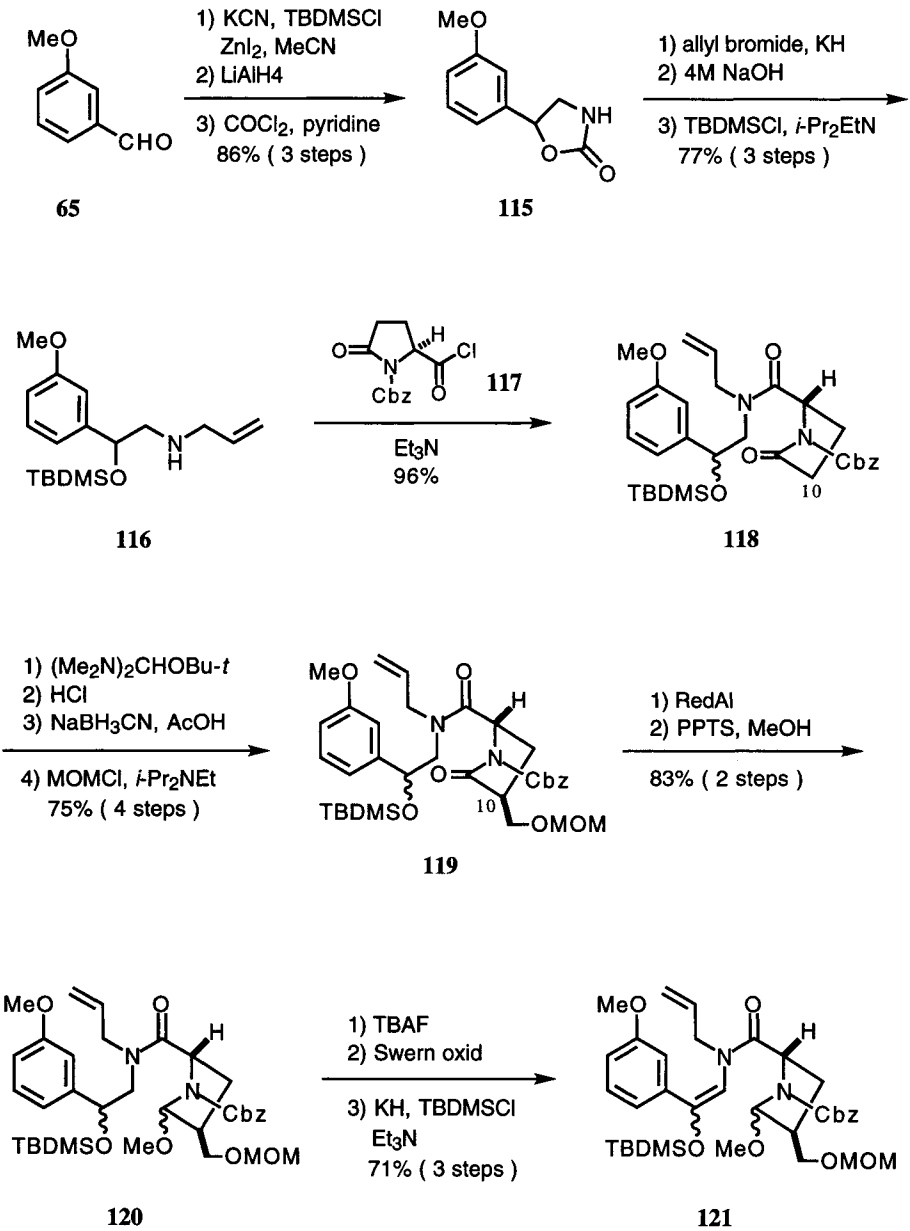
The two fragments **104** and **106** were then coupled to provide the amide **107** as a diastereomeic mixture in 69% yield. Swern oxidation of **107** resulted in the formation of the requisite tricyclic aminal **108**. As shown in **Scheme 26**, the key intramolecular cyclization of **108** could be achieved by treatment with titanium(IV) chloride-pyridine complex in tetrahydrofuran to give the tetracyclic compound **110** as an inseparable mixture of diastereomers in 46% yield. This step probably proceeds *via* the *N*-acyliminium intermediate **109**. Removal of the Cbz group in **110** by hydrogenolysis followed by reductive *N*-methylation furnished the diastereomeric compounds **111** and **112** in a ratio of *ca.* 1 : 1.4, which were easily separated by column chromatography on silica gel. Saponification of **111** followed by decarboxylation and sodium hydroxide treatment yielded the corresponding sodium salt. Further reduction of this salt with lithium aluminium hydride at 0°C and subsequent treatment with sodium cyanide provided the desired compound **113**. The structure of **113** including stereochemistry was confirmed by ^{1}H- and ^{13}C-NMR spectroscopic analysis in comparison with that of DX-52-1 (**2**). Similarly, **112** was converted to the other stereoiomer **114**.

3.3.2. Synthesis of the Optically Active ACD Ring System of Quinocarcin [Weinreb et al., 1990]

In 1990, Weinreb *et al.* disclosed the synthesis of the optically active ACD ring system **126** (**Schemes 27** and **28**),[57] which is closely related to the intermediate in Fukuyama's synthesis of racemic quinocarcin (see, section ***2.2.***). The key step in the synthesis consists of the intramolecular *N*-acyliminium ion mediated silylenol ether cyclization of **121** to construct the key diazabicyclo ring system **123** (**121**→**122**→**123**).

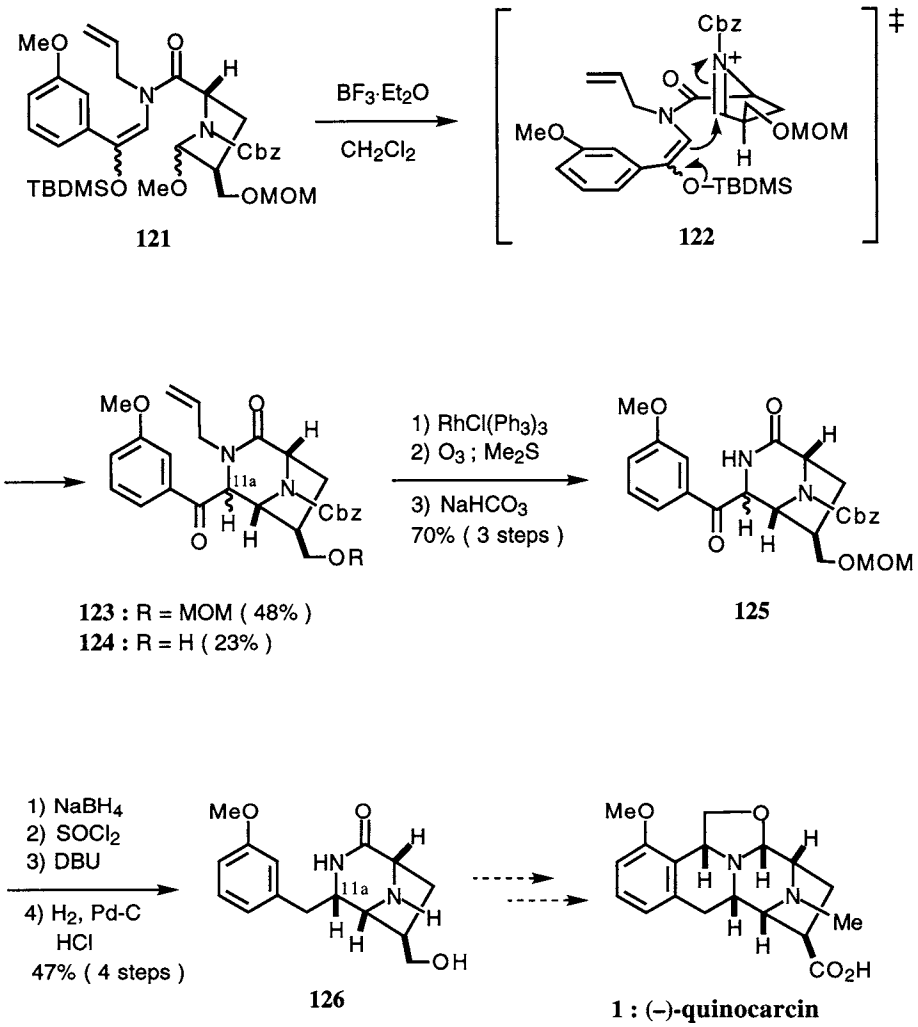
The synthesis of the substrate **121** for the key cyclization commenced with the conversion of 3-methoxybenzaldehyde (**65**) to the cyclic carbamate **115** *via* a three-step sequence involving siloxy nitrile formation,[58] reduction of the cyano functionality, and exposure to phosgene. *N*-Allylation of **115** and subsequent hydrolysis of the carbonate moiety followed by silylation of the hydroxy group afforded the amine **116**. Condensation of **116** with optically pure acid chloride **117** prepared form (*S*)-glutamic acid (**92**),[59] furnished the amide **118** in 96%

Scheme 27. Synthesis of the intermediate **121**



65 115 116 118 119 120 121

Scheme 28. Synthesis of the optically active ACD ring system **126**



yield. After stereocontrolled introduction of a hydroxymethyl group (β-CH_2OH : α-CH_2OH = 8 : 1) at the C-10 position in **118** *via* a three step operation, the resulting alcohol was then protected to give the MOM ether **119**. Chemoselective reduction of the lactam carbonyl function in **119** followed by treatment with pyridinium *p*-toluenesulphonate (PPTS) in methanol led to the methoxy carbamate

120. This compound was further transformed in three simple steps to the silylenol ether **121** as a mixture of *E* and *Z* stereoisomers.

The stage is now set for the key cyclization event. As shown in **Scheme 28**, treatment of **121** with borontrifluoride etherate at 0°C followed by warming to room temperature, provided the desired bicyclic lactam **123** (48%) along with the deprotected alcohol **124** (23%) *via* the plausible *N*-acyliminium intermediate **122**.[60] Both cyclization products **123** and **124** were 2 : 1 mixtures of epimers concerning the C-11a position. The *N*-allyl group in **123** was deleted *via* a three-step sequence involving isomerization of the *exo*-olefinc bond, oxidative cleavage of the *endo*-olefin moiety, and final removal of the resulting *N*-formyl group, furnishing the keto lactam **125** in 70% overall yield. Finally, **125** was converted to the optically active ACD ring system **126** *via* a conventional four-step sequence of reactions.

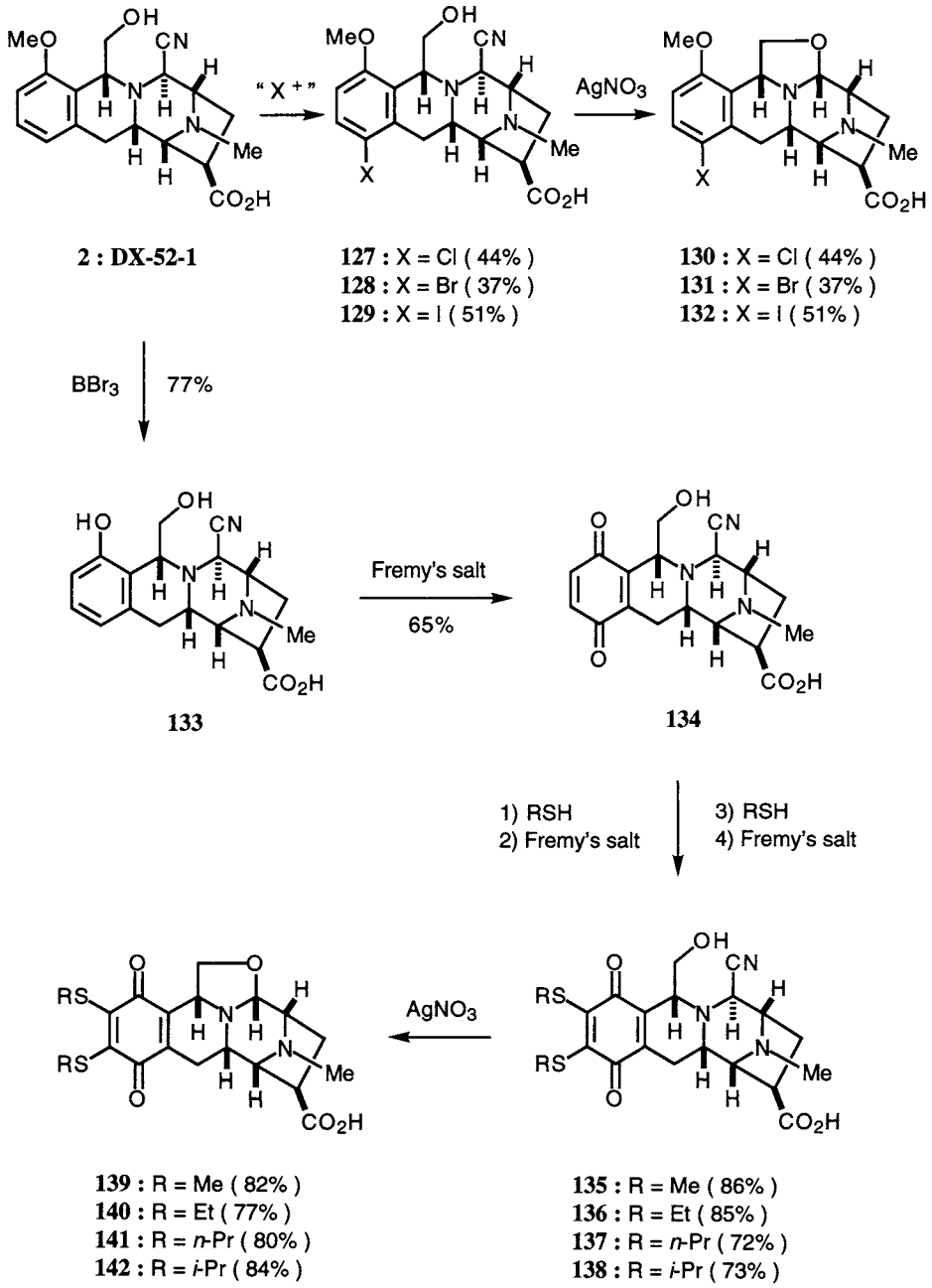
4. Synthesis and Antitumor Activity of Quinocarcin Congeners

4.1. Chemical Modifications of the A Ring of Quincarcin and their Antitumor Activity [Kyowa Hakko, 1990, 1991]

4.1.1. Synthesis of the A Ring Substituted Quinocarcin Derivatives

The Kyowa Hakko Laboratories group has synthesized various aromatic ring-substituted derivatives to improve the antitumor activity as well as to broaden the spectrum of **1**.[5,61] As summarized in **Scheme 29**, the 1-halogenated quinocarcin derivatives **130** - **132** were prepared starting with DX-52-1 (**2**) which are readily obtained from quinocarcin (**1**) as stated in section *1*. Thus, halogenation of **2** followed by treatment of the resulting halogenated compounds **127** - **129** with silver nitrate provided **130** - **132** in 14 - 26% yields for the two steps. *O*-Demethyl-DX-52-1 (**133**) derived from **2** was oxidized with Fremy's salt[62] to afford the quinone **134** in 65% yield. The quinone **134** was then converted to the bis(thioalkyl)quinones **135** - **138** by addition of mercaptan followed by oxidation with Fremy's salt and repetition of mercaptan addition and oxidation. Exposure of **135** - **138** to silver nitrate afforded the corresponding bis(thioalkyl)-containing quinocarcin derivatives **139** - **142** in 58 - 71% overall yields from **134**.

Scheme 29. Synthesis of aromatic ring-substituted quinocarcin derivatives according to the Kyowa Hakko Laboratories group



2 : DX-52-1

127 : X = Cl (44%)
128 : X = Br (37%)
129 : X = I (51%)

130 : X = Cl (44%)
131 : X = Br (37%)
132 : X = I (51%)

133

134

135 : R = Me (86%)
136 : R = Et (85%)
137 : R = *n*-Pr (72%)
138 : R = *i*-Pr (73%)

139 : R = Me (82%)
140 : R = Et (77%)
141 : R = *n*-Pr (80%)
142 : R = *i*-Pr (84%)

4.1.2. Antitumor Activitiy of the A Ring Substituted Quinocarcin Derivatives

The antitumor activity of these aromatic ring-substituted derivatives **127** - **142** was evaluated by growth inhibition against HeLa S_3 cells (*in vitro*) and increase of life span (ILS) by single and five daily administrations for mice implanted with P388 murine leukemia cells (*in vivo*). As shown in **Table 1**, 1-halogenated derivatives (**127**, **130** - **132**) and bis(alkylthio)quinones (**138**, **139**, **141**, and **142**) exhibit superior *in vitro* cytotoxicity to quinocarcin (**1**). Especially, the cytotoxicity of bis(isopropylthio)quinone **142** is 25 times more potent than that of **1**. *In vivo* experiments (**Table 2**) disclosed that 1-halogenated derivatives (**130** and **131**) and bis(alkylthio)quinones (**135** - **142**) exhibit significant antitumor activity both in a single and in five daily administrations. It is noteworthy that bis(methylthio)quinone **139** displays antitumor activity superior to that of **1** against most of the human xenografted carcinomas. Consequently, further preclinical studies of **139** (KT 6104) are in progress. For details of these works,

Table 1. *In vitro* cytotoxicity of quinocarcin derivatives against HeLa S_3 cells

Compound	IC_{50} (μg/ml)[a]	Compound	IC_{50} (μg/ml)[a]
1 (quinocarcin)	5.0×10^{-2}	**134**	>10
2 (DX-52-1)	5.0×10^{-2}	**135**	1.3×10^{-1}
127	4.2×10^{-2}	**136**	1.1×10^{-1}
128	Nt[b]	**137**	5.0×10^{-2}
129	1.1×10^{-1}	**138**	1.2×10^{-2}
130	4.0×10^{-2}	**139**	1.9×10^{-2}
131	4.8×10^{-2}	**140**	8.0×10^{-2}
132	4.0×10^{-2}	**141**	3.0×10^{-2}
133	5.3	**142**	1.9×10^{-3}

a) Concentration required for 50% inhibition of the cell growth after incubation for 72 h at 37°C (initial cell density : 5×10^4 cells/ml).
b) Not tested.

it is desirable to refer to the original papers.[5b,c,61]

Table 2. *In vivo* antitumor activity of quinocarcin derivatives against P388 murine leukemia cells

Compound	P388 ip-ip[a]			
	optimal dose (mg/ kg) x 1	ILS[b] (%)	optimal dose (mg/ kg) x 5	ILS[b] (%)
1	10-20	24-48	5-10	70-120
2	20	26	7.5	62
127	12.5	23	12.5	88
128	12.5	17	12.5	97
129	50	31	25	85
130	12.5	40	6.25	121
131	12.5	50	1.56	81
132	25	24	12.5	90
133	3.13	18	3.13	22
134	Nt[c]		Nt[c]	
135	12.5	53	6.25	71
136	12.5	50	6.25	51
137	25	56	6.25	42
138	25	65	12.5	119
139	6.25	48	3.13	91
140	6.25	64	3.13	82
141	12.5	58	6.25	86
142	6.25	69	3.13	98

a) $CD2F_1$ mice (5 mice/group) were implanted intraperitoneally (i.p.) with 1 x 10^6 cells, and a sample was dosed i.p. on day 1 and days 1-5.
b) Percent increase of life span calculated (T/C-1) x 100, where T and C are mean survival days of treated and control mice, respectively.
c) Not tested.

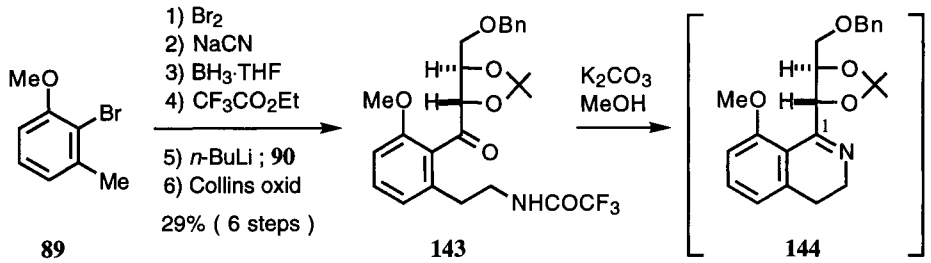
4.2. Synthesis and Antitumor Activity of Various Structural Types of Quinocarcin Congeners [Katoh-Terashima et al., 1994]

4.2.1. Synthesis of Enantiomeric Pairs of the ABE Ring Systems of Quinocarcin

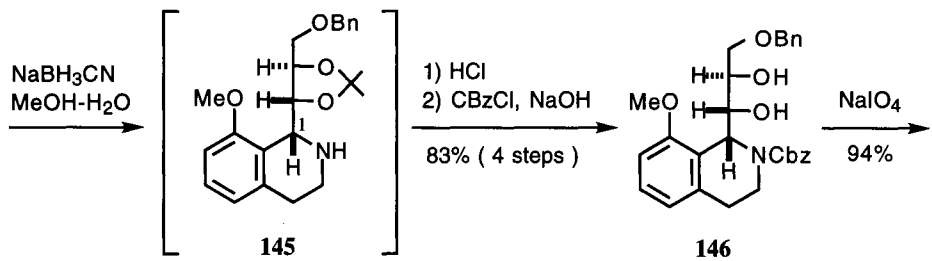
The optically active ABE ring systems (**149** - **151**) were synthesized starting with 2-bromo-3-methylanisole (**89**) as shown in **Scheme 30**.[35] Thus, **89** was first converted to the ketone **143** *via* a six-step sequence of reactions. The crucial reduction of the 3,4-dihydroisoquinoline **144**, produced *in situ* by removal of the trifluoroacetyl group in **143** with potassium carbonate in methanol, with sodium cyanoborohydide provided the tetrahydroisoquinoline **145**. This compound could be isolated in the form of the diol carbamate **146** in 83% overall yield from **143** after acidic hydrolysis of the acetonide moiety followed by protection of the amino group. The stereostructure of **146** was unambiguously confirmed based on the 400MHz ^{1}H-NMR spectra of the 2-oxazolidinone derivatives prepared from **146**. The final task remaining to complete the synthesis of **149** is the construction of the E ring in **149**. Thus, oxidative cleavage of the vicinal diol moiety in **146** afforded the aldehyde **147**, which was immediately reduced with sodium borohydride to yield the alcohol **148**. Deprotection of the Cbz group in **148** and subsequent treatment with aqueous formaldehyde furnished the ABE ring system (**149**). The optical purity of **149** was determined to be more than 95% *ee* by comparison of the 400MHz ^{1}H-NMR spectra of (*R*)- and (*S*)-MTPA esters[63] derived from **148**.

We became interested in the modification of the E ring in **149**, because the E ring in **1** plays an important role in respect of its remarkable antitumor activity as mentioned in section *1.*. Accordingly, we next undertook the synthesis of the analogues **150** and **151**, in which the E ring in **149** is replaced with larger six- and seven-membered rings, respectively. Thus, sequential olefination[64] of **147**, hydroboration, treatment with alkaline hydrogen peroxide, and removal of the Cbz group gave the corresponding amino alcohol, which was then treated with aqueous formaldehyde to give the six-membered cyclic amino acetal **150** (>95% *ee*). On the other hand, stereospecific olefin formation[65] of **146** followed by simultaneous hydrogenolysis and hydrogenation provided the corresponding amino alcohol, which was similarly condensed with aqueous formaldehyde, resulting in the formation of the seven-membered cyclic amino acetal **151** (>95% *ee*).

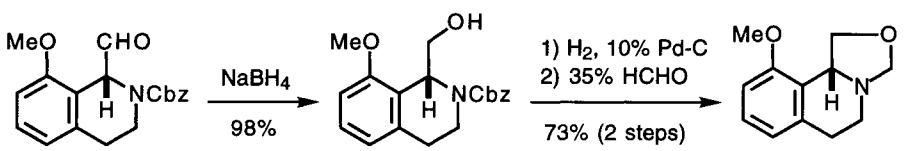
Scheme 30. Synthesis of the optically active ABE ring systems (**149** - **151**)



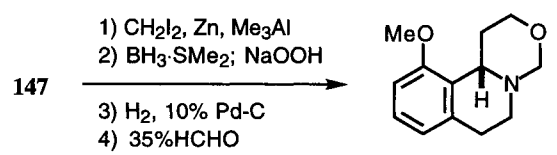
89 **143** **144**



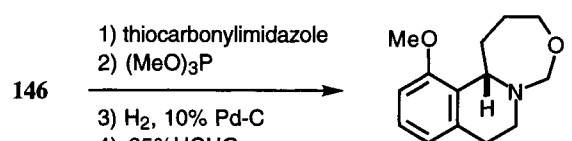
145 **146**



147 **148** **149**



150



151

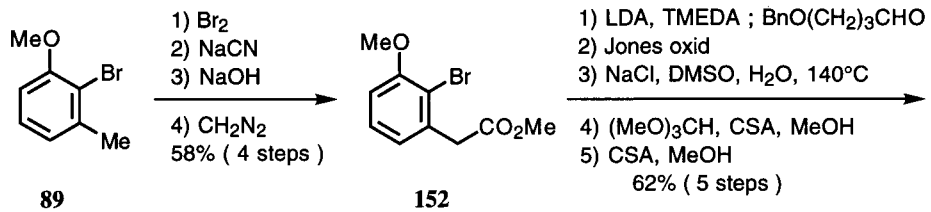
The enantiomeric ABE ring systems (*ent*-**149**, *ent*-**150**, and *ent*-**151**) were synthesized in the same manners as described above by employing 4-*O*-benzy-2,3-*O*-isopropylidene-L-threose (*ent*-**90**) instead of **90**.

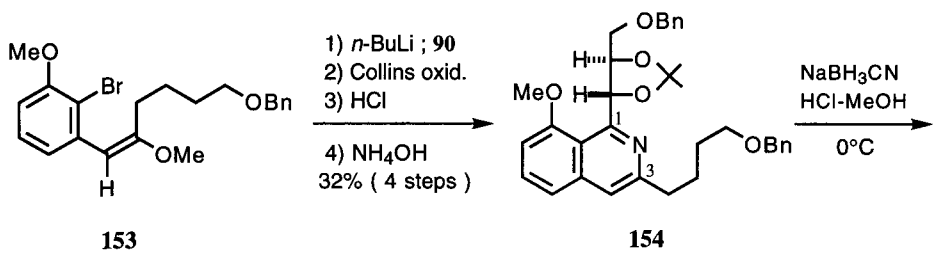
4.2.2. Synthesis of an Enantiomeric Pair of the ABC Ring System of Quinocarcin

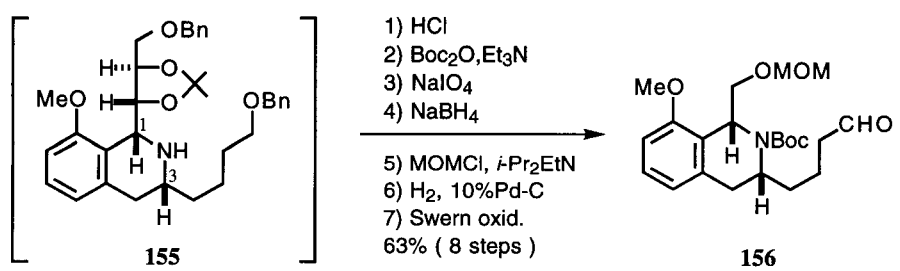
We next investigated the synthesis of an enantiomeric pair of the ABCE ring system of **1** (**157** and *ent*-**157**) as shown in **Scheme 31**.[36] After experimentation, however, **157** turned out to be too labile to be isolated. Therefore, the isolation of **157** was achieved in the form of the corresponding stable cyano derivative **158** (*vide infra*). The synthesis commenced with the conversion of **152** prepared from **89** to the methyl enol ether **153** *via* a five-step sequence of reactions involving coupling with 5-benzyloxypentanal,[66] Jones oxidation, demethoxycarbonylation, dimethylacetalization, and enol ether formation. After the coupling reaction of **153** with **90**, the coupling adduct was then transformed to 1,3-disubstituted isoquinoline **154** by sequential Collins oxidation, acidic hydrolysis of the enol ether moiety, and treatment of the resulting diketone with aqueous ammonia.

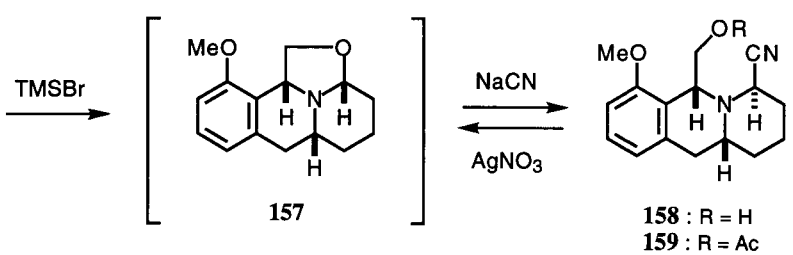
The key diastereoselective reduction of **154** to control the stereochemistries of the asymmetric centers at the C-1 and C-3 positions (isoquinoline numbering) was achieved by treating **154** with sodium cyanoborohydride in acidic media at 0°C, giving rise to the tetrahydroisoquinoline **155** as a single diastereomer. The key intermediate **156** having the requisite carbon frameworks and functional groups with correct absolute stereochemistries was elaborated from **155** through seven step operations. Upon treatment of **156** with bromotrimethylsilane, a new single compound deemed to be the ABCE ring system (**157**) was observed on TLC analysis. All attempts to isolate **157**, however, met with failure probably due to its chemical instability inherent in the oxazolidine ring of **157**. Consequently, **157** was isolated in the form of the corresponding stable cyano derivative **158** by treatment with sodium cyanide. Exposure of **158** to silver nitrate effected regeneration of **157**. However, all efforts to isolate **157** were again unsuccessful. The gross structure of **158** including stereochemistry was proven by the 400MHz ^{1}H-NMR spectral analysis of the corresponding acetate **159**.[67]

Scheme 31. Synthesis of the optically active ABC ring systems (**158** and **159**)









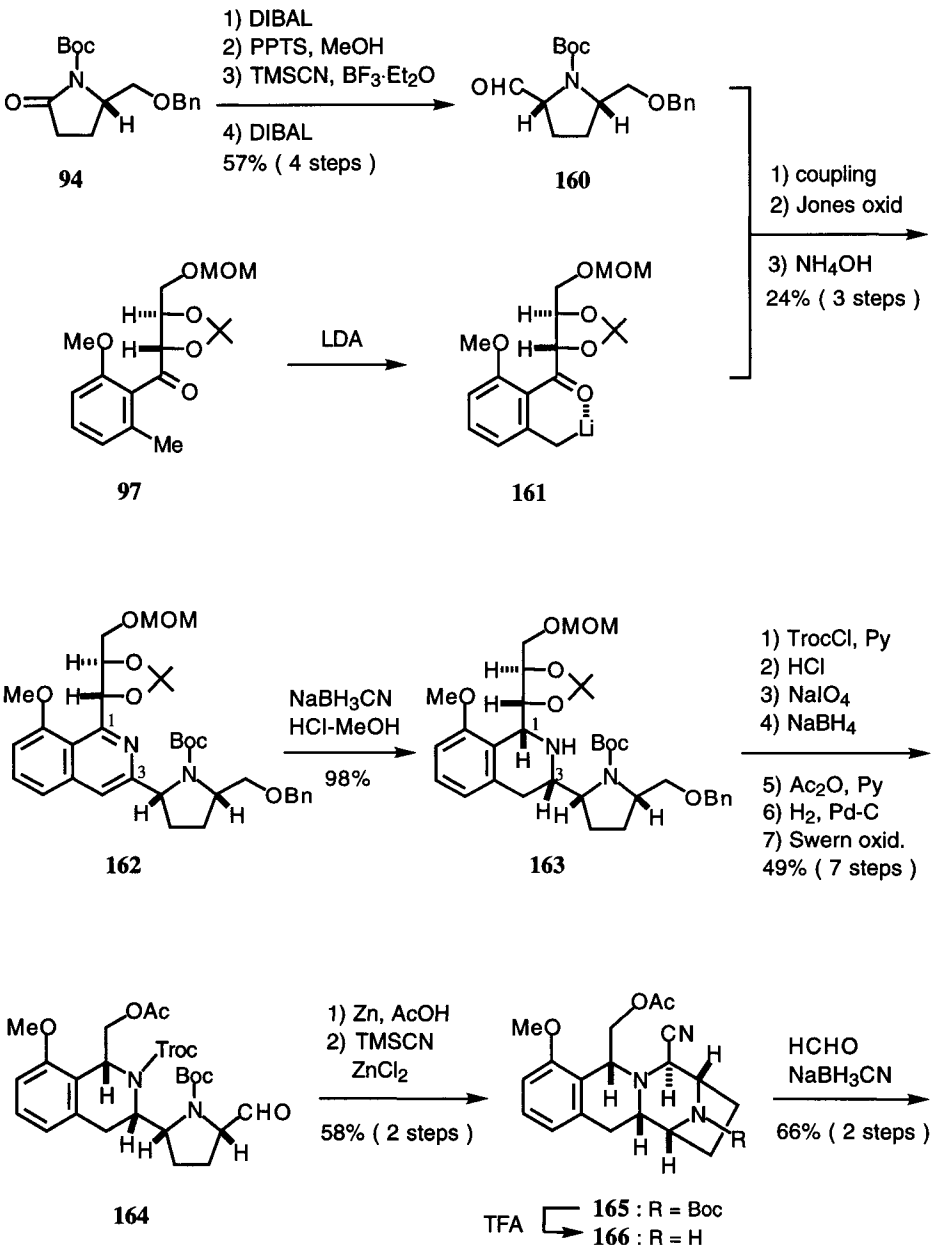
By employing *ent*-**90** instead of **90**, the enantiomeric ABC ring system (*ent*-**158**) and its acetate *ent*-**159** were prepared in the same manner as described above. Finally, the optical purity of **158** and *ent*-**158** was determined to be more than 95% *ee* by comparison of the 400MHz ^{1}H-NMR spectra of the corresponding MTPA esters.[63]

4.2.3. Synthesis of Enantiomeric Pairs of the ABCD Ring System and the ABCDE Ring System of Quinocarcin

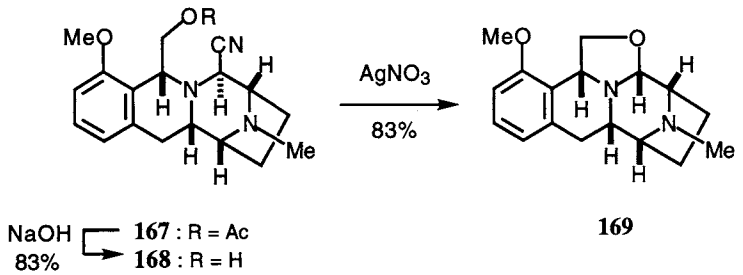
Encouraged by the successful synthesis of the ABE and the ABC ring systems, we next pursued the synthesis of both enantiomers of the ABCD ring system (**168**) (10-decarboxy-DX-52-1) and the ABCDE ring system (**169**) (10-decarboxy-quinocarcin) as shown in **Scheme 32**.[37] The optically pure D ring fragment **160** was first prepared starting with the lactam **94** *via* a four-step sequence of reactions involving DIBAL reduction of the lactam carbonyl function, acid-catalyzed aminal formation, nucleophilic addition of a cyanide anion, and further DIBAL reduction of the resulting nitrile.[38] The ketone **97** has been already prepared in section ***3.2.*** (see, **Scheme 22**). Coupling reaction of **160** with the benzyl lithium **161** *in situ* produced from **97**, followed by Jones oxidation and aqueous ammonia treatment led to the formation of the isoquinoline **162** in 24% overall yield for the three steps. Crucial reduction of **162** took place with complete diastereoselectivity similarly to those of **87** and **154**, giving rise to the 1,3-*cis*-tetrahydroisoquinoline **163** in an excellent yield. The reduction product **163** was further converted to the tetracyclic amino nitrile **165** *via* the aldehyde **164** by a sequential nine step operation. Deprotection of the Boc group in **165** and subsequent reductive *N*-methylation of the resulting amine **166** afforded the acetate **167** in 66% overall yield for the two steps. Saponification of **167** provided the ABCD ring system (**168**), which was then treated with silver nitrate to furnish the ABCDE ring system (**169**).

The enantiomeric ABCD ring systems (*ent*-**167** and *ent*-**168**) and ABCDE ring system (*ent*-**169**) were synthesized in a similar manner to that described above by employing *ent*-**94** and *ent*-**97** instead of **94** and **97**, respectively. Enantiomeric lactam *ent*-**94** was prepared from (*R*)-glutamic acid (*ent*-**92**) as detailed in section ***3.2.*** (see, **Scheme 21**).

Scheme 32. Synthesis of the optically active ABCDE ring system (**169**) (10-decarboxyquinocarcin)



Scheme 32. Synthesis of the optically active ABCDE ring system (**169**) (10-decarboxyquinocarcin) (continued)



4.2.4. In Vitro Cytotoxicity Assay of Enantiomeric Pairs of Various Structural Types of Quinocarcin Congeners Against P388 Murine Leukemia Cells

Enantiomeric pairs of various structural types of quinocarcin congeners such as the ABE ring systems (**149**, *ent*-**149**, **150**, *ent*-**150**, **151**, and *ent*-**151**), the ABC ring systems (**158**, *ent*-**158**, **159**, and *ent*-**159**), the ABCD ring systems (**165**, *ent*-**165**, **166**, *ent*-**166**, **167**, *ent*-**167**, **168**, and *ent*-**168**), and the ABCDE ring system (10-decarboxyquinocarcin) (**169** and *ent*-**169**) were first subjected to *in vitro* cytotoxicity assay against P388 murine leukemia cells along with **1**, **2**, **102** and their antipodes (*ent*-**1**, *ent*-**2**, and *ent*-**102**).[13]

The IC_{50} values collected are shown in **Table 3**. These results clearly disclosed that 10-decarboxyquinocarcin (**169**) and its 7-cyano congeners **167** and **168** were 10^{1-3} times more cytotoxic than the corresponding 10-carboxy compounds **1**, **102**, and **2**. It is also noteworthy that **1**, **2**, **102**, **167** - **169** bearing natural absolute configurations were found to be 10^{2-4} times more cytotoxic than the corresponding enantiomers (*ent*-**1**, *ent*-**2**, *ent*-**102**, and *ent*-**167** - *ent*-**169**) possessing unnatural absolute configurations. The N_{13}-H derivative **166** and the N_{13}-Boc derivative **165** were considerably inferior to the corresponding N_{13}-Me derivative **167**. The compounds **149**, **158**, and **159** consisting of the partial structures showed no potent cytotoxicity. The ABE ring systems (**150** and **151**) in which the E ring of **149** was replaced with six- and seven-membered rings, respectively, turned out to be one order of magnitude more cytotoxic than the

Table 3. *In vitro* cytotoxicity of various structural types of quinocarcin congeners against P388 murine leukemia cells

Compound	IC_{50} (μg/ml)[a]	Compound	IC_{50} (μg/ml) [a]
1 (quinocarcin)	3.3×10^{-2}	*ent*-**1**	3.2
2 (DX-52-1)	3.6×10^{-2}	*ent*-**2**	5.1
102	1.0×10^{-1}	*ent*-**102**	>100
149	4.5	*ent*-**149**	4.5
150	6.6×10^{-1}	*ent*-**150**	6.6×10^{-1}
151	6.8×10^{-1}	*ent*-**151**	6.8×10^{-1}
158	13	*ent*-**158**	8.4
159	1.1	*ent*-**159**	6.6
165	3.0×10^{-1}	*ent*-**165**	>4.4
166	1.5	*ent*-**166**	3.4
167	2.0×10^{-4}	*ent*-**167**	>3.6
168	8.2×10^{-4}	*ent*-**168**	>3.1
169	3.9×10^{-3}	*ent*-**169**	34

a) Concentration required for 50% inhibition of the cell growth after incubation for 96 h at 37°C (initial cell density : 1×10^4 cells/ml).

corresponding five-membered **149**. Among the compounds tested, **167** - **169** were found to be most promising. Consequently, further evaluation of the antitumor activity for these compounds was performed (see, section ***4.2.6.***).

4.2.5. Synthesis and In Vitro Cytotoxicity of Various 10-Substituted Quinocarcin Congeners

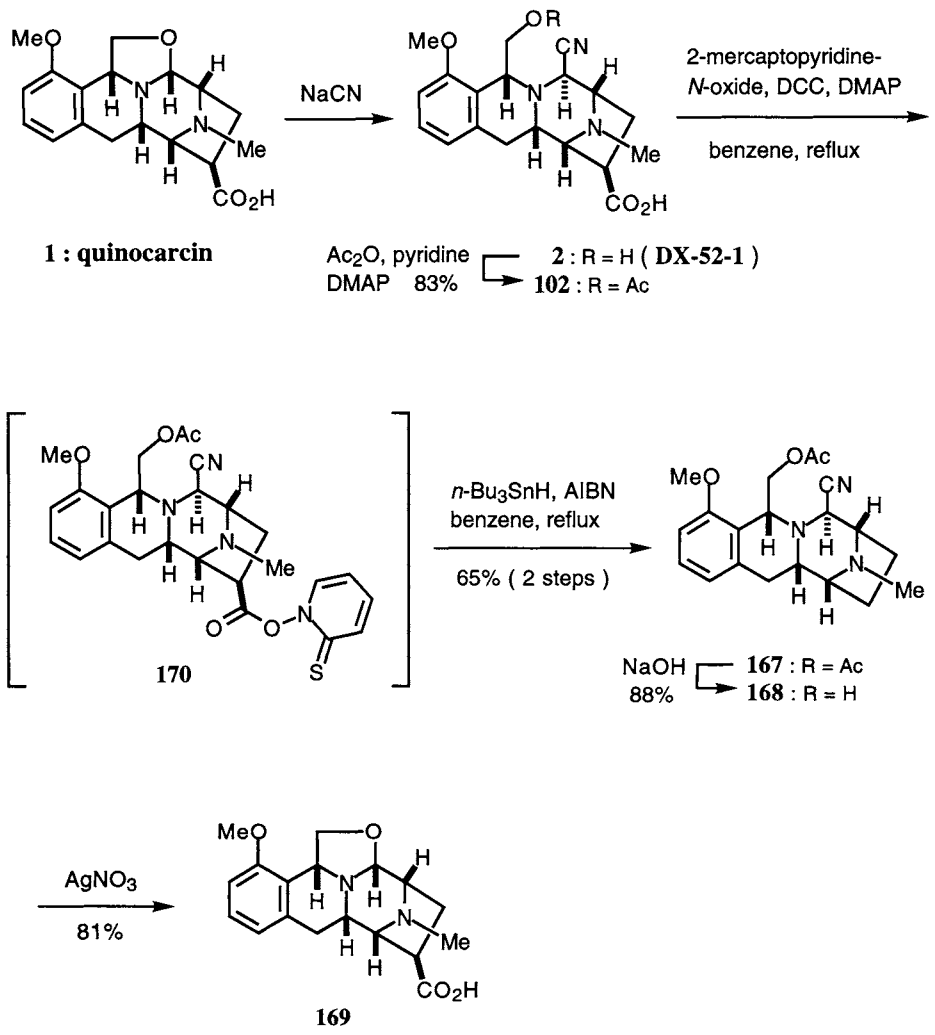
At first, in order to evaluate characteristics of *in vivo* antitumor activity of 10-decarboxyquinocarcin (**169**) and its 7-cyano congeners **167** and **168**, a novel preparation method was sought which could afford these compounds more

expeditiously than the total synthesis achieved by us. We have found that **167** - **169** can be synthesized in a straightforward manner starting with naturally occurring quinocarcin (**1**) by employing the Barton radical decarboxylation[68] as a key step. A large quantity of **1** is readily available from the culture broth of *Streptomyces melanovinaceus*.[69] As shown in **Scheme 33**, treatment of **1** with sodium cyanide according to the reported method[5] provided the amino nitrile, DX-52-1 (**2**), which was further acetylated to furnish the acetate **102** in 61 % overall yield from **1**.

Crucial decarboxylation of **102** turned out to be effected by employing the protocol of Barton.[68] Thus, **102** was initially esterified with 2-mercaptopyridine-*N*-oxide in the presence of DCC and 4-dimethylamino-pyridine (DMAP) in refluxing benzene to afford the corresponding 2-thiopyridon-1-yl ester **170**. Without isolation, **170** was immediately subject to radical decarboxylation using α,α'-azobisisobutyronitrile (AIBN) and tributyltin hydride, giving rise to the 10-decarboxy derivative **167** in 65% overall yield from **102**. Saponification of **167** followed by treatment of the resulting alcohol **168** with silver nitrate gave **169**. The compounds **167** - **169** showed identical spectral properties (IR, ^{1}H-NMR, MS) with those of authentic samples prepared in section ***4.2.3***. With large quantities of **167** - **169** in hand, investigations aiming at characterizing the *in vivo* antitumor activity of these compounds were undertaken (see, section ***4.2.6.***).

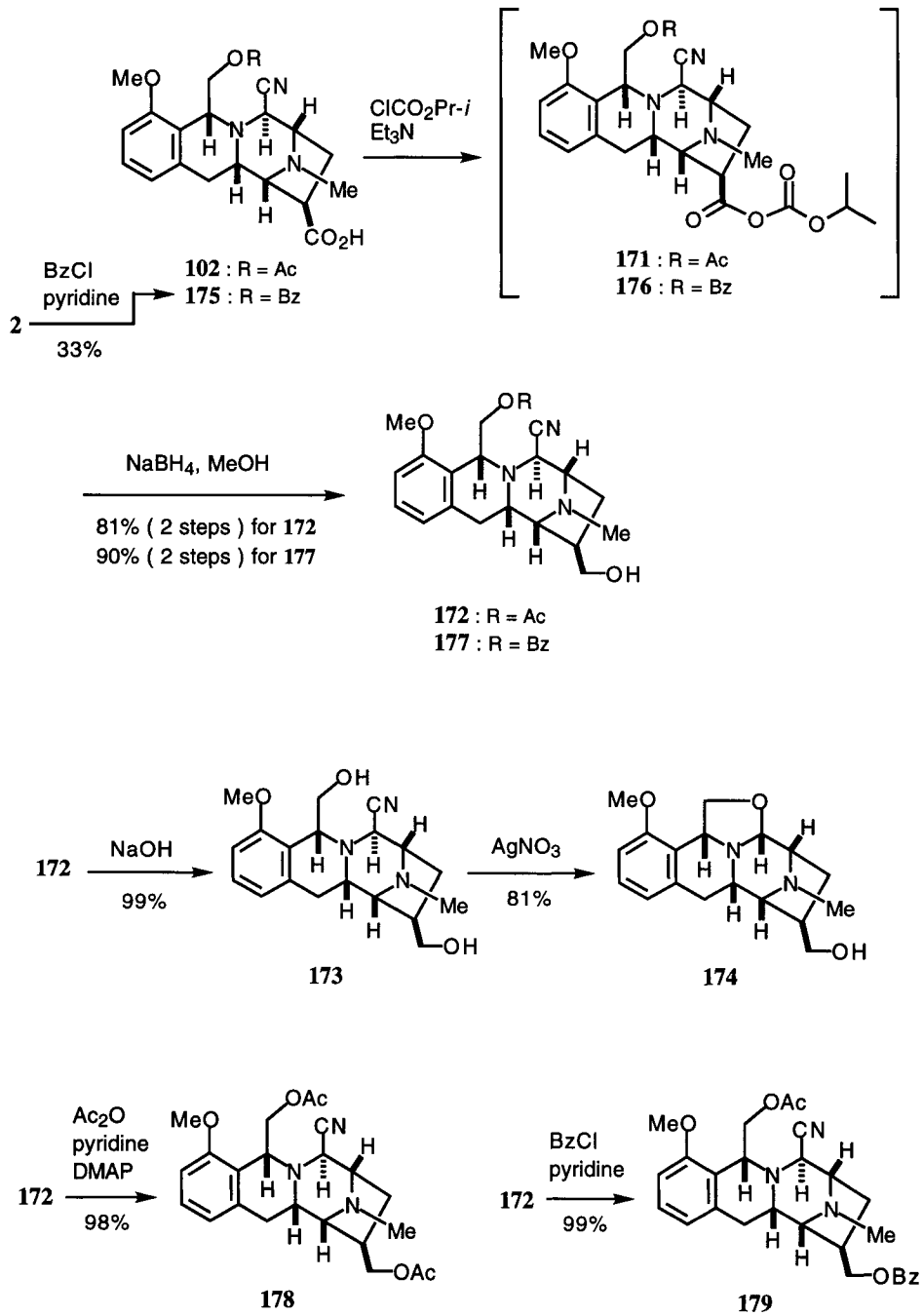
In the light of the results collected by *in vitro* cytotoxicity assay against P388 murine leukemia described in section ***4.2.4.***, it was of interest to examine the antitumor activity of the quinocarcin congeners lacking the C-10 carboxyl group. Therefore, as shown in **Scheme 34**, we next designed and synthesized quinocarcin congeners **172** - **174** and **177** - **184**, which possess various functionalities at their C-10 positions. Thus, the mixed acid anhydride **171** derived by treatment of **102** with isopropyl chloroformate in the presence of triethylamine was allowed to react with sodium borohydride, providing the alcohol **172** in 81% yield from **102**. After saponification of **172**, further treatment of the resulting diol **173** with silver nitrate gave the desired 10-hydroxymethyl derivative **174**. By employing the reaction sequence similar to that described for the preparation of the acetate **172**, the benzoate **177** was prepared starting with **2** *via* carboxylic acid **175** and mixed anhydride **176**. The 10-acetoxymethyl derivatives **178** and **180** were prepared by

Scheme 33. Synthesis of 10-decarboxyquinocarcin (**169**) from quinocarcin (**1**)

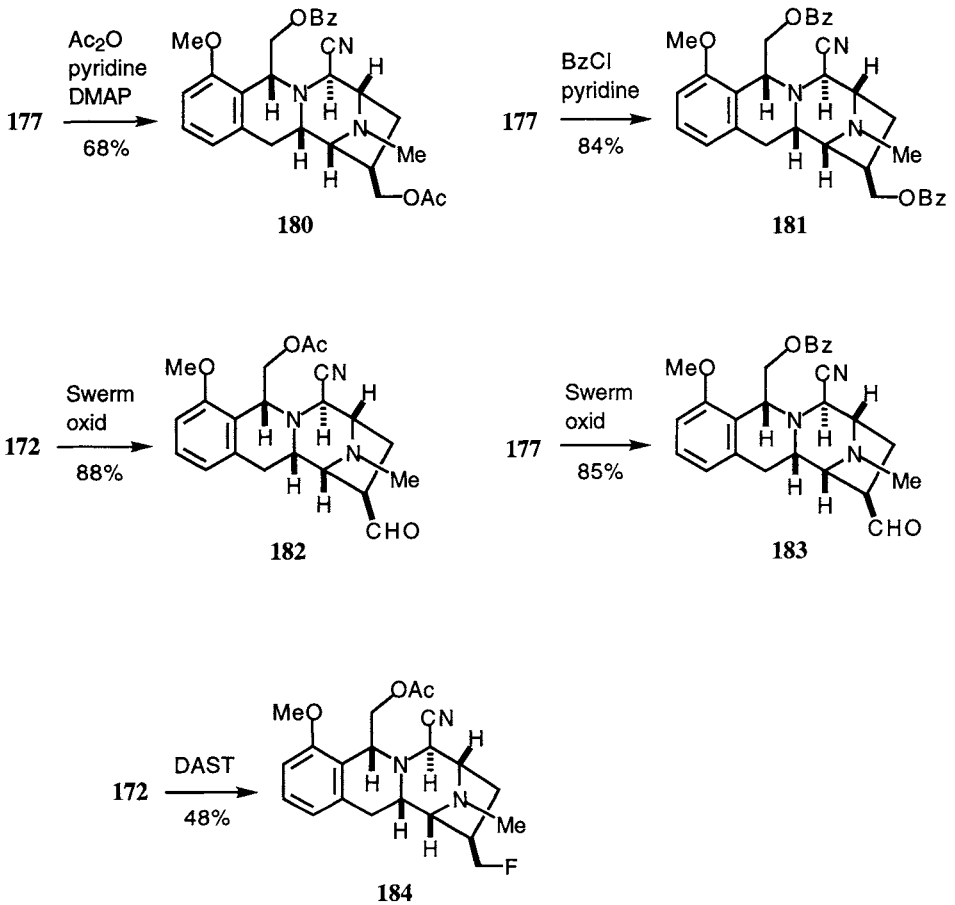


acetylation of **172** and **177**, respectively. Benzoylation of **172** and **177** cleanly provided the corresponding 10-benzoyloxymethyl derivatives **179** and **181**. The 10-formyl derivatives **182** and **183** were synthesized by Swern oxidation of **172** and **177**, respectively. Furthermore, treatment of **172** with diethylaminosulfur trifluoride (DAST) provided the 10-fluoromethyl derivative **184**.

Scheme 34. Synthesis of various 10-substituted quinocarcin derivatives **172** - **174** and **177** - **184**



Scheme 34. Synthesis of various 10-substituted quinocarcin derivatives **172** - **174** and **177** - **184** (continued)



With various 10-substituted congeners **172** - **174** and **177** - **184** in hand, the *in vitro* cytotoxicity of these compounds against P388 murine leukemia was investigated in a similar manner to that described in section ***4.2.4.*** The IC_{50} values collected are shown in **Table 4.** From these results, it was revealed that almost all of these congeners exhibit superior cytotoxicity to **1**. It is noteworthy that the cytotoxicity of **177** is 10^3 times more potent than that of **1**. Taking into account both the potent cytotoxicity and chemical stability, **174**, **177**, and **184** were further subjected to *in vivo* antitumor activity assay (see, section ***4.2.6.***).

Table 4. *In vitro* cytotoxicity of various 10-substituted quinocarcin congeners against P388 murine leukemia cells

Compound	IC_{50} (μg/ml)[a]	Compound	IC_{50} (μg/ml)[a]
1 (quinocarcin)	3.3×10^{-2}	**179**	5.6×10^{-3}
2 (DX-52-1)	3.6×10^{-2}	**180**	3.1×10^{-2}
172	3.4×10^{-3}	**181**	3.1×10^{-2}
173	3.2×10^{-3}	**182**	3.2×10^{-2}
174	7.2×10^{-3}	**183**	3.0×10^{-3}
177	1.0×10^{-5}	**184**	1.6×10^{-2}
178	1.3×10^{-3}		

a) Concentration required for 50% inhibition of the cell growth after incubation for 96 h at 37°C (initial cell density : 1×10^4 cells/ml).

4.2.6. Antitumor Activity of Various Highly Cytotoxic Quinocarcin Congeners

The antitumor activity of highly cytotoxic quinocarcin congeners **167 - 169**, **174**, **177**, and **184** primarily screened with *in vitro* cytotoxicity assay against P388 murine leukemia cells, was further evaluated by both growth inhibition against HeLa S3 cells (*in vitro*) and increase of life span (ILS) by single and five daily administrations for mice implanted with P388 murine leukemia cells (*in vivo*) in a similar manner to that described in ***4.1.2.***

The results shown in **Table 5** disclosed that **167 - 169**, **174**, and **177** exhibit *in vitro* cytotxicity superior to that of **1**. The cytotoxicity of **184** was approximately 5 times less than that of **1**. *In vivo* experiments revealed that all of the tested compounds except for **184** show appreciable antitumor activity in a single administration or in five daily administrations, while they are a little less effective than **1**. Only marginal antitumor activity was observed for **184**. These results obviously suggest that the C-10 carboxy group is not always indispensable for potent antitumor activity of quinocarcin congeners.

Table 5. *In vitro* cytotoxicity against HeLa S_3 cells and *in vivo* antitumor activity against P388 murine leukemia cells

Compound	HeLa S_3	P388 ip-ip[b]			
	IC_{50} (μg/ml)[a]	optimal dose (mg/ kg) x 1	ILS[c] (%)	optimal dose (mg/ kg) x 5	ILS[c] (%)
1	1.0×10^{-1}	25.0	33-39	6.25	67-85
167	6.4×10^{-2}	12.5	6	12.5	33
168	7.5×10^{-2}	12.5	8	12.5	29
169	5.4×10^{-2}	12.5	20	3.13	37
174	4.1×10^{-2}	12.5	17	6.25	61
177	3.6×10^{-2}	25.0	27	3.13	21
184	5.0×10^{-1}	25.0	12	12.5	13

a) Concentration required for 50% inhibition of the cell growth after incubation for 72 h at 37°C (initial cell density : 5×10^4 cells/ml).
b) $CD2F_1$ mice (5 mice/group) were implanted intraperitoneally (i.p.) with 1×10^6 cells, and a sample was dosed i.p. on day 1 and days 1-5.
c) Percent increase of life span calculated (T/C-1) x 100, where T and C are mean survival days of treated and control mice, respectively.

Summarizing the results of *in vitro* cytotoxicity and *in vivo* antitumor activity assay for various structural types of quinocarcin congeners described above, it appears evident that *(i)* all the carbon framework (the ABCDE ring system or the ABCD ring system bearing the 7-cyano group) with natural absolute configurations is indispensable for significant cytotoxicity, wherein the absolute configuration inherent in **1** might provide a key structural feature for molecular recognition by DNA, *(ii)* the N_{13}-Me group plays an important role to exhibit pronounced cytotoxicity because removal of the N_{13}-methyl group causes almost complete loss of inhibitory activity regardless of the presence of all the carbon framework with natural absolute configuration, and *(iii)* the C-10 carboxyl group is not always necessary for potent antitumor activity.

5. Conclusion

In this review the synthetic studies on quinocarcin (**1**) and its related compounds including three independent total synthesis of **1** (and one formal total synthesis of **1**) have been summarized with particular focus on their strategies. It is of interest to look at the various synthetic methodologies devised for constructing the requisite ring systems and functional groups with correct stereochemistry. Among them, our synthetic method which can provide both enantiomers of **1** as well as various structural types of quinocarcin congeners with definite absolute configurations seems to be superior to those explored by other research groups in both efficiency and flexibility. Our work described in the foregoing section including studies of the structure-activity relationships should hold promise for both investigating the mode of action of **1** and developing novel anticancer agents related to **1**.

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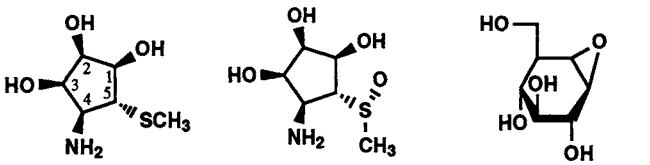
Synthesis of Mannostatins and Cyclophellitols, New Cyclitol Inhibitors for Glycoside Metabolism of Glycoproteins and Glycolipids

Yoshio Nishimura

1. INTRODUCTION

Glycoproteins and glycolipids are ubiquitous in nearly all forms of life and involved in cell-to-cell recognition, adhesion, transport, *etc.* (ref. 1) Specific inhibitors of glycosidases aid in developing an understanding of the metabolism of oligosaccharides in glycoconjugates, functional domains for carbohydrate protein interactions involved in a variety of biological functions such as immune response, oncogenesis, metastasis of tumors, viral infection, differentiation of neuronal cell and so forth. The possible applications in antimetastatic (ref. 2), antitumor (ref. 3), antiviral (ref. 4), or immunoregulatory agents (ref. 5) stimulate interest into the relationships between structures and specific biological functions (ref. 6). Glycosidase inhibitors have generally structural homology with the natural glycosides, and are traditionally polyhydroxylated six- and five-membered heterocyclic rings—often azacyclic rings (ref. 7). Two new classes of natural cyclitol inhibitors have been isolated from the culture filtrates of microorganisms with pentacarbocyclic or hexacarbocyclic rings which demonstrate powerful and specific inhibitory effects for glycoside metabolism of glycoproteins or glycolipids. Mannostatins A and B (***1***, ***2***) isolated from the culture filtrate of *Streptoverticillium verticillus* var. *quintum* ME 3-AG3 in 1989 by Aoyagi *et al.* (ref. 8), have been proved to be a potent inhibitor of the Golgi glycoprotein-processing enzyme mannosidase II (ref. 9). In cell culture, ***1*** blocked the normal processing of influenza viral glycoproteins, resulting in the accumulation of hybrid types of oligosaccharides (ref. 9). On the other hand, cyclophellitol (***3***), isolated from a culture filtrate of a mushroom of the *Phellinus* genus in 1990 by Umezawa *et al.*, has

been found to inhibit Molt-4 β-glucocerebrosidase (refs. 10a,b). A single administration of ***3*** induced severe abnormality of the nervous system known as Gaucher's disease in mice with accumulation of glucosylsphingosine in the brain, and also liver and spleen by inhibition of glucosylceramidase (refs. 10c,d). In view of their characteristic inhibitory effects and/or intriguing chemical structure, they have become attractive synthetic targets, and general and flexible approaches to their syntheses have resulted. Several analogues and derivatives have also been designed and synthesized for a mechanistic investigation and the potential therapeutic applications.



Mannostatin A (*1*) **Mannostatin B (*2*)** **Cyclophellitol (*3*)**

Fig. 1

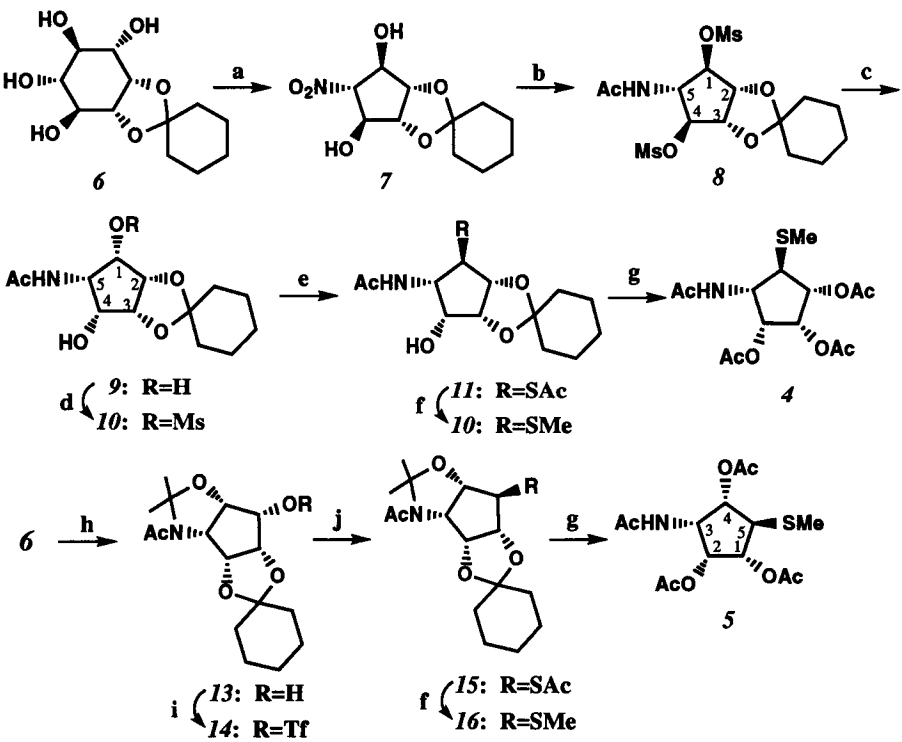
The present review discusses mainly the synthesis of mannostatins, cyclophellitol and their analogues, focusing on the progress in the stereoselective, enantioselective and chiral synthesis. This review covers the literature that appeared from 1989 to the middle of 1996.

2. SYNTHESIS OF MANNOSTATINS

The structure of mannostatin A (*1*) was first clarified to be (1,2,3,4/5)-4-amino-5-(methylthio)-1,2,3-cyclopentanetriol by nuclear magnetic resonance and mass spectrometry (ref. 11). This assignment and the absolute stereochemistry was later confirmed by X-ray crystallographic analysis of mannostatin B tetraacetate (ref. 11). Mannostatin B (*2*) also contains a *R* configurational methylsulfinyl group. The high density and juxtaposition of functionality with the five chiral centers in the cyclopentane ring present an attractive synthetic target, and four independent syntheses (refs. 12-15), an improved synthesis (ref. 16) and two syntheses of analogues (refs. 17,18) have been reported.

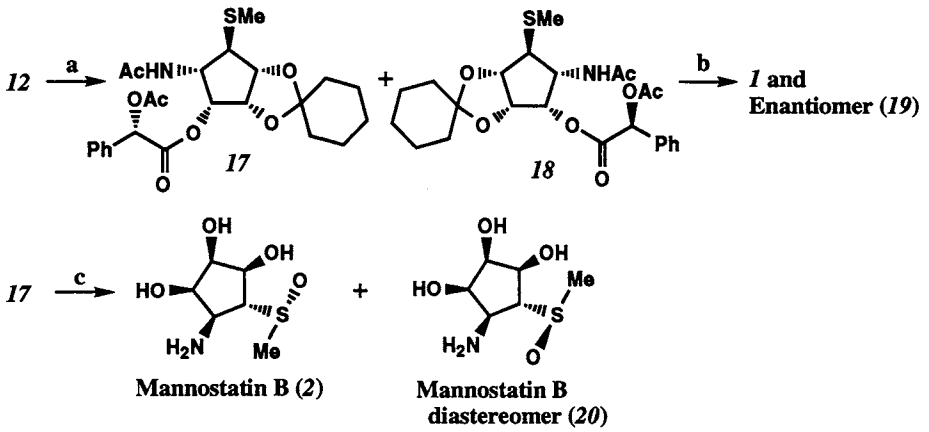
A synthesis of (±)-mannostatin A and its positional isomer as the tetraacetyl derivative (***4*** and ***5***) was first achieved from *myo*-inositol by Ogawa and Yuming

(ref. 12). The *meso* (1,4,2,3,5/0)-5-acetamido-2,3-*O*-cyclohexylidene-1,4-di-*O*-mesylcyclopentane-1,2,3,4-tetraol (***8***) prepared from (±)-1,2-*O*-cyclohexylidene-*myo*-inositol (***6***) by the method of Angyal *et al.* (ref. 19) was converted into racemic (1,2,3,4,5/0)-5-acetamido-2,3-*O*-cyclohexylidenecyclopentane-1,2,3,4-tetraol (***9***) by a procedure involving the configurational inversions at C-1 and C-4 *via* neighbouring-group participation of the 5-acetamido group developed by Suami *et al.* (ref. 20) Successive treatment of ***9*** by mono-mesylation, SN2 displacement with thioacetate, *S*-deacetylation and methylation resulted in the thiomethyl compound ***12***. Acid hydrolysis of ***12*** followed by acetylation yielded ***4***. The key intermediate ***9*** was also transformed into the acetate of DL-(1,2,3,4/5)-3-acetamido-5-methylthio-1,2,4-cyclopentanetriol (***5***) *via* the *N*,*O*-isopropylidene derivative ***13***.



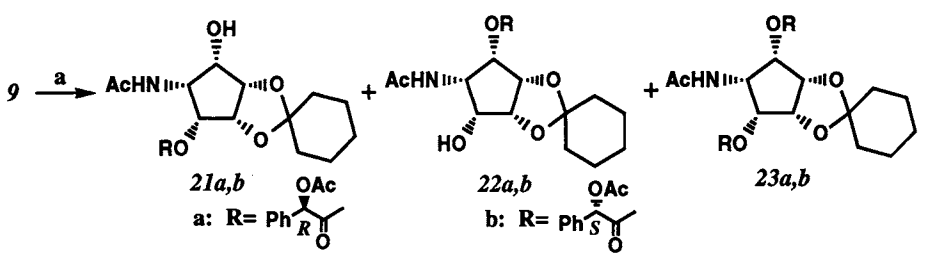
a) $NaIO_4$, 0°C; $MeNO_2$, MeONa, MeOH, 0°C (ref. 19) b) H_2, Raney Ni, EtOH; Ac_2O, pyr; sat. NH_3/MeOH; MsCl, pyr c) AcONa, aq 80% 2-methoxyethanol, reflux d) $MeSO_2Cl$, pyr e) KSAc, DMF f) MeONa, MeOH; MeI, MeOH g) aq 80% AcOH; Ac_2O, pyr h) $Me_2C(OMe)_2$, *p*-TsOH, DMF i) $(CF_3SO_2)_2O$, pyr, $ClCH_2CH_2Cl$ j) KSAc, DMF

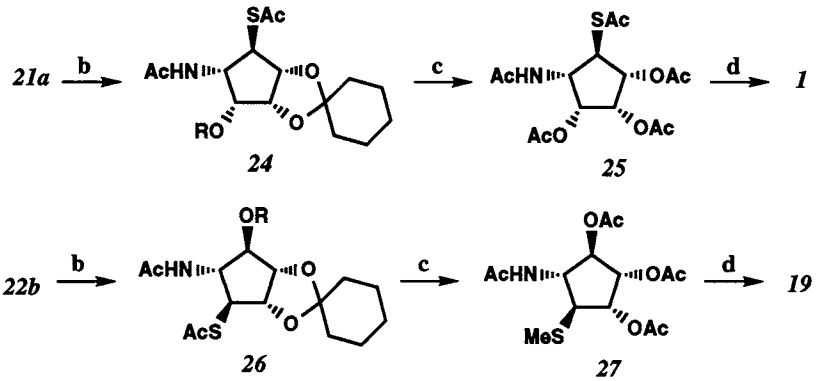
The authors (ref. 12b) later reported the optical resolution of ***12*** led to mannostatin A (***1***) and its enantiomer (***19***) using (*S*)-(+)-*O*-acetylmandelic acid. Mannostatin B (***2***) and its diastereomer (***20***) of (*S*)-sulfinyl function were also prepared by oxidation of ***17*** with sodium metaperiodate. While mannostatin A enantiomer (***19***) weakly affected jack bean α-mannosidase, mannostatin B diastereomer (***20***) of (*S*)-sulfinyl function showed potent inhibition.



a) (*S*)-*O*-acetylmandelic acid, DMAP, DCC, CH_2Cl_2 b) 1M HCl, then Dowex 50W-X2 (H^+) c) $NaIO_4$, MeOH; aq 80% AcOH/1M HCl; acetylation; aq 10% NaOH

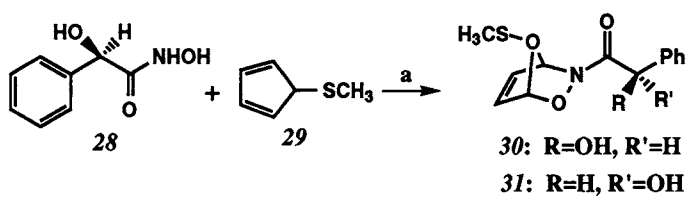
They also described an alternative approach to ***1*** and ***19*** utilizing optically active derivatives of (1,2,3,4,5/0)-5-aminocyclopentane-1,2,3,4-tetrol (***21a***, ***22b***) prepared by diastereoselective acylation of ***9*** with (*R*)-(-)- and/or (*S*)-(+)-*O*-acetylmandelic acid. Acylation of ***9*** with (*R*)-(-)- and (*S*)-(+)-*O*-acetylmandelic acid gave predominantly ***21a*** and ***22b***, respectively.





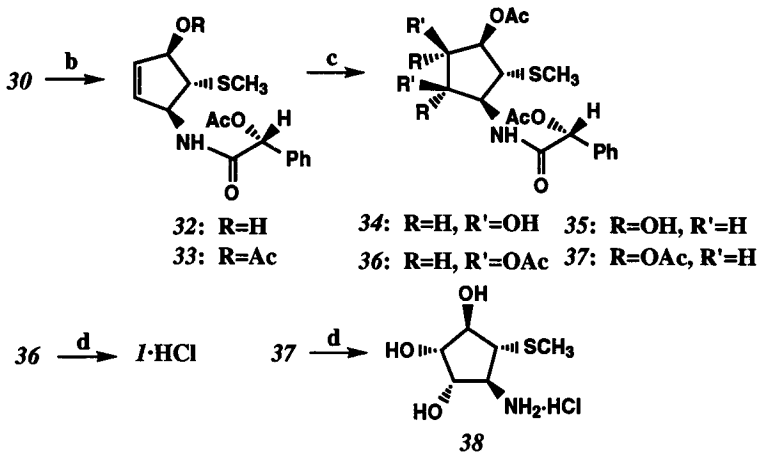
a) (*R*)-(-)-*O*-acetylmandelic acid, DMAP, DCC, CH_2Cl_2, -45°C b) Tf_2O, pyr, CH_2Cl_2; KSAc, 18-crown-6-ether c) MeONa, MeOH; MeI, MeOH; 2M HCl; acetylation d) 2M HCl, then Dowex 50W-X2 (H^+)

The first enantioselective synthesis of ***1*** by asymmetric acylnitroso cycloaddition has been reported by King and Ganem (ref. 13). Cycloaddition of (*R*)-mandelohydroxamic acid (***28***) derived from (*R*)-mandelic acid with 1-(methylthio)cyclopenta-2,4-diene (***29***)(ref. 21) in the presence of Bu_4NIO_4 afforded a 2.6:1 ratio of the adducts. Reductive cleavage of the major bicyclic adduct ***30*** using aluminum amalgam gave ***32***. Vicinal hydroxylation of the bicyclic adduct was successfully achieved by an unusual, syn-stereoselective osmylation noted in the osmylation of bis-allylically substituted cyclopentenes by Trost *et al.* (ref. 21) Thus, stoichiometric osymylation of the acetate ***33*** afforded high facial selectively triols ***34*** and ***35*** in a 20:1 ratio. The acetates ***36*** and ***37*** were easily separated by chromatography. Acid hydrolysis of the major tetraacetate ***36*** resulted in the hydrochloride of ***1***. 2,3-Di-*epi* analogue ***38*** was also synthesized at this stage.



***30*: R=OH, R'=H**

***31*: R=H, R'=OH**

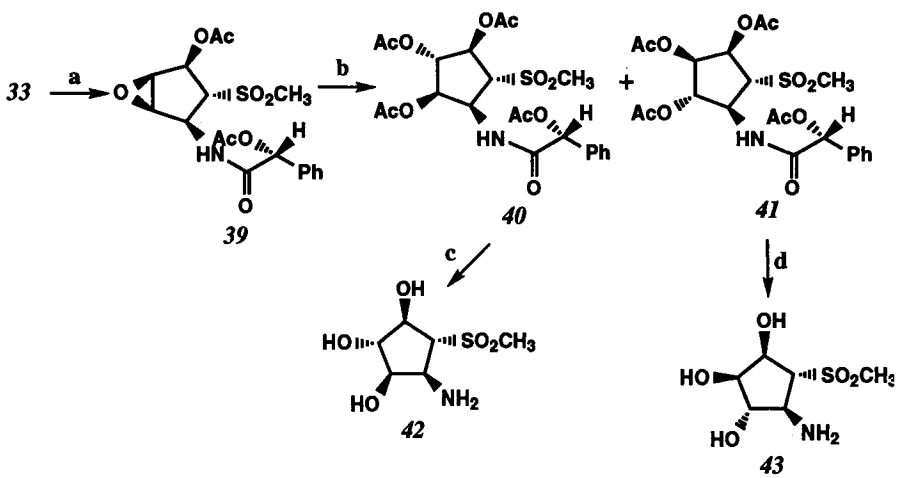


32: R=H **34**: R=H, R'=OH **35**: R=OH, R'=H
33: R=Ac **36**: R=H, R'=OAc **37**: R=OAc, R'=H

38

a) Bu_4NIO_4, MeOH, 0°C b) Al(Hg); Ac_2O, DMAP, pyr
c) OsO_4-pyr; Ac_2O, DMAP, pyr d) HCl/MeOH

The authors also synthesized 2-*epi*-mannostatin A sulfone ***42*** as well as 3-*epi* analogue ***43*** for the purpose of transforming ***1*** from a mannosidase inhibitor to a glucosidase inhibitor.

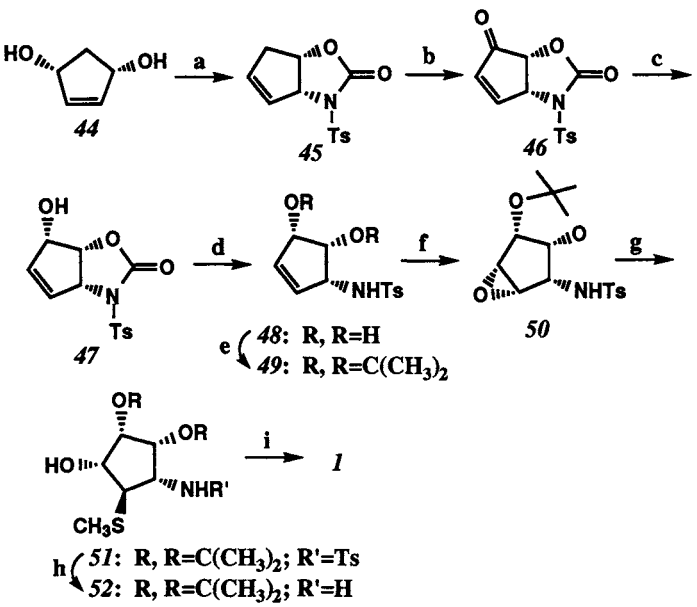


a) dimethyldioxiran, Me_2CO b) 10% TFA/H_2O; Ac_2O, DMAP, pyr. c) 6M HCl, reflux d) 0.4M HCl/MeOH, 65°C

While 2,3-di-*epi*-mannostatin A (***38***) was proved to be a competitive inhibitor of jack bean α-mannosidase (Ki=~16 μM), 2-*epi*- and 3-*epi*-mannostatin A

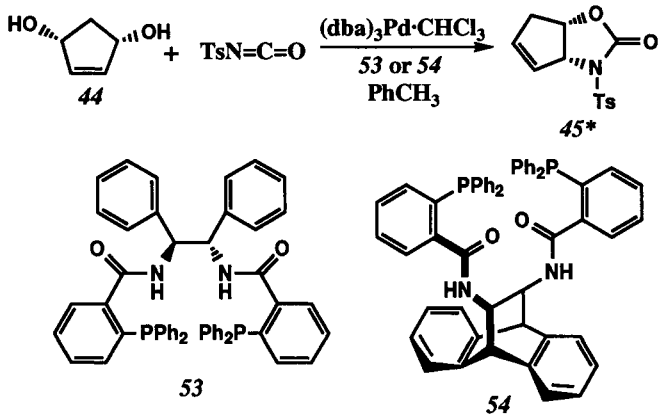
sulfones (***42***, ***43***) showed no effect on almond β-glucosidase, *Aspergillus niger* amyloglucosidase, and jack bean α-mannosidase. Clearly, the relative configuration at C-2 and C-3 of the mannostatin skeleton is the main determinant of the specificity and potency of its inhibitor.

Trost and Van Vranken (ref. 14) demonstrated a highly flexible strategy to ***1*** by controlled introduction of heteroatoms around a cyclopentane nucleus. The strategy involves the palladium-catalyzed oxazolidinone-forming reaction developed for the general synthesis of aminocyclopentitol glycosidase inhibitors. Treatment of *meso*-diol ***44*** with 2 equivalents of toluenesulfonyl isocyanate in the presence of 1.8 mol% tetrakis(triisopropyl phosphite)palladium generated from $(dba)_3Pd \cdot CHCl_3$ and $(iC_3H_7O)_3P$ *in situ* gave oxazolidin-2-one ***45***. Allylic oxidation without rearrangement of the double bond was best achieved by use of selenium dioxide in which quartz sand was added to maintain dispersion of the reactants in refluxing diglyme to afford the β-allylic alcohol admixed with ketone ***46***. The mixture was oxidized to ***46*** with the Dess-Martin periodinate (ref. 22) buffered with sodium bicarbonate. The reduction of the enone ***46*** under Luche conditions ($NaBH_4$, $CeCl_3$) (ref. 23) gave the α-allylic alcohol ***47*** and its epimer in a ratio of 7:1. The introduction of the sensitive sulfur substituent, the most difficult aspect of this synthesis, was best achieved by the regioselective opening of the epoxide of a bicyclo[3.3.0] ring system. Thus, the α-allylic alcohol ***47*** was converted into the bicyclo[3.3.0] epoxide ***50*** by hydrolysis, ketalization and epoxidation. The epoxide ***50***, upon treatment with lithium thiomethoxide, generated the desired product ***51*** along with the isomeric ring-opening product in a ratio of 4.6:1. This noticeable regioselectivity may be caused by a Fürst-Plattner effect (ref. 24) which is well established in the reactions of six-membered ring epoxides. Detosylation of ***51*** followed by hydrolysis yielded (±)-***1***.

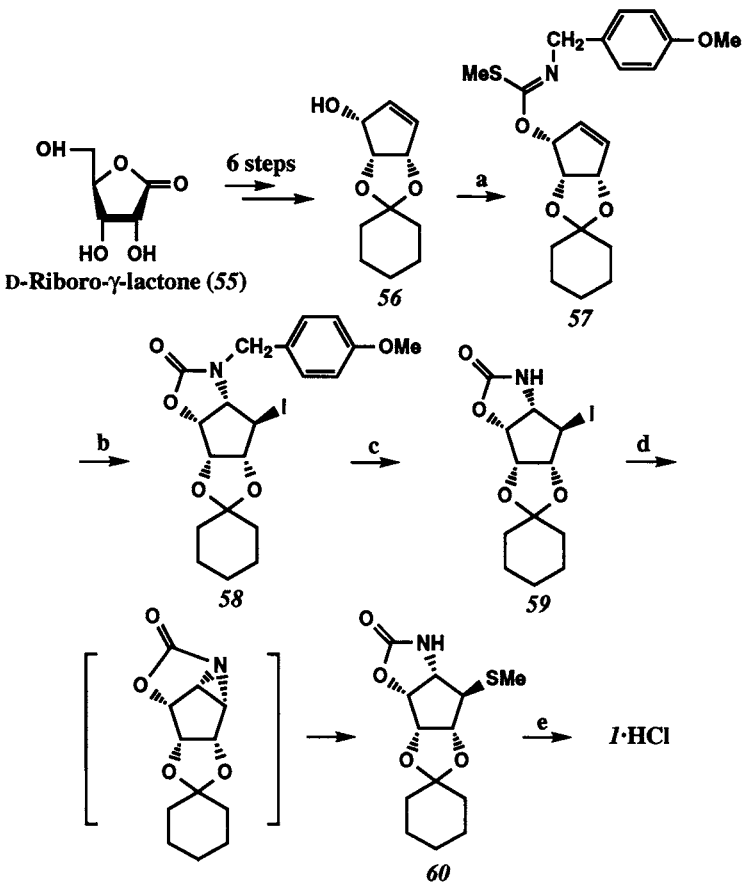


a) TsNCO (2 eq), THF, then $(dba)_3Pd_2{\cdot}CHCl_3$, $(iC_3H_7O)_3P$, reflux b) SeO_2, Na_2HPO_4, quartz sand, diglyme, 170°C; Dess-Martin periodinate, $NaHCO_3$, CH_2Cl_2, rt c) $NaBH_4$, $CeCl_3$, MeOH, EtOAc, -5°C d) K_2CO_3, MeOH/H_2O, rt e) $Me_2C(OMe)_2$, Me_2CO, CSA, rt f) CF_3CO_3H, Na_2HPO_4, CH_2Cl_2 g) MeSLi, THF, -78°C → rt h) Na, NH_3 i) 60% aq. CF_3CO_3H, 60°C

The authors (ref. 25) have also shown the asymmetric synthesis of the carbamate ***45**** by a palladium-catalyzed ionization/cyclization reaction using chiral ligands such as ***53*** and ***54*** instead of triisopropyl phosphate. Thus, the above synthetic route provides the optically active ***1***.



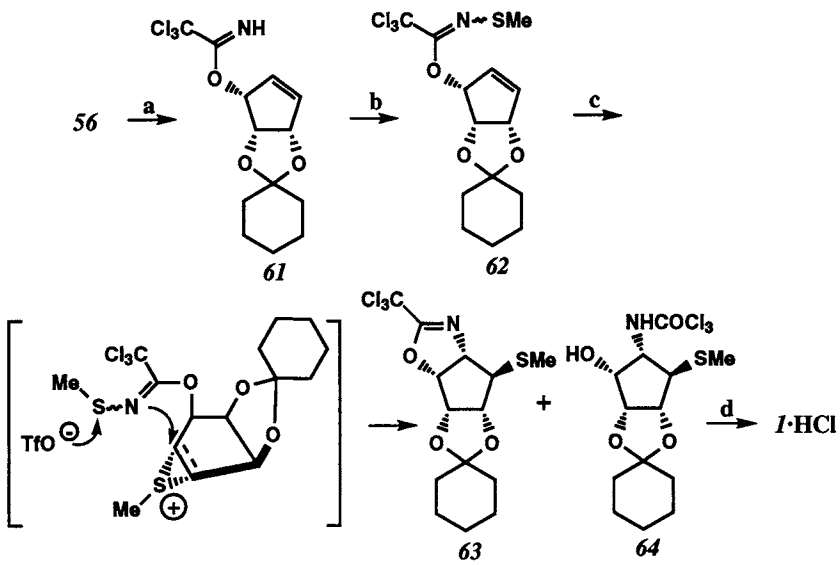
A stereocontrolled route to the individual enantiomers of ***1*** separately from D- or L-ribonolactone *via* iodocyclization of unsaturated thiocarbamidate as a key step has been described by Knapp and Dhar (ref. 15). Treatment of the sodium salt of the known cyclopentenol ***56*** prepared from D-ribono-γ-lactone (***55***)(ref. 26) with *p*-methoxybenzyl isothiocyanate followed by iodomethane afforded the key intermediate carbon-imidothioate ***57*** (ref. 27). Cyclization of ***57*** using iodine (ref. 28) gave the oxazolidinone ***58*** through a stereocontrolled trans alkene addition (refs. 27,29). Removal of the *N*-*p*-methoxybenzyl group with ceric ammonium nitrate afforded the iodide ***59***, which was converted into the methylthio derivative ***60*** upon treatment of sodium methylmercaptide *via* overall retention of configuration at C-1 by the neighbouring group participation of the oxazolidinone nitrogen (refs. 28,29). Sequential removal of the protecting groups of ***60*** by alkaline and acid hydrolysis resulted in ***1*** as its hydrochloride. The enantiomer of ***1*** was also prepared by an identical route starting with the antipode of ***56***, readily available from L-ribono-γ-lactone.



a) NaH, THF; (*p*-MeO)C_6H_4·CH_2NCS; CH_3I b) I_2, THF, MS 4A; aq. Na_2SO_3 c) CAN, aq. CH_3CN d) $NaSCH_3$, DMF e) 2M KOH, reflux; 6M HCl

Li and Fuchs (ref. 16) have achieved a short stereospecific route to ***1*** in optically active form *via* methanesulfenyl triflate-mediated intramolecular cyclization of allylic *N*-sulfenylimidate ***62***. The synthesis of the key intermediate ***62*** began with the same allylic alcohol ***56*** used in the synthesis of Knap and Dhar (ref. 15). The allylic alcohol ***56*** was transformed into the allylic trichloroacetimidate ***61*** with trichloroacetonitrile by the method of Overman (ref. 30). Imino-sulfenylation of the allylic trichloroacetimidate ***61*** was best achieved by treatment with 3.4 equivalents of *N,N*-diisopropylethylamine and 3.3 equivalents of CH_3SOTf in three equal portions to afford the allylic *N*-sulfenylimidate ***62*** in an excellent yield.

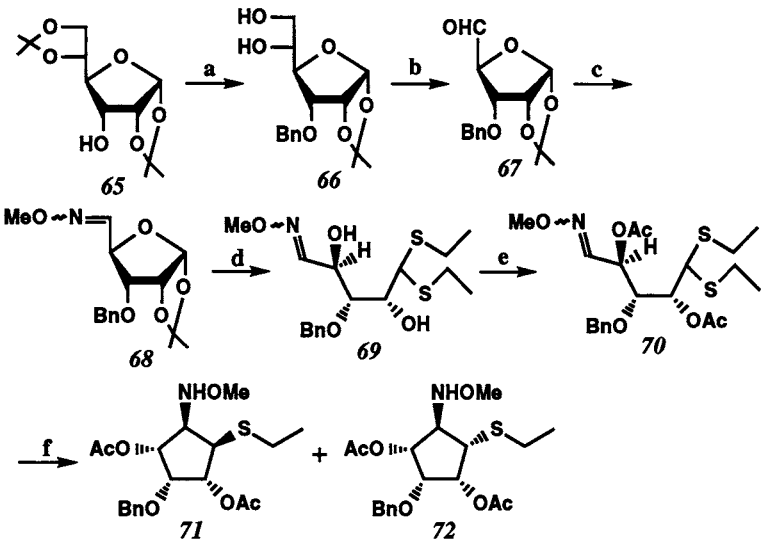
Subsequent treatment of ***62*** with 3.5 equivalents of CH_3SOTf and 1.5 equivalents of *N,N*-diisopropylethylamine in dichloromethane gave the oxazoline ***63*** along with the trichloroacetamide derivative ***64*** by hydrolysis of ***63*** during the aqueous workup or column chromatography. Hydrolysis of ***63*** and ***64*** with acidic methanol resulted in ***1*** as its hydrochloride. Thus, mannostatin A (***1***) has been efficiently synthesized in ten steps from D-ribono-γ-lactone (***55***) (~39% overall yield).



a) NaH, Cl_3CCN, CH_2Cl_2 b) 3.4 eq. *i*-Pr_2NEt, 3.3 eq. CH_3SOTf, CH_2Cl_2, -72 to 0°C c) 1.5 eq. *i*-Pr_2NEt, 3.5 eq. CH_3SOTf, CH_2Cl_2, -72 to 20°C d) 7M HCl/MeOH (50:50)

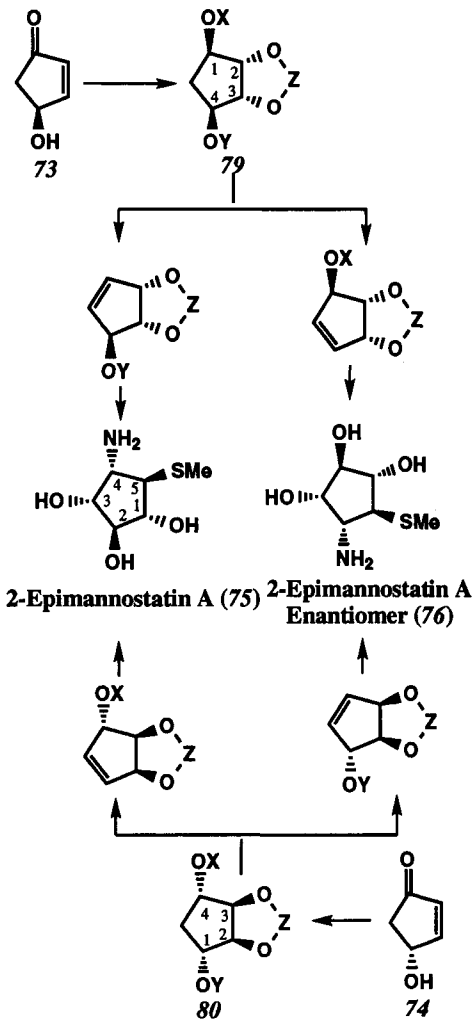
An intramolecular free radical cyclization of a radical derived from a dithioacetal onto an oxime in the synthesis of carbocyclic compounds (ref. 31) has been applied to the synthesis of mannostatin A (***1***) analogues by Ingall *et al.* (ref. 17). Sodium metaperiodate oxidation of 3-*O*-benzyl-1,2-*O*-isopropylidene-D-allofuranose (***66***) prepared from D-allose afforded the aldehyde ***67***, which was converted into the oxime ***68*** upon treatment with *O*-methyl hydroxylamine hydrochloride. Thioacetal formation of ***68*** with ethanethiol in the presence of anhydrous zinc chloride gave the dithioacetal ***69*** which was proved ineffective for radical cyclization. On the other hand, the radicals derived from the peracetylated

dithioacetal ***70*** can be effectively cyclized in a 5-*exo* fashion onto *O*-protected oximes to form mannostatin analogues ***71*** and ***72*** in high yield.



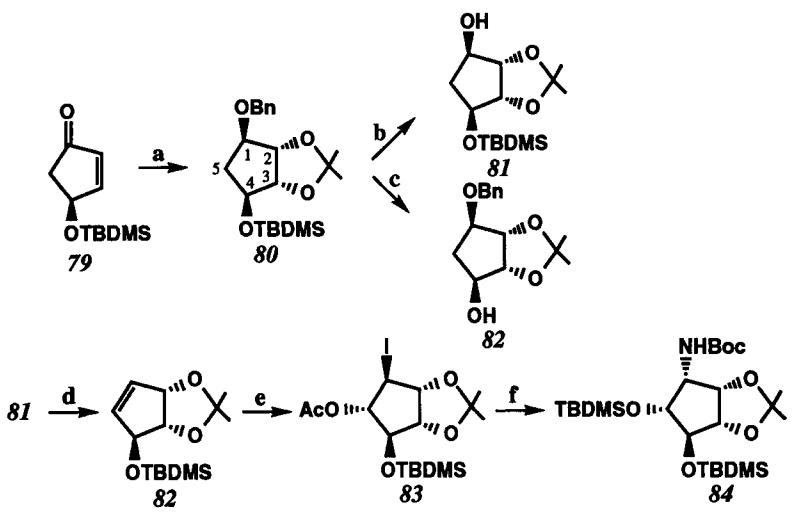
a) $PhCH_2Br$, NaH, THF; HCl, MeOH b) $NaIO_4$, H_2O/MeOH c) $MeONH_2$·HCl, pyr., CH_2Cl_2 d) EtSH, $ZnCl_2$ e) Ac_2O, DMAP, pyr. f) 6 eq. Bu_3SnH, 2.5 eq. AIBN, $PhCH_3$, 110°C

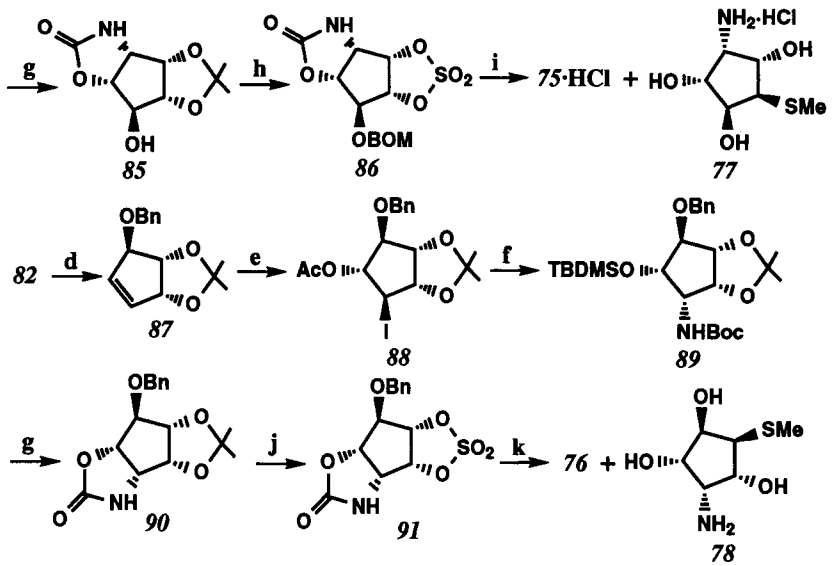
Nishimura *et al.* (ref. 18) have introduced the highly flexible enantiodivergent strategy in a totally stereospecific fashion in their synthesis of mannostatin analogues utilizing both (*S*)-4-hydroxy-2-cyclopentenone (***73***) and its 4*R*-isomer ***74*** as outlined in Scheme 11. 2-*Epi*-mannostatin A (***75***) and its enantiomer (***76***) as well as their positional isomers (***77***, ***78***) were designed for probing structure-activity relationships in this class of glycosidase inhibitors.



This approach to the analogues involves, as the key steps, the regio- and stereoselective introduction of three different heteroatom functions on each carbon of the same *meso*-intermediates, ***79*** and ***80***, by differentiation of these positions. An enantiodivergent route to the compounds (***75***, ***76***, ***77***, ***78***) was demonstrated starting from (*S*)-4-((*tert*-butyldimethylsilyl)oxy)-2-cyclopentenone (***79***). Compound ***79*** was effectively transformed into the pivotal intermediate, mesotetrol ***80***, by a sequence of reduction with diisobutylaluminum hydride, Woodward reaction (ref. 32), methanolysis and protection with an isopropylidene group. Selective

removal of the protecting groups in ***80*** by either catalytic hydrogenolysis with Pd/C or treatment with tetrabutylammonium fluoride provided 1-hydroxy derivative ***81*** and 4-hydroxy derivative ***82***. Elimination of the hydroxy group of ***81*** was achieved by treatment with triphenylphosphine and diethyl azodicarboxylate to give ***82***. The amino-hydroxylation of ***82*** was successfully carried out by a silver acetate-iodine reaction to ***83***, displacement of the iodo group of the appropriately protected derivative with an azido group, and hydrogenolysis. The last issue, the introduction of the methylthio group, was best achieved by nucleophilic substitution of the cyclic sulfate function of the fused tricyclic system with sodium thiomercaptide. The key compound ***86*** was obtained by removal of the *O-tert*-butyldimethylsilyl group, cyclic carbamate formation, protection of the hydroxy group, acid hydrolysis, and cyclic sulfate formation (ref. 33) by treatment with $SOCl_2$ and pyridine followed by oxidation with $RuCl_3$ and $NaIO_4$ in CCl_4-CH_3CN-H_2O. Treatment of ***86*** with sodium thiomercaptide and removal of the protecting groups resulted in 2-*epi*-mannostatin hydrochloride ***75*** and its positional isomer (***77***). Their enantiomers ***76*** and ***78*** were also synthesized from ***82*** by a similar sequence of reactions.





a) DIBALH, PhH; BnBr, NaH, Bu_4NI, THF; AgOAc, I_2, wet AcOH; MeONa, MeOH; $Me_2C(OMe)_2$, D-camphor-10-sulfonic acid, CH_2Cl_2 b) H_2, Pd/C, MeOH c) Bu_4NF, THF d) Ph_3P, DEAD, PhH e) AgOAc, I_2, PhH f) NH_3/MeOH; TBDMSCl, imidazole, DMF; NaN_3, DMSO; H_2, Pd/C, AcOEt-MeOH; (t-$BuOCO)_2O$, i-Pr_2NEt, CH_2Cl_2 g) Bu_4NF, THF; NaH, DMF h) BOMCl, i-Pr_2NEt, DMF; aq. AcOH; $SOCl_2$, py, CH_2Cl_2; $RuCl_3$, $NaIO_4$, CCl_4-CH_3CN-H_2O i) NaSMe, DMF; H_2SO_4, H_2O-THF; $Ba(CH)_2$, H_2O; HCl/dioxane j) aq. AcOH; $SOCl_2$, py, CH_2Cl_2; $RuCCl_3$, $NaIO_4$, CCl_4-CH_3CN-H_2O h) NaSMe, DMF; H_2SO_4, H_2O-THF; Me_3SiI, CH_2Cl_2; $Ba(OH)_2$, H_2O

In contrast with α-mannosidase inhibitor ***1***, the antipode ***76*** among the analogues ***75***, ***76***, ***77*** and ***78*** affected almond β-glucosidase (IC_{50} 8 μg/mL). Molecular modeling (PM3/MOPAC) revealed that ***76*** superimposes well on the chair form of β-D-glucopyranose and has its three hydroxy groups lying in the same region of space as C2, C3 and C4 of the glucose to mimic glucopyranoside in ground-state binding to β-glucosidase.

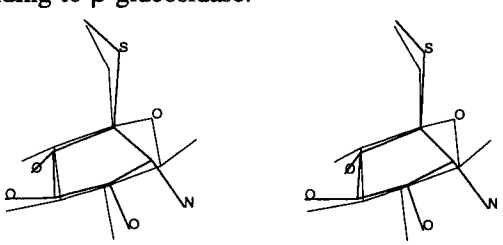
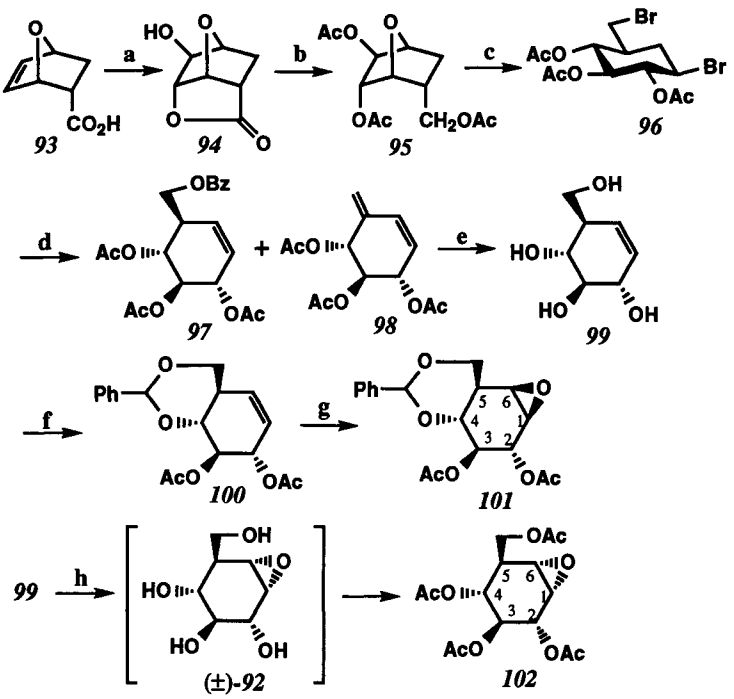


Fig. 2. Superimposition of 2-Epimannostatin A Enantiomer (*76*) and β-D-Glucopyranose, Hydrogen Atoms are Omitted for Clarity

3. SYNTHESIS OF CYCLOPHELLITOLS

The structure of cyclophellitol (***3***) was determined to be (1*S*,2*R*,-3*S*,4*R*,5*R*,6*R*)-5-hydroxymethyl-7-oxabicyclo[4.1.0]heptane-2,3,4-triol by spectroscopic and crystallographic analysis (ref. 10a). Cyclophellitol (***3***) has the fully oxygenated carbocyclic analogue of D-glucopyranose with an epoxide ring on the β-face of the molecule. The natural product currently attracts additional synthetic interest in that its diastereomeric oxirane has been proved to be a potential therapeutic agent against tumor metastasis (ref. 2e). Most significantly, it should be noted that protected derivatives of (±)-cyclophellitol (***3***) and (±)-1,6-*epi*-cyclophellitol (***92***) were prepared by Ogawa *et al.* (ref. 34) in the course of their synthesis of valienamine seven years earlier than the discovery of ***3*** from nature. Several syntheses forcused on ***3*** and its diastereomeric oxirane, *epi*-cyclophellitol (***92***), have been reported commencing with sugars (ref. 35-38), cyclitols (refs. 39,40), and racemic and nonracemic Diels-Alder adduct (refs. 41-43). The epimer of ***3*** has also been synthesized starting from a cycloaddition adduct (ref. 44).

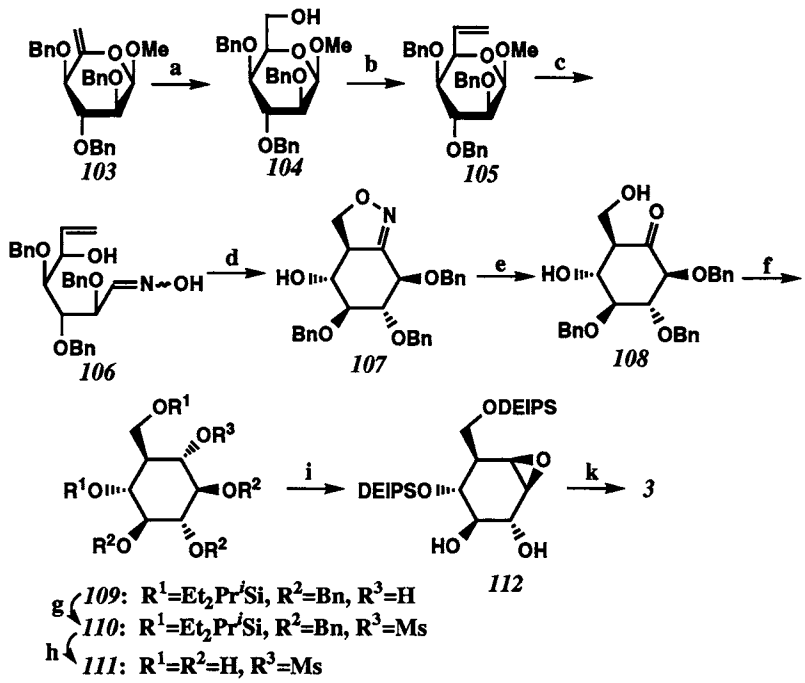
Ogawa *et al.* (ref. 34) prepared racemic 2,3-di-*O*-acetyl-4,5-*O*-benzylidenecyclophellitol ***101*** and 2,3,4,5-*tetra*-*O*-acetyl-1,6-*epi*-cyclophellitol ***102*** in the synthesis of valienamine from the Diels-Alder adduct of furan to acrylic acid. Oxidation of the readily available 7 *endo*-oxabicyclo[2.2.1]heptane-2-carboxylic acid (***93***)(ref. 44) with hydrogen peroxide followed by reduction with lithium aluminum hydride, and then acetylation gave *exo*-3-acetate ***95***. The cleavage of the anhydro ring with hydrogen bromide afforded a single crystalline bromide ***96***, which furnished the elimination product ***97*** together with ***98*** upon treatment with sodium benzoate. Removal of the protecting groups of ***97*** yielded the tetrol ***99***, which was converted into the benzylidene-diacetate ***100*** by acetal formation and acetylation. Oxidation of ***100*** with mCPBA resulted in the protected (±)-cyclophellitol ***101***. On the other hand, the protected (±)-1,6-*epi*-cyclophellitol ***102*** was obtained upon oxidation of ***99*** with mCPBA.



a) 30% H_2O_2, HCO_2H b) $LiAlH_4$; THF; Ac_2O, pyr. c) 15% HBr/AcOH, 75°C in a sealed tube d) NaOBz, DMF, 110-115°C e) NaOMe, MeOH f) PhCH(OMe), *p*-TsOH, DMF; Ac_2O, pyr. g) mCPBA, phosphate buffer (pH 8), CH_2Cl_2 h) mCPBA, AcOH, then Ac_2O, pyr.

The synthesis of the free cyclophellitol (***3***) was first achieved by Tatsuta and co-workers (ref. 35) from L-glucose *via* an intramolecular [3+2]-cycloaddition of a nitrile oxide derived from an oxime to alkene as a key step. Stereoselective hydroboration of *xylo*-hex-5-enopyranoside ***103*** derived from L-glucose by Sepulchre's method (ref. 45) afforded the D-idopyranoside ***104***, which was transformed into the key intermediate, oxime-olefin ***106*** by the subsequent sequence of oxidation, Wittig reaction, hydrolysis to an idopyranose and oxime formation with $NH_2OH \cdot HCl$. Stereospecific intramolecular cycloaddition of ***106*** was successfully achieved by utilizing NaOCl through the intermediary nitrile oxide to give the isoxazoline (***107***). The isoxazoline opening of ***107*** by hydrogenolysis followed by silylation gave the ketone ***108***, which was converted into the α-alcohol ***109*** upon reduction with $BH_3 \cdot Me_2S$. Mesylation of ***98***, removal of the *O*-benzyl

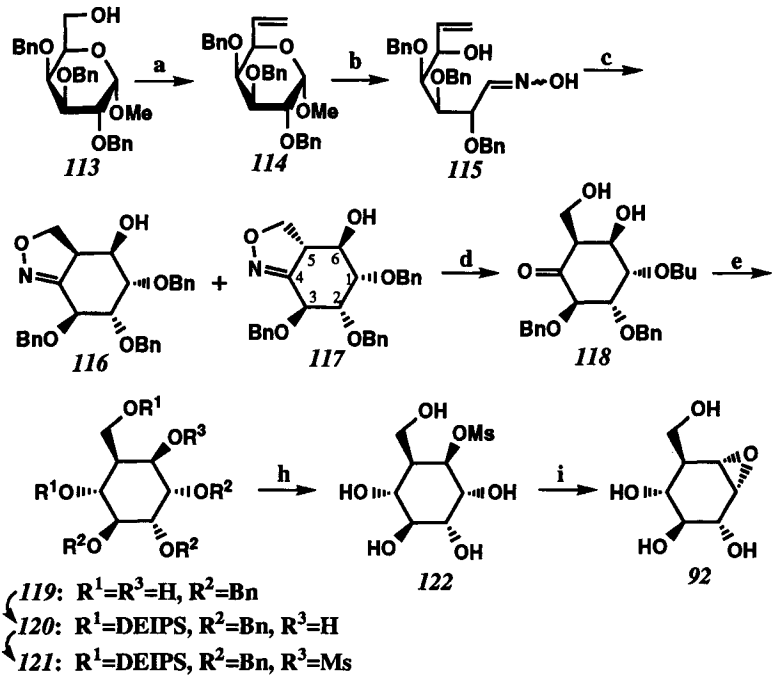
groups and the subsequent epoxidation furnished the labile epoxide ***112***. Removal of the protecting groups of ***112*** resulted in ***3***.



a) dicyclohexylborane, THF; H_2O_2/NaOH b) $(COCl_2)_2$, DMSO, Et_3N, CH_2Cl_2; $Ph_3P{=}CH_2$, PhH c) HCl/aq. dioxane; $NH_2OH{\cdot}HCl$, py d) NaOCl, CH_2Cl_2 e) H_2/Raney Ni, AcOH-aq. dioxane f) DEIPS-OTf, 2,6-lutidine, CH_2Cl_2; $BH_3{\cdot}Me_2S$, THF g) MsCl, py h) H_2/$Pd(OH)_2$, MeOH i) MeONa, $CHCl_3$ k) *n*-Bu_4NF, THF

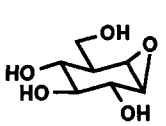
The authors (ref. 35b) also prepared the diastereomeric epoxide, 1,6-*epi*-cyclophellitol (***92***) starting from D-galactose by use of a similar intramolecular cycloaddition of a nitrile oxide for purposes of biological evaluation. The key oxime ***115*** derived from methyl 2,3,4-tri-*O*-benzyl-α-D-galactopyranoside (***113***) was subjected to the intramolecular cycloaddition utilizing NaOCl in toluene at 100°C to afford the desired isoxazoline ***116*** and its epimer ***117*** in a ratio of 1:5. Acidic hydrogenolysis of both ***116*** and ***117*** resulted in the desired keto-alcohol ***118***. Hydrogenolysis of the undesired isoxazoline ***117*** was favorably carried out *via*

epimerization at the C-5 position by the keto-enol tautomerization. The keto-alcohol ***118*** was converted into the crystalline 1,6-*epi*-cyclophellitol ***92***.

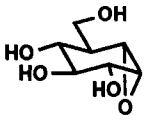


a) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2; $Ph_3P{=}CH_2$, PhH b) Ac_2O, cat. H_2SO_4; MeONa, MeOH; $NH_2OH{\cdot}HCl$, py c) 5% aq. NaOCl, $PhCH_3$, 100°C d) H_2/Raney Ni, dioxane-aq. AcOH e) $BH_3{\cdot}Me_2S$, THF f) DEIPSCl, imidazole, DMF g) MsCl, py h) $H_2/Pd(OH)_2$, MeOH i) MeONa, MeOH

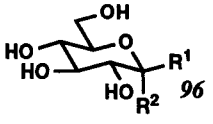
In contrast with the characteristic β-glucosidase inhibitor ***3***, ***92*** affected both α- and β-glucosidases (ref. 2e). However, inhibition against almond β-glucosidase by ***102*** (IC_{50} 300 μM) was much weaker than that by ***3*** (IC_{50} 1.4 μM)(ref. 2e). Cyclophellitol (***3***) has a similar configuration of the C1-O bond to β-gluco-pyranoside (***123***) while 1,6-*epi*-cyclophellitol (***92***) has a similar configuration of the C1-O bond to α-glucopyranoside (***124***). These results emphasized that α- and β-glucosidases recognize specifically the C1-O configuration of glucopyranoside for enzyme-substrate interaction.



Cyclophellitol (*3*)



1,6-*epi*-Cyclophellitol (*92*)



β-Glucopyranoside (*123*): R^1=OH, R^2=H

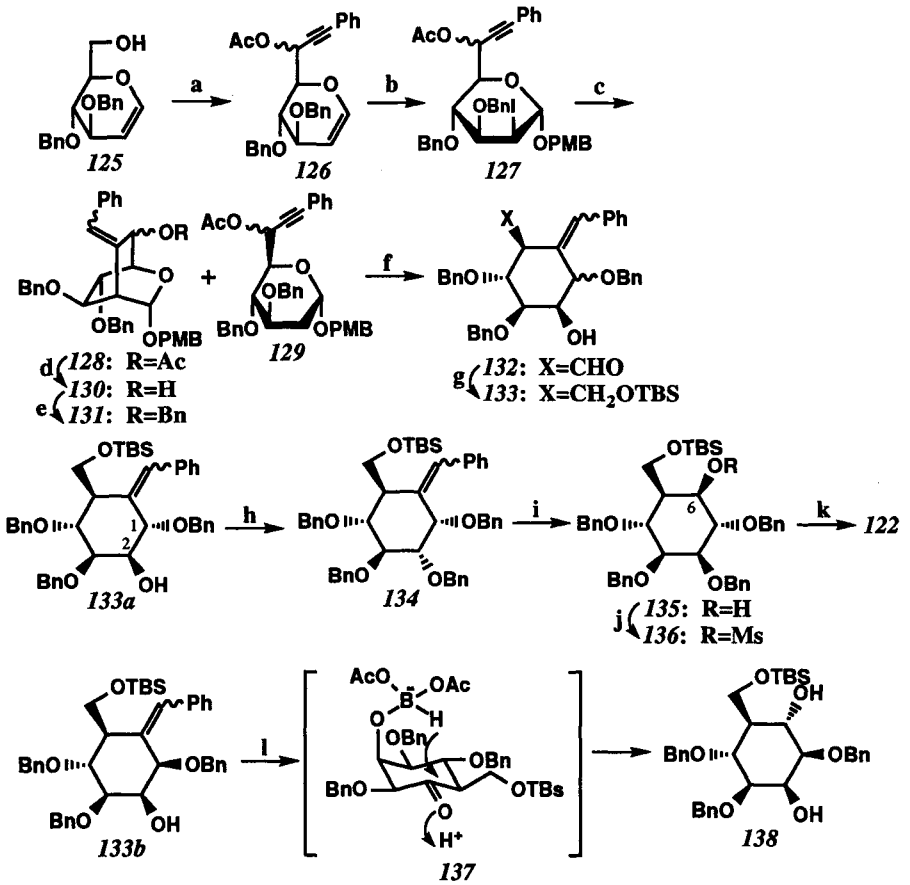
α-Glucopyranoside (*124*): R^1=H, R^2=OH

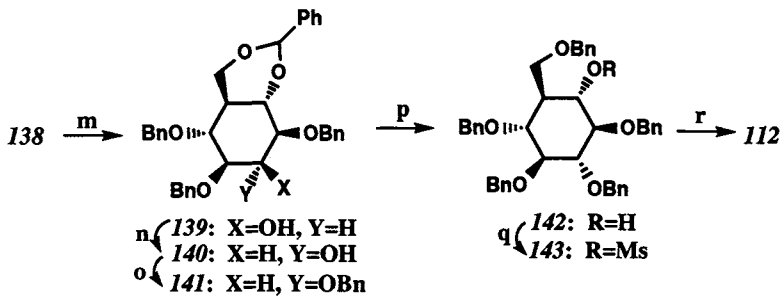
Fig. 3

Umezawa and co-workers (ref. 2e) have found that 1,6-*epi*-cyclophellitol (***92***) also inhibited α-glucosidase in cultured B16/F10 cell lysate. Compound ***92*** was inhibitory against B16/F10 cell invasion through reconstituted basement membranes at the doses effective in inhibiting α-glucosidase *in situ*. Pulmonary colonization after intravenous transplantation of B16/F10 cells into the tail veins of mice was significantly suppressed dose-dependently by *in vitro* pretreatment with ***92***. It is likely that ***92*** suppresses metastasis of tumor cells by inhibition of glucosidase I and/or II, *N*-linked oligosaccharide-processing enzyme and/or by glycolipid metabolism to disrupt the balance of the oligosaccharide organization.

McDevitt and Fraser-Reid (ref. 36) have reported a divergent route to ***3*** and ***92*** from a [2.2.2]oxabicyclic glycoside *via* a 6-*exo-trig* radical cyclization of 2-deoxy-2-iodo-6-alkynyl glycoside prepared from D-glucal. The glucal ***125*** was transformed upon oxidation, direct alkynylation and then acetylation into the epimeric mixture ***126***, which was subjected to iodoalkylation by Thiem's procedure (ref. 46) to the 2-deoxy-2-iodo-6-alkynyl glycoside ***127***. Radical cyclization of ***127*** with Bu_3SnH and AIBN in benzene at reflux gave the diastereomeric [2.2.2]oxabicycloglycosides ***128*** and the 2-deoxy epimers ***129*** in 4:1 ratio. The benzyl ether of ***128*** was converted into the mono-alcohol ***133*** by oxidative hydrolysis with DDQ to the hydroxy aldehyde ***132***, immediate reduction so as to avoid epimerization and selective silylation. The thus obtained diastereomeric mixture was divided into two sets, ***133a*** and ***133b***, related to the intermediates ***122*** and ***112*** in the synthesis of *epi*-cyclophellitol (***92***) and cyclophellitol (***3***), respectively, by Tatsuta *et al.* (ref. 35) The C2-OH inversion of ***133a*** was best achieved by a sequence of Dess-Martin oxidation and $NaBH_4$ reduction to give ***134***

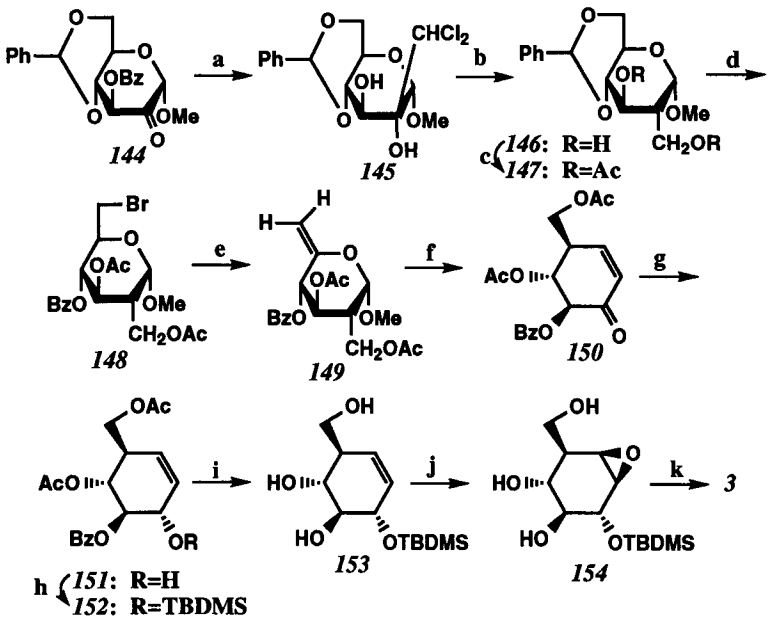
after benzylation. The benzyl ether gave the desired mono-alcohol ***135*** and its 6-*epi* derivative in a ratio of 1:1 upon ozonolysis and $BH_3 \cdot Me_2S$ reduction. Mesylation of ***135*** followed by hydrogenolysis afforded the intermediate ***122*** for *epi*-cyclophellitol (***92***). On the other hand, ozonolysis of ***133b*** followed by stereospecific reduction with sodium triacetoxyborohydride *via* the complex ***137*** gave the diol ***138*** as the sole product. Dess-Martin oxidation of the conformational rigid benzylidene derivative ***139***, $BH_3 \cdot MeS$ reduction and benzylation then afforded ***141***. Chemoselective cleavage of the benzylidene ring in ***141*** by Garegg's procedure (ref. 47) gave the desired mono-alcohol ***142***, which was transformed upon mesylation and hydrogenolysis into the other intermediate ***112*** for cyclophellitol (***3***).





a) $(COCl)_2$, DMSO, THF; BuLi, ≡—Ph , THF; Ac_2O b) NIS, PMBOH, CH_3CN c) AIBN, nBu_3SnH, PhH, reflux d) deacetylation e) BnBr f) DDQ, CH_2Cl_2/H_2O g) reduction, then silylation h) Dess Martin oxidation; NaBH4; BnBr i) O_3, PPh_3; $BH_3{\cdot}Me_2S$ j) MsCl k) H_2, Pd-C/$Pd(OH)_2$ l) O_3, PPh_3; $NaB(OAc)_3H$ m) 1M HCl; $PhCH(OMe)_2$ n) Dess Martin oxidation; $BH_3{\cdot}Me_2S$ o) BnBr p) $NaCNBH_3$; HCl q) MsCl r) H_2, Pd-C

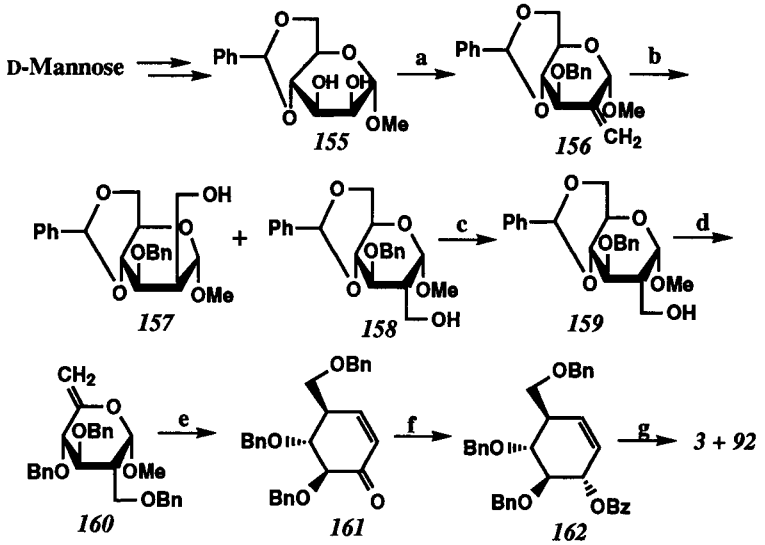
A branched-chain 6-deoxyhex-5-enopyranoside prepared from D-glucose was involved as a key intermediate in the synthesis of ***3*** by Sato *et al.* (ref. 37) Treatment of methyl 3-*O*-benzoyl-4,6-*O*-benzylidene-α-D-*arabino*-hexo–pyranoside-2-ulose (***144***) with dichloromethyllithium gave stereoselectively methyl 4,6-*O*-benzylidene-2-*C*-dichloromethyl-α-D-glucopyranoside (***145***). Hydride reduction of ***145*** followed by acetylation afforded 2-*C*-acetoxymethyl-2-deoxy-α-D-glucopyranoside ***147***, which was converted into 2-*C*-acetoxymethyl-2-deoxy-α-D-*xylo*-hex-5-enopyranoside ***149*** upon oxidative ring opening of the benzylidene acetal with NBS followed by displacement of bromine with iodine, and treatment with DBU. Ferrier reaction (ref. 48) of ***149*** with $HgCl_2$ and subsequent elimination of its mesilate gave the key 5-cyclohexen-1-one ***150***. Luche reduction (ref. 23) of ***150*** resulted stereoselectively in 5-cyclohexene-1,2,3-triol ***151***. Stereoselective epoxidation was best achieved by treatment of 1D-(1,3/2,4)-1-*O*-*t*-butyldimethylsilyl-4-hydroxymethyl-5-cyclohexene-1,2,3-triol ***153*** with mCPBA to give the desired single epoxide ***154***. Acid hydrolysis of ***154*** yielded ***3***.



a) LDA, CH_2Cl_2/THF, -78°C b) $NaBH_4$, DMSO c) Ac_2O, py d) NBS, $BaCO_3$, CCl_4, reflux e) NaI, acetone reflux, then DBU, MS 4A, DMSO, 80°C f) $HgCl_2$, acetone/H_2O, reflux, then MsCl, Et_3N, CH_2Cl_2, 0°C g) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, EtOH/CH_2Cl_2, -78°C h) TBDMSCl, imidazole, DMF, 40°C i) KOH/EtOH, rt j) mCPBA, $ClCH_2CH_2Cl$, 40°C h) 70% AcOH, r.t.

An analogous route to cyclophellitol (*3*) and *epi*-cyclophellitol (*92*) from D-mannose has been reported by Jung and Tina Choe (ref. 38). The alkene ***156*** was prepared from methyl benzylidenemannoside (***155***) by a subsequent sequence of benzylation of the equatorial alcohol, oxidation and Wittig reaction. Hydroboration-oxidation of ***156*** furnished a 1:1 mixture of the two isomeric hydroxymethyl compounds ***157*** and ***158***, which was transformed into the required equatorial hydroxymethyl compound ***158*** upon Swern oxidation, equilibration to the β-aldehyde with a mild base and reduction. The alkene ***160*** obtained by benzylation, reductive opening of the benzylidene acetal, iodide formation, and elimination was subjected to Ferrier rearrangement (ref. 49) and elimination to give the key intermediate enone ***161***. Luche reduction (ref. 23) followed by benzoylation afforded ***162***. Epoxidation of ***162*** with mCPBA resulted in a

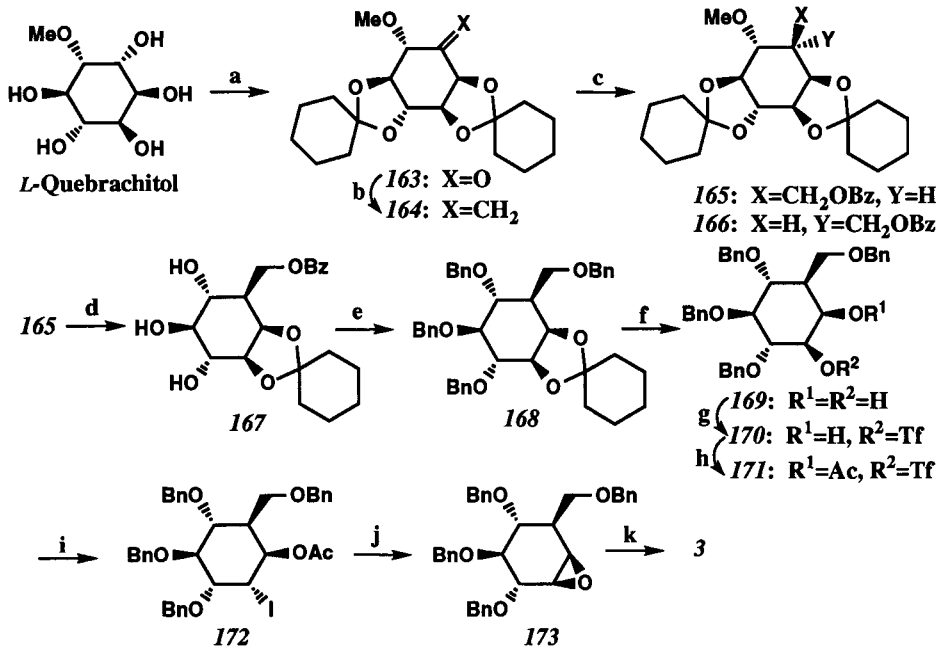
mixture of epoxides, which was converted into *3* and *92* by removal of the protecting groups.



a) Bu_2SnO, MeOH; BnBr, DMF; DMSO, TFAA, CH_2Cl_2/Et_3N; $Ph_3P{=}CH_2$ b) BH_3·DMS, then, H_2O_2, NaOH c) Swern oxid., then Et_3N; $NaBH_4$ d) BnBr, NaH; $LiAlH_4$, $AlCl_3$; PPh_3, I_2; DBU e) aq. $HgCl_2$; MsCl, pyr. f) $NaBH_4$, $CeCl_3$; BzCl, pyr. g) mCPBA; K_2CO_3, MeOH; H_2/Pd

Ozaki and his co-workers (ref. 39) have presented a route to cyclophellitol (*3*) from a naturally abundant cyclitol, L-quebrachitol. Olefination of the ketone ***163*** readily available from L-quebrachitol (ref. 49) was best achieved by Peterson olefination to give the exo olefin ***164***. Hydroboration of ***164*** followed by benzoylation furnished a 1:1 mixture of ***165*** and ***166***. Chemoselective removal of both methyl ether and *trans*-cyclohexylidene functions of ***165*** with a combination of $AlCl_3$ and *n*-Bu_4NI gave the triol ***167***, which was transformed into the tetra-benzyl ether ***168*** in a one-pot procedure upon treatment with NaH and benzyl bromide followed by the addition of NaOMe and NaH/benzyl bromide. A subsequent sequence of acid hydrolysis, regioselective triflation and acetylation afforded compound ***171***. Replacement of the triflate group of ***171*** by iodide was efficiently achieved by the SN2 reaction with *n*-Bu_4NI to give the axial iodide ***172***. Upon treatment with NaOMe the iodide ***172*** gave the epoxide ***173***, which was converted

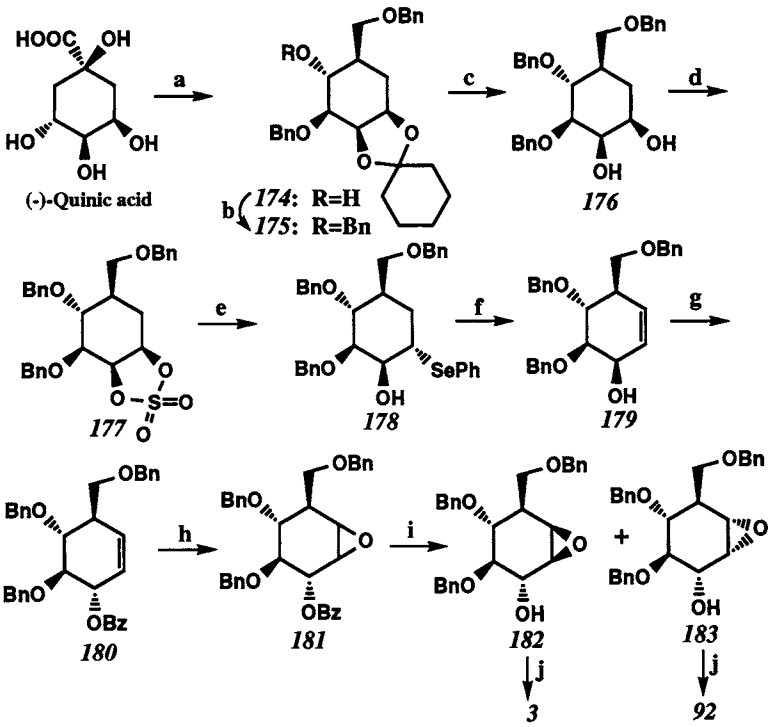
into *3* by removal of the protecting groups.

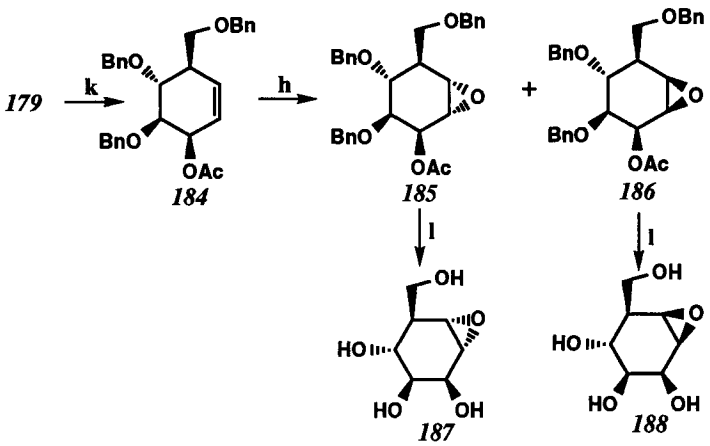


a) ref. 49 b) Me_3SiCH_2MgCl, THF, r.t. 9h, then reflux 2h; KH, THF, r.t. c) BH_3·THF, r.t., then H_2O_2, NaOH, 50°C; Bz_2O, pyr d) $AlCl_3$, *n*-Bu_4NI, CH_3CN e) BnBr, NaH, NaOMe, DMF f) CF_3CO_2H, MeOH g) $(CF_3SO_2)_2O$, pyr h) Ac_2O, r.t. i) *n*-Bu_4NI, PhH, reflux j) NaOMe, MeOH/THF k) H_2, Pd/C

Shing and Tai (ref. 40) have demonstrated another route to cyclophellitol (*3*) together with *epi*-cyclophellitol, (1*R*,2*S*,6*S*)- and (2*S*)-diastereoisomers (***92***, ***187*** and ***188***) from a natural (-)-quinic acid. Benzylation of compound ***174***, readily available from (-)-quinic acid (ref. 50) followed by acid hydrolysis gave the diol ***176***, which was transformed into the cyclic sulfate ***177*** by the Sharpless protocol (ref. 33). Opening of the cyclic sulfate ***177*** with selenide anion was regioselectively carried out to afford the *trans*-diaxial seleno-alcohol ***178*** after acid hydrolysis. Oxidative elimination of ***178*** followed by inversion of configuration of the hydroxy group *via* the Mitsunobu reaction (ref. 51) furnished the β-benzoate ***180***. Epoxidation of ***180*** resulted in an inseparable mixture of oxiranes ***181*** which provided the alcohols ***182*** and ***183*** by deprotection of the benzoyl group. Removal of the protecting groups of ***182*** and ***183*** yielded cyclophellitol (*3*) and

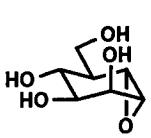
epi-cyclophellitol (***92***), respectively. On the other hand, acetylation of ***179*** followed by epoxidation gave the oxiranes ***185*** and ***186***. Deprotection of ***185*** and ***186*** resulted in (1*R*,2*S*,6*S*)- and (2*S*)-diastereomers (***187*** and ***188***), respectively.



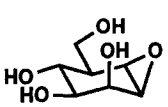


a) ref. 50 b) NaH, THF, then BnBr, *n*-Bu_4NI, reflux c) CF_3CO_2H, CH_2Cl_2, r.t. d) $SOCl_2$, Et_3N, CH_2Cl_2, then $RuCl_3$, $NaIO_4$, CCl_4-MeCN-H_2O e) Ph_2Se_2, $NaBH_4$, EtOH, then H_2SO_4, H_2O f) mCPBA, CH_2Cl_2, then *i*-Pr_2NEt, $PhCH_3$ g) $PhCO_2H$, PPh_3, diethylazodicarboxylate, $PhCH_3$ h) mCPBA, CH_2Cl_2, reflux i) cat. K_2CO_3, MeOH, r.t. j) H_2, Pd/C, EtOH k) Ac_2O, pyr., cat. DMAP, CH_2Cl_2 l) cat. K_2CO_3, MeOH; H_2, Pd/C, EtOH

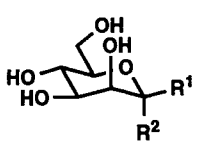
The thus obtained cyclophellitol (*3*), *epi*-cyclophellitol (***92***), (1*R*,2*S*,6*S*)- and (2*S*)-diastereomers (***187*** and ***188***) were evaluated for inhibitory activity against glycosidases (ref. 52). While the epoxide, the three hydroxy groups and the hydroxymethyl group in *3* and ***92*** constitute the β- and α-D-*gluco*-configuration, respectively, the diastereomers ***187*** and ***188*** have the β- and α-D-manno-configuration, respectively (Fig. 4). As expected, compounds ***187*** and ***188*** inhibited β-D-mannosidase (*A. oryzae*, IC_{50} 28 μg/ml) and α-D-mannosidase (jack beans, IC_{50} 9 μg/ml), respectively. These results confirmed that glycosidases recognize precisely not only the C1-O configuration but also the configurations of other functions of the glycopyranoside skelton for enzyme-substrate interaction.



(1*R*,2*S*,6*S*)-Cyclophellitol (*187*)



(2*S*)-Cyclophellitol (*188*)

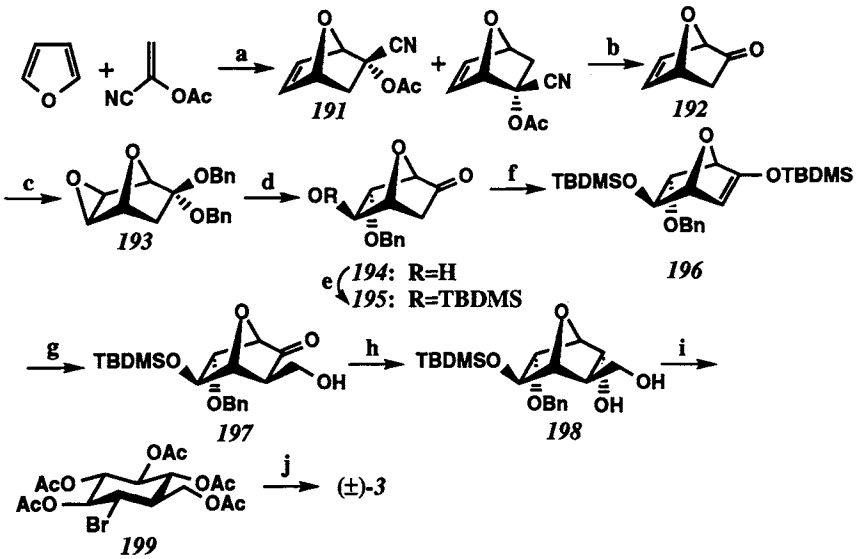


β-D-Mannopyranoside (*189*): R^1=H, R^2=OH

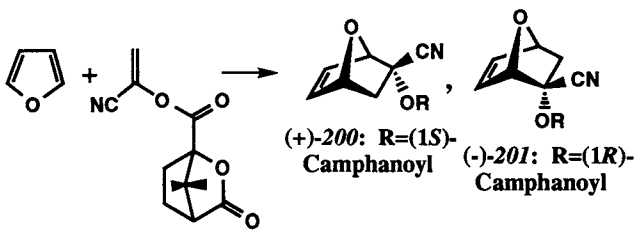
α-D-Mannopyranoside (*190*): R^1=OH, R^2=H

Fig. 4

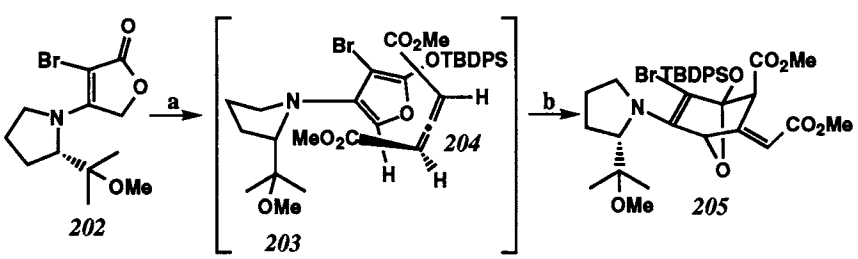
Moritz and Vogel (ref. 41) have described a highly stereoselective route to racemic cyclophellitol (*3*) *via* the Diels-Alder adduct of furan to 1-cyanovinyl acetate. The starting racemic Diels-Alder adduct (±)-***191*** (ref. 53) was transformed into the 7-oxonorbornanone ***194*** by the procedures of Le Drian *et al.* (ref. 54) Silylation of ***194*** followed by treatment with $(Me_3Si)_2NK$ and *t*-$BuMe_2SiCl$ afforded the silyl enol ether ***196***. Addition of monomeric formaldehyde to ***196***, and a subsequent addition of $TiCl_4$ furnished the *exo* alcohol ***197***, which was converted into *endo* alcohol ***198*** upon reduction with $NaBH_4$. Heating ***198*** in 30% HBr/AcOH yielded the bromocyclohexylacetate ***199***. Treatment of ***199*** with NaOMe in methanol resulted in (±)-*3*. The synthesis described here can be applied to the preparation of cyclophellitol (*3*) and its enantiomer since the authors have already developed the preparation of the optically pure 7-oxabicyclo[2.2.1]hept-5-en-2-yl derivatives (+)-***200*** and (-)-***201*** (ref. 55).

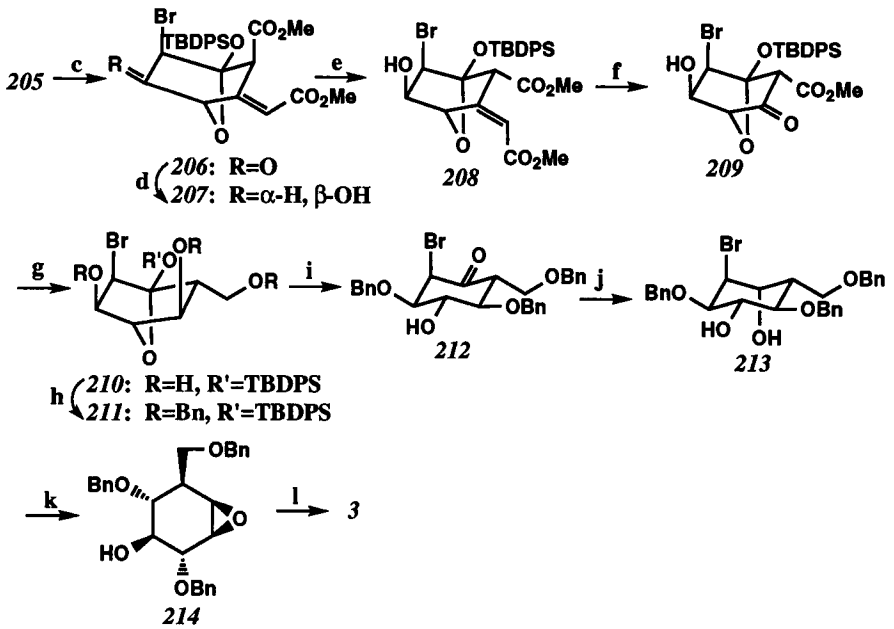


a) $Cu(OAc)_2 \cdot H_2O$, $Cu(BF_4)_2 \cdot 6H_2O$, C_6H_4-1,4-$(OH)_2$, proylene oxide, ref. 42 b) NaOMe; H_2CO c) BnOTMS, mCPBA d) HSO_3F e) TBDMSCl, imidazole, DMF f) $(Me_3Si)_2NK$, TBDMSCl, THF, -78°C g) CH_2O, then $TiCl_4$ h) $NaBH_4$, MeOH i) 30% HBr/AcOH j) NaOMe, MeOH



Schlessinger and Bergstrom (ref. 42) have developed an elegant route to the optical active (+)-cyclophellitol (*3*) *via* nonracemic Diels-Alder [2:2:1]oxabicyclic enamine adduct. The synthesis of optical active [2:2:1]oxabicyclic enamine adduct ***205*** began with the vinylogous urethane lactone ***202*** which was transformed to the nonracemic furan ***203*** by deprotonation and subsequent treatment of the resultant enolate with *tert*-butyldiphenylsilyl chloride. The thus obtained nonracemic furan ***203*** was treated with racemic dimethyl 2,3-pentadienedioate ***204***. Kinetic resolution of the allenic diester ***204*** by a double diastereofacially selective reaction led to the adduct ***205***. Hydrolysis of ***205*** followed by stereoselective reduction furnished the *syn* bromohydrin ***207***, which was converted into the C_5 equatorial ester ***208*** by epimerization with 4-dimethylaminopyridine. Ozonolysis of ***208*** and subsequent reduction of the resulting unstable β-keto ester ***209*** afforded the triol ***210*** possessing the correct stereochemistry at C_4. Cleavage of the oxabicyclic ketal residue of the tribenzyl ether ***211*** was smoothly carried out by treatment with $BF_3 \cdot Et_2O$ to give the cyclohexanone ***212***. Reduction of ***212*** with diisobutyl-aluminum 2,6-di-*tert*-butyl-4-methylphenoxide (ref. 56) followed by treatment with KHMDS yielded the epoxide ***214***. Hydrogenolysis of ***214*** resulted in ***3***.

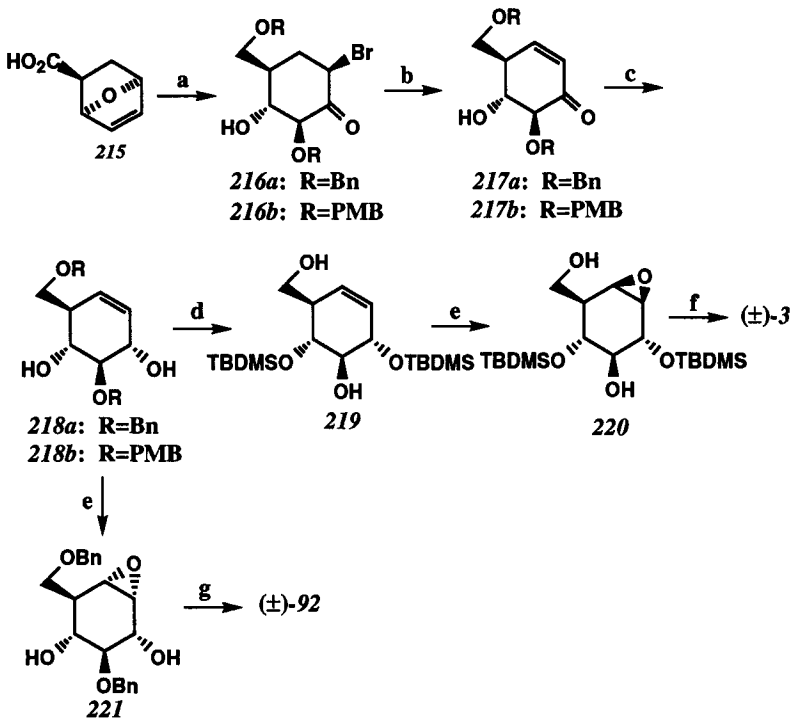




a) KHMDS, THF, 0°C, then TBDPSCl, 0°C b) THF, -100°C c) 2M HCl, CH_3CN d) $NaBH_4$, EtOH, -78°C e) DMAP, THF, 23°C f) O_3, CH_2Cl_2, -78°C; DMS, -78°C g) DIBALH, $PhCH_3$, -78°C h) $BnOC(NH)CCl_3$ i) $BF_3{\cdot}Et_2O$, CH_2Cl_2, -20°C j) Diisobutylaluminum 2,6-di-*tert*-butyl-4-methylphenoxide, $PhCH_3$, 22°C k) KHMDS, THF, -78°C l) H_2, Pd/C, MeOH

The Diels-Alder adduct of furan and acrylic acid has been involved in a concise, stereodivergent synthesis of (±)-cyclophellitol (***3***) and (±)-*epi*-cyclophellitol (***92***) by Acena and co-workers (ref. 43). The Diels-Alder adduct ***215*** available from furan and acrylic acid was transformed into the bromoketone ***216*** in seven steps *via* a sequence developed by the authors (ref. 57). Dehydrohalogenation of ***216a*** to the enone ***217a*** followed by reduction under Luche's conditions (ref. 23) afforded the diol ***218a***. When the diol ***218a*** was epoxidized with mCPBA, the single oxirane ***221*** was produced by the participation of the free allylic hydroxyl group (ref. 58). Removal of the protecting groups by hydrogenolysis yielded (±)-***92***. On the other hand, in order to inverse the selectivity of the epoxidation, the diol ***219*** was used for the synthesis of (±)-cyclophellitol (***3***). Thus, the diol ***219*** readily available from ***215*** was epoxidized with mCPBA to give the oxirane ***220*** as a single product. Removal of the protecting groups with tetrabutylammonium fluoride resulted in (±)-***3***. The

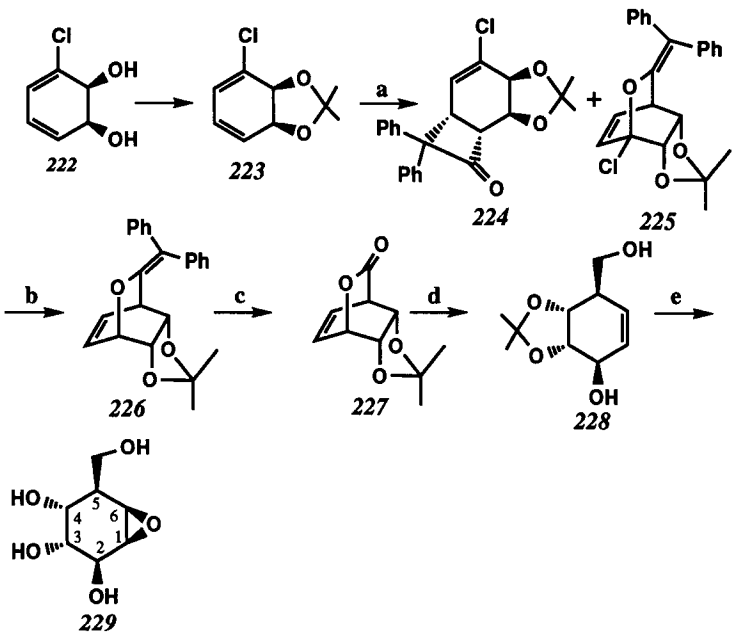
optically active cyclophellitol (*3*) and 1,6-*epi*-cyclophellitol (*92*) can be prepared by similar sequences described here since the authors have already achieved the optical resolution of *215*.



a) ref. 59 b) $CaCO_3$, DMF, 150°C c) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, -78°C to r.t. d) TBDMSOTf, Et_3N, THF, -78°C; DDQ, CH_2Cl_2/H_2O, r.t. e) mCPBA, CH_2Cl_2, r.t. f) nBu$_4$NF, THF, r.t. g) H_2, 10% Pd/C, MeOH, r.t.

[2+2] and [4+2]cycloaddition reaction were utilized effectively for the synthesis of 2,3-di-*epi*-cyclophellitol (*229*) by Roberts and Sutton (ref. 44). Treatment of the acetal *223* with diphenylketene afforded a mixture of [2+2]- and [4+2]-adducts (*224*, *225*) in a ratio of 65:16. The isomers *224* and *225* also equilibrate to give two compounds in a ratio of 66:19, respectively, under reflux in THF. Treatment of the tertiary chloride *225* with *tri*-butyltinhydride furnished (-)-*226* by hydrodechlorination, while [2+2]-adduct *224* was stable under these reaction conditions. Thus, a mixture of *224* and *225* resulted in the single enol ether *226* *via* a process of equilibration and selective hydrodechlorination upon

treatment with *tri*-butyltinhydride and AIBN in THF. Oxidation of ***226*** with mCPBA followed by reduction gave the diol ***228***, which was transformed into 2,3-di-*epi*-cyclophellitol (***229***) by epoxidation and removal of the isopropylidene group.



a) Diphenylketene, THF, reflux b) *n*-Bu_3SnH, THF, reflux, and then AIBN, reflux c) mCPBA, CH_2Cl_2, 0°C d) $LiAlH_4$, THF, -15°C e) mCPBA, CH_2Cl_2, r.t.; Amberlyst 15 (H^+), H_2O, r.t.

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Molecular Rearrangements in the Derivatives of Grandiflorenic Acid [(-)-kaura-9(11),16-diene-19-oic Acid] and Some Related Diterpenes

Tatsuhiko Nakano

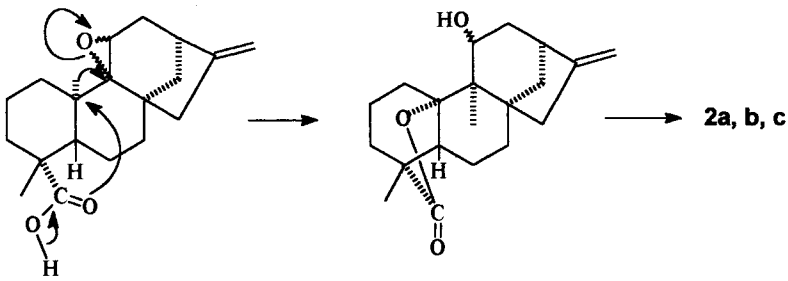
1 INTRODUCTION

Various molecular rearrangements are known in almost all kinds of natural products, such as alkaloids, terpenoids, steroids, aminoacids, peptides and carbohydrates, and are well documented.[1] By these rearrangements a variety of products whose basic skeletons are different from the original substances are often produced. The studies of the structures and stereochemistries of these rearrangement products and the modes of their formation have long attracted interest among natural product chemists.

The terpenoids were among those natural products in which rearrangements were earliest detected and well studied. The contributions in this field, particularly those directed to the investigation of reactions now known to involve cationic species, served greatly to promote the general knowledge of reaction mechanism.

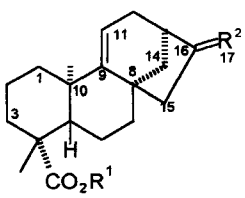
This article aims to summarize the molecular rearrangement studies in the derivatives of grandiflorenic acid [(-)-kaura-9(11),16-diene-19-oic acid] (section 2)[2] and some related diterpene ring systems (sections 3 and 4), which we have carried out in our own laboratory. The structures and stereochemistries of all rearrangement products were determined on the basis of ^{1}H, ^{13}C-NMR spectroscopic evidence and some of them were further confirmed by X-ray crystallographic analysis. In this review only the mechanisms for their formation are presented in detail.

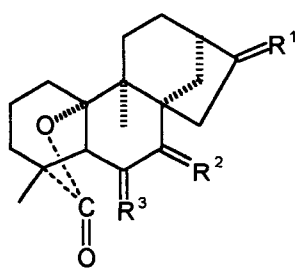
Grandiflorenic acid is widely distributed in several species of *Espeletia* of the family Compositae and its structure and absolute stereochemistry have been unambiguously determined as **1a** by Piozzi and his coworkers. [3] Venezuela is rich in the *Espeletia* species and grandiflorenic acid can be obtained in large quantities from the acid fractions of the resins of several species. Our initial interest was in transforming this diterpene acid to zoapatline **2a** and its analogs **2b** and **2c**[4] which had been isolated from the native Mexican shrub "Zoapatle" (*Montanoa tomentosa* Cerv.). These diterpene lactones possess a basic skeleton in which the methyl groups at C-10 in the natural (-)-kaurane skeleton migrates to C-9. We assumed that they might be derived chemically from grandiflorenic acid *via* the acid-catalyzed rearrangement of its 9,11-epoxide, as illustrated in Scheme 1.



Scheme 1

Before attempting to transform grandiflorenic acid **1a** to zoapatline **2a**, we first investigated the rearrangement of the epoxides of both (-)-kaur-9(11)-en-19-oic acid **1b** and methyl (-)-kaur-9(11)-en-19-oate **1c** to see whether or not the methyl group at C-10 would migrate to C-9.





1a; R^1=H, R^2=CH_2

1b; R^1=H, R^2= 

1c; R^1=Me, R^2= 

1d; R^1=Me, R^2=O

2a; R^1=CH_2, R^2=R^3=H_2

2b; R^1=β-CH_3, α-OH, R^2=α-OH, β-H, R^3=H_2

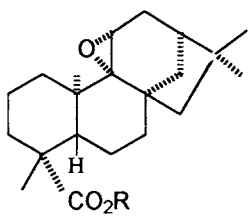
2c; R^1=β-CH_3, α-OH, R^2=H_2, R^3=α-OH, β-H

During the course of this work we discovered some very interesting rearrangement reactions and it prompted us to study further the other molecular rearrangements in several derivatives of grandiflorenic acid **1a**. As a result, we were able to prepare eight diterpenes **4**, **5**, **8**, **17**, **19, 20, 21** and **22**, with new ring systems which have not been encountered in nature.

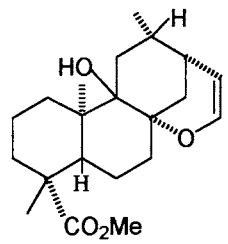
2 STUDIES ON THE REARRANGEMENTS IN DERIVATIVES OF GRANDIFLORENIC ACID

2.1 *Reaction of the Epoxides of Methyl (-)-Kaur-9(11)-en-19-oate and (-)-kaur-9(11)-en-19-oic Acid with Boron Trifluoride-Diethyl Ether in the Absence or in the Presence of N-Nitrosomethylurea. Formation of Two Diterpenes of a New Skeletal Type*[5]

On methylation with diazomethane compound **1b** afforded compound **1c**. Epoxidation of compound **1b** with *m*-chloroperbenzoic acid (MCPBA) in chloroform took place stereoselectively at the less sterically hindered α-side and yielded solely the α-epoxide **3b.**

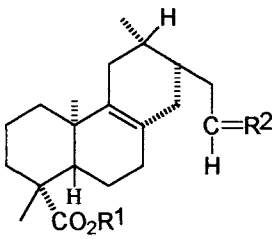


3a ; R=H
3b ; R=Me



4

During this epoxidation we found that when compound **1c** was contaminated *N*-nitrosomethylurea as an impurity,[6] the expected epoxide was not obtained but instead compound **4** was formed with a backbone rearranged skeleton. We postulated that an intermediate for the formation of compound **4** from compound **1b** in the above reaction is the epoxide **3b** and we studied in detail how the reaction proceeded. Immediately after compound **1c** (1 mol equiv.) was mixed in chloroform with *N*-methylnitrosourea (1 mol equiv.) and MCPBA (2 mol equiv.) at room temperature, the reaction was monitored by t.l.c. After 1 h, almost all of compound **1c** disappeared and a product of polarity identical with that of epoxide **3b** appeared. Discontinuation of the reaction at this point afforded epoxide **3b**. However, when the above reaction was allowed to continue for an additional 3 h, epoxide **3b** disappeared and two products, both of which are more polar than epoxide **3b**, appeared on t.l.c. The more polar of these was compound **4** while the other proved to be compound **5a**.



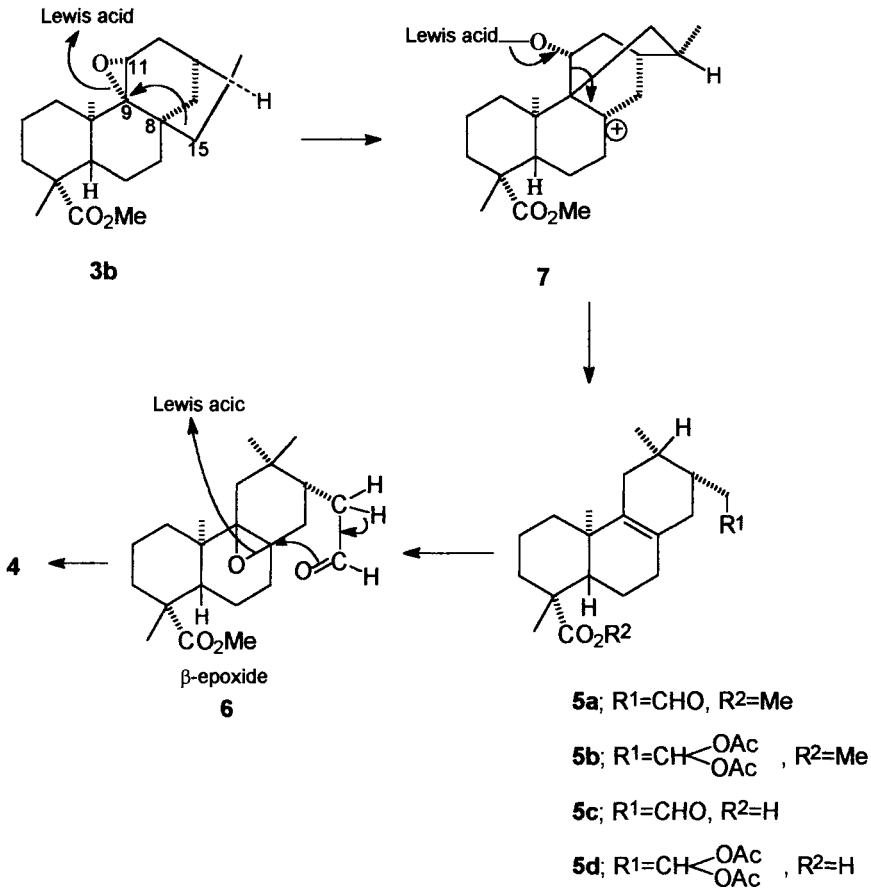
5a; R^1=Me, R^2=O

5b; R^1=Me, R^2=C(OCOMe)(OCOMe)

5c; R^1=H, R^2=O

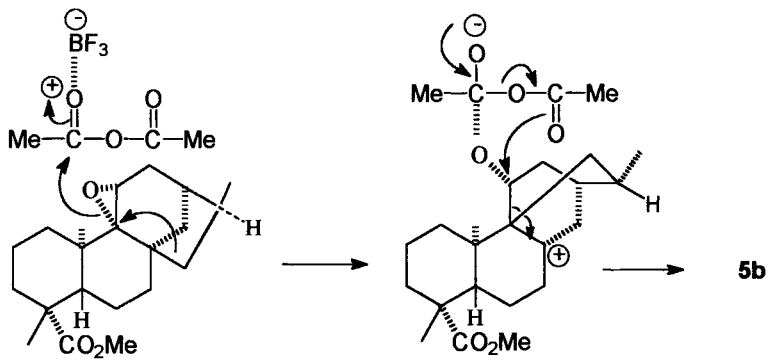
5d; R^1=H, R^2=C(OCOMe)(OCOMe)

That epoxide **3b** was indeed an intermediate was further supported by a separate experiment in which when it was treated with *N*-nitrosomethylurea (1 mol. equiv.) and MCPBA (2 mol equiv.) under the same conditions as above, compound **4** was obtained. Epoxide **3b** is stable in chloroform solution towards either *N*-nitrosomethylurea alone or a mixture of MCPBA and *m*-chlorobenzoic acid alone. Therefore, one reasonable guess for the formation of compound **4** would be that *N*-nitrosomethylurea may generate in the presence of MCPBA some nitric acid (from oxidation and cleavage of the nitroso group), but when epoxide **3b** was treated with MCPBA and one drop of either 65% or 10% aqueous nitric acid, compound **4** was not obtained, and instead the reaction afforded a mixture of unidentifiable products. However, on treatment with MCPBA and one drop of nitric acid in the presence of urea, epoxide **3b** afforded compound **4** in small yield. Therefore, it is reasonable to suppose that when nitric acid is generated, the amides such as *N*-nitrosomethylurea or urea may act as a buffer so as not to allow the medium of the reaction mixture to be too acidic. We consider a plausible mechanism for the formation of compound **4** to be depicted in Scheme 2. The initially formed epoxide **3b** undergoes ring opening by the mixture of *N*-nitrosomethylurea and MCPBA which then acts as a Lewis acid to cause rupture of the epoxide ring. As illustrated by arrows, the cleavage of the epoxide ring would be followed by the migration of the C-8-C-15 bond to C-9. Rupture of C-9-C-11 bond in compound **7** and concurrent formation of the aldehyde group would then give rise to compound **5a**, which would further react with MCPBA to form the epoxide **6**. Subsequent nucleophillic attack from the α-side at C-8 by the aldehyde carbonyl oxygen, followed by rupture of the epoxide ring, would then lead to compound **4**. This mechanistic pathway may also verify the formation of compound **5a** mentioned earlier.

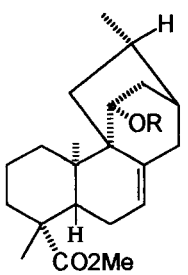


Scheme 2

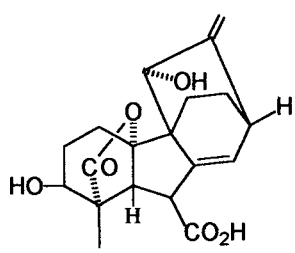
On treatment with boron trifluoride-etherate in benzene, epoxide **3b** afforded intractable mixtures. After chromatography over silica gel, aldehyde **5a** was obtained in some amounts as the only identifiable product. We reasoned that the complexity of this reaction might be avoided in the presence of acetic anhydride, because in this case if any alcohol **7** were formed, it might be acetylated *in situ* so that the resultant product might be compound **8a** rather than aldehyde **5a**. If that were the case, it might be possible to obtain a ring C/D-analog of antheridiogen, A_{An} **9**, the antheridium-inducing factor from *Anemia phyllitidis*.[7] However, when epoxide **3b** was treated with boron trifluoride-etherate in acetic anhydride, the expected product **8a** was not formed, but compound **5b** (acylal) was obtained as the sole product in over 50% yield. This compound would be generated according to the mechanism shown in Scheme 3.



Scheme 3



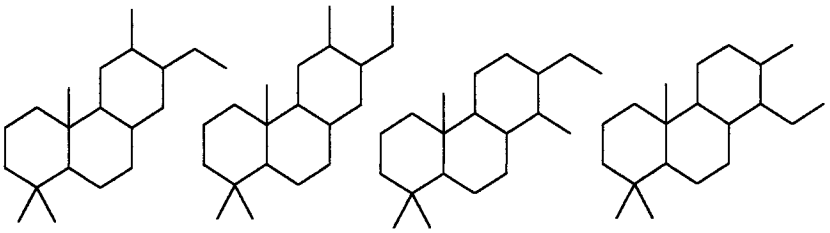
8a; R=Ac
8b; R=H



9

Epoxidation of compound **1b** with MCPBA in chloroform gave the α-epoxide **3a** as the sole product. Epoxide **3a** is very reactive and when set aside in benzene solution over silica gel, it undergoes cleavage of the epoxide ring to afford aldehyde **5c**. The same aldehyde was also obtained by the rupture of epoxide **3a** with boron trifluoride-etherate in benzene. On treatment with boron trifluoride-etherate in acetic anhydride, aldehyde **5c** afforded acylal **5d**, which on methylation with diazomethane led to compound **5b**. We then attempted to verify the previously postulated mechanistic pathway (see Scheme 2) by converting compound **5a** into compound **4.** Epoxidation of compound **5a** with MCPBA in chloroform yielded epoxide **6** which, on treatment with one drop of conc. hydrochloric acid in chloroform, led to a product identical with compound **4**.

The rearrangement products **4** and **5** represent new types of diterpene skeleton **10**. So far, only diterpenes that possess a pimarane **11**, a cassane **12** or a cleistanthane **13** skeleton have been discovered in nature.



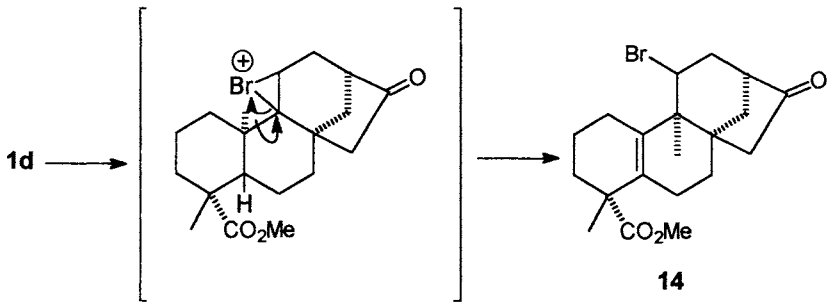
10 **11** **12** **13**

2.2 *Rearrangement of Methyl Grandiflorenate 17-Nor-ketone with N-Bromosuccinimide in Aqueous Acetone*[8]

In section 2.1 it has been shown that the epoxidation of compounds **1b** and **1c** afforded exclusively the α-epoxides **3a** and **3b**, respectively. Since the epoxides ring and the C (20)-methyl group possessed the same α-stereochemistry, upon cleavage of these epoxide rings the concerted migration of this methyl group to C(9) did not proceed and instead the C(8)-C(15) bond with the β-configuration migrated to this position.

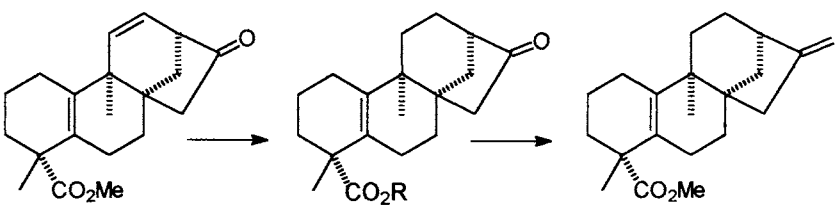
MacMillan and his coworker[9] reported that on treatment with acetyl hypobromite or bromine, the 17-nor-ketone **1d** underwent **10α** → **9α**-methyl migration to afford the **11β**-bromo derivative **14**.

Construction of the molecular models of compounds **1b,c** and **1d** indicates that the steric environments about the olefinic double bond are rather different. The C(16)-endo methyl group in structure **1b, c** would provide an effective shield over the β-face of the olefinic double bond, an observation in accord with the observed direction of epoxidation. The lack of this sterically shielding methyl group in **1d** would allow a bromine molecule (or a bromine ion) to attack from the β-side in essentially the spot where the C(16)-methyl group is located. The β-stereochemistry of the intermediate bromonium ion would greatly facilitate subsequent preferential migration of the α-oriented C(20)-methyl group to C(9) (formation of compound **14**), as illustrated in Scheme 4.



Scheme 4

Indeed, on being treated with *N*-bromosuccinimide in acetone-water, compound **1d**, afforded a bromo derivative **14** with the rearranged skeleton whose structure was confirmed after conversion *via* compound **15** to compound **16a**.



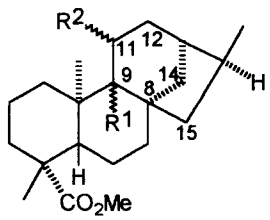
15 **16a** ; R=Me **17**
16b ; R=H

Attempts to lactonize compound **16b** under a variety of conditions reported failed. Wittig reaction of compound **16a** with methylenetriphenylphosphorane led to the new diterpene **17**.

2.3 *Rearrangements of the Derivatives of Methyl 9,11- Dihydroxy-(-)-kauran-9-oate*[10]

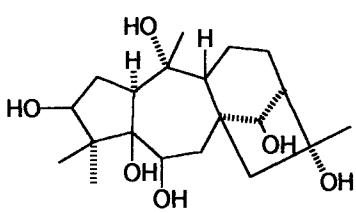
In section 2.1 it has also been shown that an attempt to synthesize a new diterpene **8a** in which the C/D ring system was analogous to that of antheridiogen, A_{An} **9**, *via* the rearrangement of the 9,11-epoxide of compound **1c**, was not successful. Therefore, we sought an alternative possibility, that is, the acid-catalyzed rearrangement of the 11α-acetoxy-9α-hydroxy derivative **18a** of compound **1c**.

The *cis*-dihydroxylation of compound **1c** with osmium tetroxide in pyridine afforded the α-glycol **18b** and the β-glycol **18c** (1 :1.8). Aceylation of the α-glycol **18b** under ordinary conditions gave the monoacetate **18a**. On treatment with a catalytic amount of concentrated sulfuric acid in methylene chloride at room temperature, compound **18a** rearranged to afford the desired compound **8a** in 96% yield. It was apparent that the rearrangement was initiated by the elimination of the 9α-OH group, followed by concerted migration of the C(8) - C(15) bond to C(9) and the formation of a double bond between C(7) and C(8). On the other hand, the rearrangement of α-glycol **18b** under identical conditions afforded two products. One (13% yield) was identified as compound **5a**, while the other (8% yield) was assigned structure **8b**.



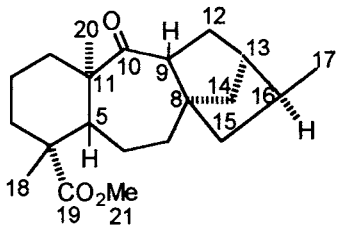
18 a ; R^1=α-OH, R^2=α-OAc
18 b ; R^1=R^2=α-OH
18 c ; R^1=R^2=β-OH
18 d ; R^1=α-OH, R^2=α-OMs
18 e ; R^1=β-OH, R^2=O

Our next interest turned to a pinacol-type rearrangement of α-glycol **18b** in order to construct a ring B-homo derivative of the kaurane-type diterpene. The toxic substances, grayanotoxins[11] I to XVIII are the highly oxygenated derivatives with a rearranged basic kaurane skeleton.



Grayanotoxin-I

Treatment of compound **18b** with mesyl chloride and triethylamine in ether at room temperature afforded the monomesylate **18d**. When compound **18d** was treated with potassium t-butoxide in t-butanol at room temperature, the desired ring B-homo derivative **19** was obtained in 100% yield.



19

The stereochemistry of the 9β-hydrogen was established by NOE difference experiments (Bruker AM-300 spectrometer) in which strong responses between the 9-proton and the 5β-proton as well as between the 9-proton and the C(17)-methyl protons were observed, but no NOE existed between the C(20)-methyl protons and the 9-proton. The retention of the stereochemistry [β-H on C (11) of **18d** → **β-H** on C (9) of **19**] during this B-homo annulation indicated that the migration of the C(9) - C(8) to C(11) did not proceed in a concerted manner upon departure of the leaving methanesulfonyloxy group, but it occurred from the less congested α-face of the resulting C(11)-carbonium ion intermediate.

In order to determine the preferred conformation[12] of compound **19** we made the NOE measurements in more detail by means of a Varian Unity 600 instrument. The 600 MHz two dimensional NMR spectrum showed clearly the following additional NOE effects between relevant neighboring protons: 5β- and 6β-; 9β- and 5β; 9β- and 1β; 9β- and 6β-; 9β- and 16β- methyl; 9β- and 12β-; 9β- and 15β-; 7α- and 11α-methyl protons. However, no NOE effects were observed between the 6α- and 9β-protons and between the 9β- and 7β-protons. The NOE is a function of internuclear distance and the requisite interatomic distance satisfying the observed NOE is less than 3.9 Å.

The molecular modeling study using molecular mechanics, quantum mechanics and molecular dynamic calculation indicated the preferred conformation for compound **19**, as depicted in Fig. 1. This preferred conformation represents a conformer of the lowest energy among the various conformations which vary within the energy difference of less than 5 kcal/mol. The interactomic distances between relevant neighboring protons for each one of these conformers are in good agreement with the data obtained by the NOE spectroscopic measurements.

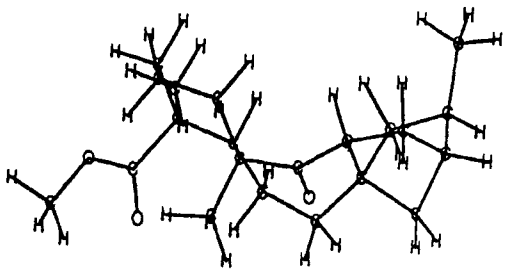
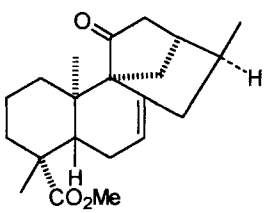


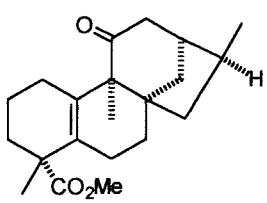
Figure 1

2.4 *Rearrangement of Methyl 9β-Hydroxy-11-oxo-(-)-kauran-19-oate*[13]

We also studied a pinacolic type of rearrangement of the 9β-hydroxy-11-oxo derivative **18e** of compound **1c**. Oxidation of compound **18c** with pyridinium dichromate afforded 9β-hydroxy 11-oxo derivative **18e**. On treatment with boron trifluoride-etherate-acetic anhydride-acetic acid[14] under standardized conditions, this compound did not undergo a pinacolic type of rearrangement leading to the formation of a ring B-homo derivative, but it yielded instead compounds **20** (14%) and **21** (33%).



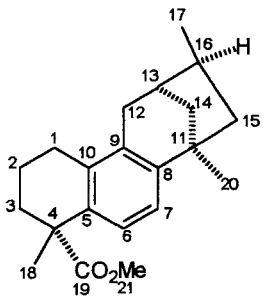
20



21

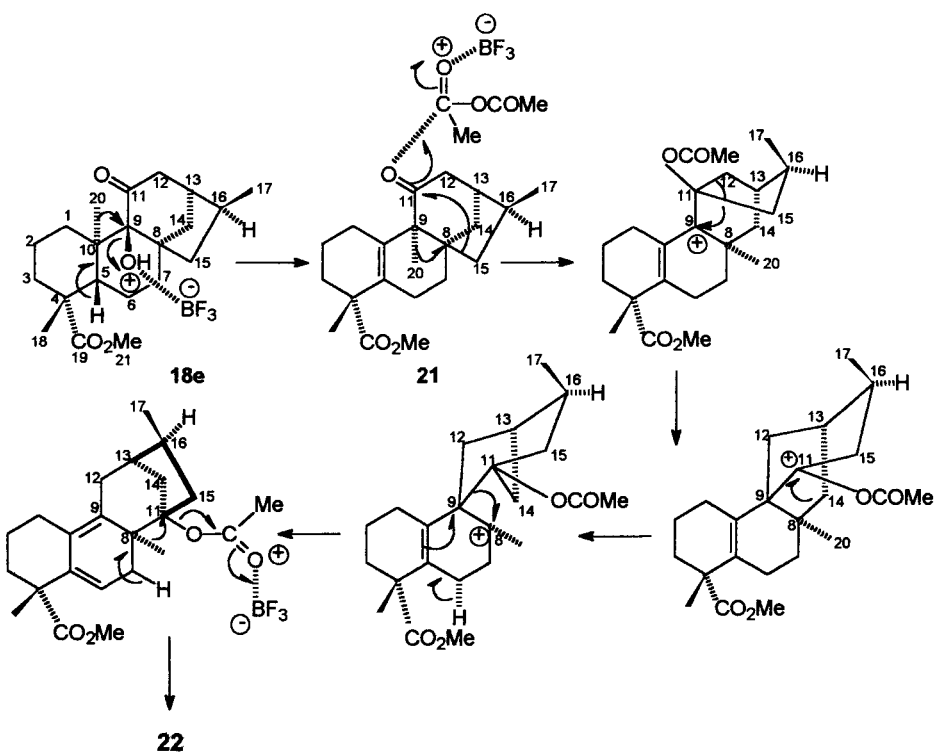
It was evident that compound **20** was formed by the elimination of the 9β-OH group, followed by concerted migration of the C(8)-C(14) bond to C(9), and subsequent production of a double bond between C(7) and C(8). Compound **21** was derived from the elimination of the 9β-OH group, followed by concerted migration of the C(20) angular methyl to C(9) with concomitant formation of a double bond between C(5) and C(10).

This rearrangement was further investigated in the absence of acetic acid. When treated with boron trifluoride etherate-acetic anhydride, compound **18e** suffered a profound backbone rearrangement to form compound **22** with an aromatic B-ring skeleton.



22

A plausible mechanistic pathway for the formation of compound **22** can be visualized in Scheme 5. A series of the following rearrangement processes are considered to take place all at once in a concerted manner.



Scheme 5

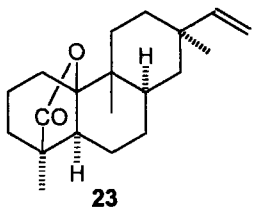
That compound **21** was indeed an intermediate in this reaction path was verified by an independent experiment in which when compound **21**, obtained earlier from compound **18e** *via* a different route, was treated with boron trifluoride-etherate-acetic anhydride, it formed compound **22**.

The mechanistic pathway postulated above was verified by means of the ^{13}C NMR spectroscopy in combination with deuterium labeling technique.[15] In Scheme 5 all the carbon atoms in compound **18e** are marked according to the customary numbering system so as to see how each carbon atom migrates during this rearrangement process. In this case, if the carbon-12 in **18e** is labeled by deuterium, then the deuterium must be detected in the same numbered carbon atom in the rearrangement product **22**, provided that our proposed mechanistic pathway is correct. For this purpose the complete assignment of chemical shifts of all carbon atoms in these compounds **18e** and **22** was made by the 2D-^{1}H, ^{1}H and ^{13}C, ^{1}H NMR single bond and multiple bond correlation studies.

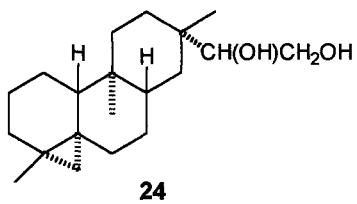
On refluxing with CD_3OD, D_2O and sodium, compound **18e** afforded a mixture of 12-d_0-compound **18e** (66%), 12-d_1-derivative (27%) and 12,12-d_2-derivative (6%), as indicated by mass spectral analysis. In its broad band proton decoupled ^{13}C NMR spectrum, besides all carbon resonances in d_0-compound **18e**, monodeuterated carbon-12 signal appeared at δ 44.81 as a broad triplet of low intensity. Its neighboring carbons-13 and -16 resonated at δ 40.39 (s) and δ 34.99 (s), respectively. The signal positions of these carbons are slightly upfield relative to the corresponding carbons in d_0-compound **18e**, due to the isotope effect (-0.33 ppm in carbon-12; -0.05 ppm in carbon-13; -0.04 ppm in carbon-16). The carbonyl carbon-11 underwent a downfield shift (+ 0.07 ppm) to δ 215.87 (s). which is also in accord with the isotope effect. On treatment with boron trifluoride-etherate in acetic anhydride under the same conditions as mentioned earlier, the above mixture of deuterated compounds afforded a mixture of d_0-compound **22** and its d_1-derivative. Its broad band proton decoupled ^{13}C NMR spectrum was identical with that of d_0-compound **22** except that in this case three additional carbon signals were observed. A deuterated carbon was found at δ 30.57 as a broad triplet peak of low intensity, which resonated upfield by 0.29 ppm , as compared with the corresponding carbon-12 in d_0-compound **22**. Its adjacent carbons-13 and -16 resonated at δ 39.02 (s) and δ 36.50 (s), respectively, exhibiting an expected upfield isotope shift (-0.09 ppm in carbon-13; -0.04 ppm in carbon-16). All these NMR results attested to the correctness of the mechanistic pathway (Scheme 5) which we have proposed for the formation of compound **22** from compound **18e**.

3 REARRANGEMENT OF 8α,9α-EPOXY-12,12-ETHYLENEDIOXYPODOCARPAN-16-OL ACETATE BY BORON TRIFLUORIDE-ETHERATE[16]

Rearrangements of the epoxides of pimarane derivatives have been exploited in biogenetically modelled synthesis of rosenololactone **23.**[17] In connection with the synthetic study[18] of erythroxydiol X **24** which possesses a rosane-type structure containing a cyclopropane ring, we sought an intermediate which might be utilized for the construction of its basic skeleton.

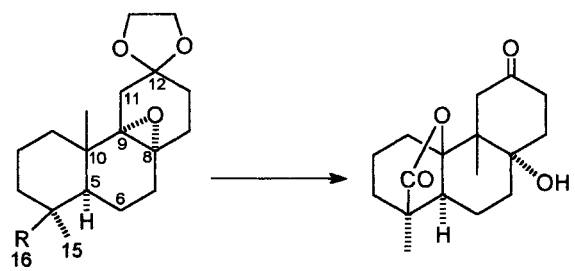


23



24

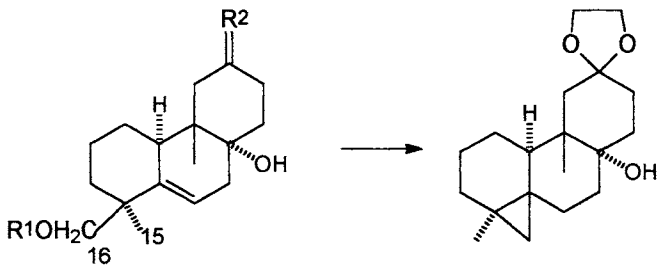
Mander and his co-workers[19] prepared the epoxide **25a** of the $\Delta^{8(9)}$-ethylene acetal derived from 12-oxopodocarp-9(11)-en-16-oic acid, and by cleavage with boron trifluoride-etherate obtained the hydroxy-lactone **26** for the construction of the rosenololactone skeleton.

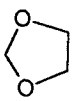


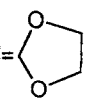
25a; R=CO_2H **26**

25b; R=CH_2OAc

Our plan was based upon the assumption that if the acetate **25b** in place of the acid **25a** was used, then a product such as **27a** or **27b** might be formed upon rupture of the epoxide ring with boron trifluoride-etherate. Once compound **27a** is formed, we might expect that its conversion into the 16-toluene-*p*-sulfonyl derivative and subsequent reductive cyclization[20] with lithium aluminum hydride would give a cyclopropane derivative such as **28**.



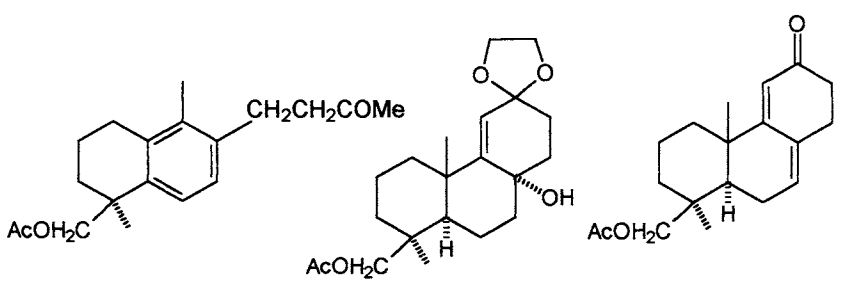
27a; R^1= Ac, R^2=  , $\Delta^{5(10)}$

27b; R^1= Ac, R^2=  , $\Delta^{5(6)}$

27c; R^1= Ac, R^2=O

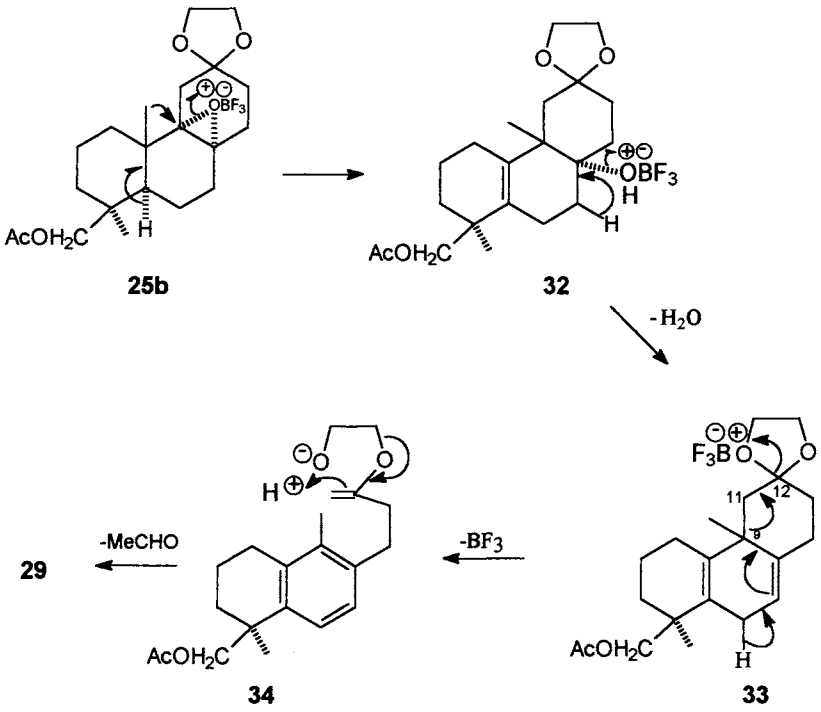
27d; R^1=Ac, R^2=O, $\Delta^{5(10)}$

We investigated the rearrangement of the epoxide **25b** in both polar and non-polar solvents. Contrary to our expectations, on treatment with boron trifluoride-etherate in nitromethane, it suffered a profound backbone rearrangement to afford compound **29** as a major product (56%), together with compounds **30** (5%) and **31** (6%).



29 **30** **31**

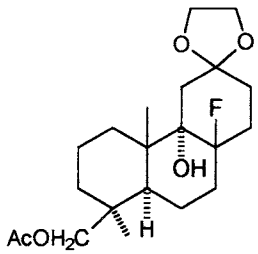
The formation of this ring B aromatic cmpound may be rationalized by postulating the following reaction pathway shown in Scheme 6.



Scheme 6

Upon cleavage of the epoxide ring, followed by migration of the methyl group from C-10 to C-9, compound **25b** would afford **32.** Dehydration of the HO-BF_3 complex and subsequent addition of BF_3 to one of the acetal oxygens would lead to **33**. The rupture of the C(12) - O bond, followed by that of the doubly allylic C(9) - C(11) bond, and aromatization of ring B would then take place in a concerted manner to form **34**. Compound **34**, in the presence of a proton, would further decompose to **29** by loss of MeCHO.

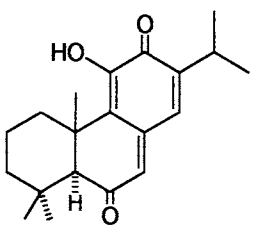
On treatment with boron trifluoride-etherate in benzene, the epoxide **25b** afforded four products. One was identified as the fluorohydrin **35** (23%) and the other three were found to be the α,β-unsaturated ketone **31** (3%), the rearranged hydroxy-ketone **27c** (2%) and its isomeric hydroxy-ketone **27d** (4%).



35

4 OXIDATION OF METHYL PODOCARPATE AND METHYL 12-HYDROXY-ABIETAN-8,11,13-TRIEN-18-OATE WITH THALLIUM (III) PERCHLORATE[21]

In addition to naturally occurring quinoid diterpenes, such as taxodione **36**, taxodone **37**,[22] fuerstione **38**[23] and its congeners, compound **39**[24] was also found in *Austrocedrus chilensis* (D. Don) Florin and Boutelje (Cupressaceae). These quinoids may be regarded as being formed from their phenolic precursors by oxidation under biogenetic conditions. In view of the observed antitumor activity of these compounds and other quinone methides,[25] we were interested in transforming podocarpic acid and its related compounds into other biologically active derivatives.



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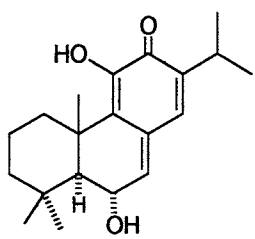
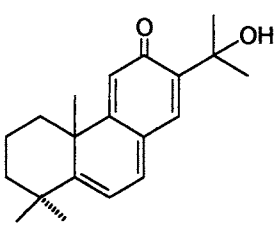
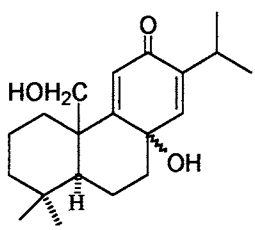


37

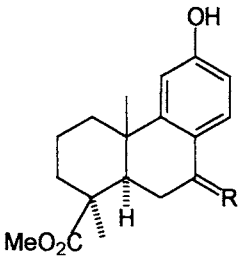


38

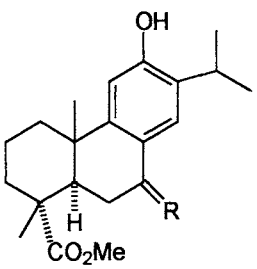


39

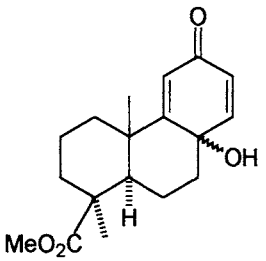
The utility of thallium(III) salts as excellent agents for the oxidation of a variety of organic substrates is well demonstrated.[26] Interesting reports on the synthesis of *p*-quinols from some phenolic compounds by thallium(III) perchlorate encouraged us to investigate the oxidation of methyl podocarpate **40a** and methyl 12-hydroxyabietan-8,11,13-trien-18-oate **41a** with this reagent.



40a ; R=H_2
40b ; R=O

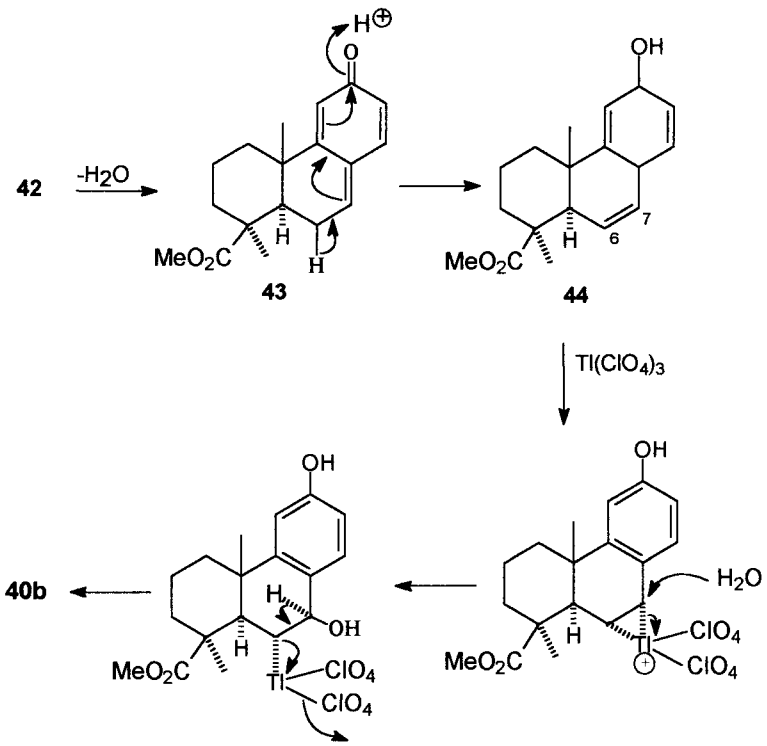


41a ; R=H_2
41b : R=O



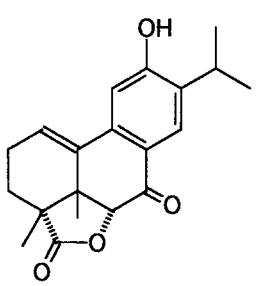
42

On treatment with thallium(III) perchlorate in methylene chloride-aqueous perchloric acid at room temperature, compound **40a** afforded three products. One was unreacted starting material (24%) and the other two were identified as the 7-oxo-derivative **40b** (11%) and the *p*-quinol **42** (19%). The formation of compound **40b** in this thallium(III) perchlorate oxidation needs comment. A similar remote oxidation during the reaction of estrone with thallium(III) perchlorate was reported by Yamada and his coworkers.[27] Compound **40b** may be derived from compound **42**, which, in acidic medium, would be dehydrated to the quinone methide **43**. It should be noted that most of the known naturally occurring quinone methides[28] contain an oxygen or other carbonyl function at the end of this quinone methide chromophore. In the remaining compounds, carbon atoms terminating the conjugating system either lack hydrogen[29] or form part of an aromatic system. This feature, which isolates the quinone methide chromophore from labile hydrogen atom(s), presents their tautomeric rearrangements to phenols. Compound **43** bears labile hydrogen atoms adjacent to the quinone methide chromophore, and hence it must be very sensitive to acids and would be expected to undergo dienone-phenol rearrangement to give the phenol **44**. Thallium(III) perchlorate, which is present in excess in the reaction medium, would then attack the 6,7-double bond in compound **44** from the less congested a-side to give a transient bridged organothallium adduct which, on reaction with water, would change to an intermediate possessing a weak C-Tl bond. Heterolysis of the C-Tl bond would proceed with the formation of a 6,7- double bond (loss of a proton from C-7), followed by ketonization of the resulting enol, to afford compound **40b**. A series of these reactions are illustrated in Scheme 7.



Scheme 7

On treatment with thallium(III) perchlorate in diethylether- aqueous perchloric acid at 0°C, compound **41a**[30] afforded two products, together with unreacted starting material (60%). One was identified as **41b** (6%) and the other was assigned structure **45** (12%). As in the case of the reaction of compound **40a** with thallium(III) perchlorate, compound **41b** must also have been formed by oxidation at C-7 remote from aromatic ring C.



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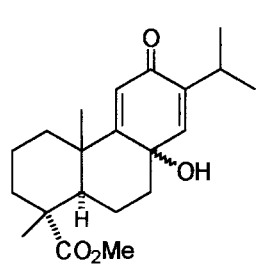
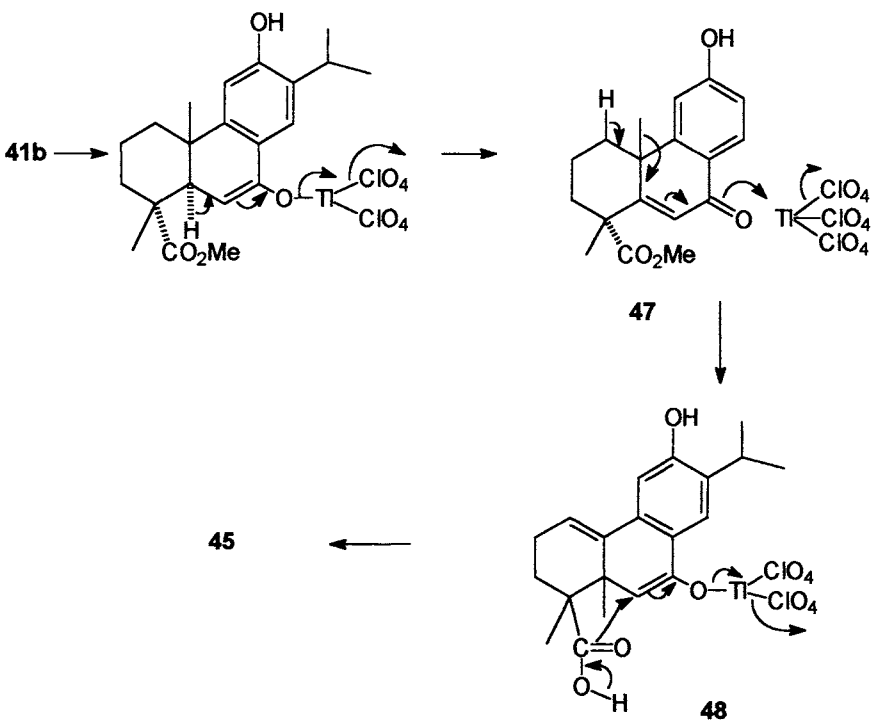


46

A plausible precursor for the formation of the lactone **45** with a rearranged skeleton would be compound **41b**, which would presumably arise from the as yet unisolable *p*-quinol **46** *via* a similar reaction mechanism (see Scheme 7) as proposed for the formation of compound **40b**. In the presence of excess thallium(III) perchlorate, compound **41b** would be converted into a thallium enolate from which the enone **47** would be derived. Further reaction of this enone with thallium(III) perchlorate would be followed by migration of the angular methyl group from C-10 to C-5 with concomitant formation of a double bond between C-1 and C-10 (formation of a thallium enolate[31] **48**). Subsequent internal nucleophilic attack at C-6 by the acid carbonyl group[32] would lead to the lactone **45**. The foregoing rearrangement pathway is depicted in Scheme 8.



Scheme 8

Acknowledgements

I wish to thank all my coworkers who have participated in this work and whose names are given in the references cited.

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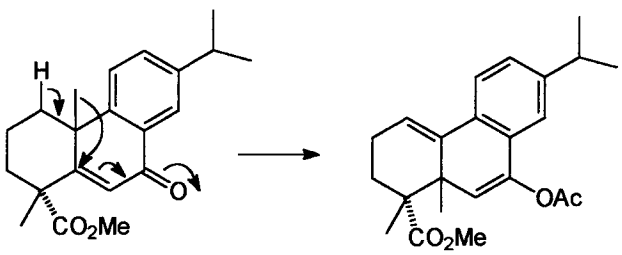
31 It is relevant to note that methyl 13-isopropyl-7-oxopodocarp-5,8,11,13-tetraen-15-oate **49** undergoes a similar rearrangement with concentrated sulfuric acid-acetic anhydride to afford compound **50** (see H. Akita and A. Tahara, *Chem. Pharm. Bull.*,1975, **23**, 2660)



49 **50**

32 During the course of conversion of **47** to **48** the methoxycarbonyl group in **47** is presumed to undergo hydrolysis in the acidic medium to give a carboxyl group which would induce lactonization more efficiently.

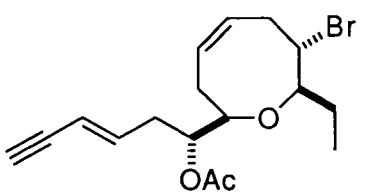
Atta-ur-Rahman (Ed.) *Studies in Natural Products Chemistry, Vol. 19*

Total Syntheses of Cyclic Halo Ether Compounds from Marine Origin

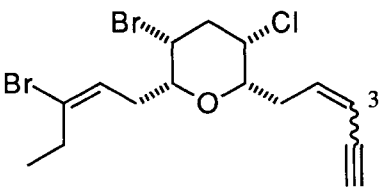
A. Murai

1. INTRODUCTION

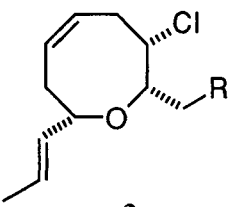
A variety of structurally unusual C_{15} nonisoprenoid metabolites have been isolated from red algae as well as the molluscs that feed on them (1). The representatives are laurencin (**1**), dactylynes (**2**), laurenyne (**3**), isolaurepinnacin (**4**), and dactomelynes (**5**). Of these, laurencin (**1**) is the most typical compound in respect of being the first natural product having an eight-membered cyclic ether isolated by Irie and co-workers in 1965 (2). The pioneering total synthesis of **1** in a racemic form was achieved in 1977 by us (3). Since that time, many groups have accepted the challenge of aiming at the total synthesis of these compounds in optically active forms. In 1988, Overman's group has carried out the total synthesis of (-)-**3** (4). We have also continued studies on the total syntheses of (+)-**1** and (-)-**2** keeping our developed procedures in mind and could complete the first syntheses of both compounds in chiral forms in 1992 (5,6). Further total syntheses of (+)-**1** have been reported by Holmes in 1993 (7) and Overman in 1995 (8). Furthermore, the first total syntheses of (-)-**4** and (+)-**5** have been published by Overman in 1993 (9) and E. Lee in 1995 (10), respectively.



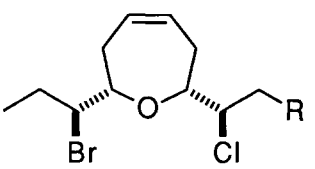
1



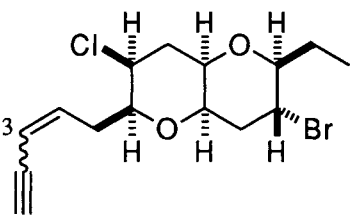
2 (3*E* and 3*Z*)



3,
R=(*E*)-CH=CH-C≡CH



4,
R=(*E*)-CH=CH-C≡CH



5 (3*E* and 3*Z*)

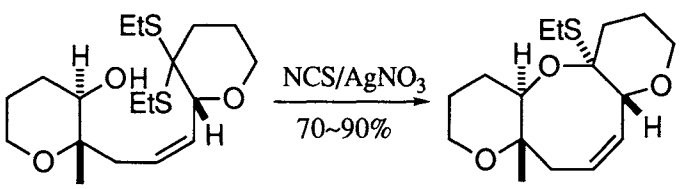
The author has surveyed these results, here focusing on our synthetic studies.

2. TOTAL SYNTHESIS OF (+)-LAURENCIN

2.1 General Strategy for the Construction of Medium Sized Cyclic Ethers

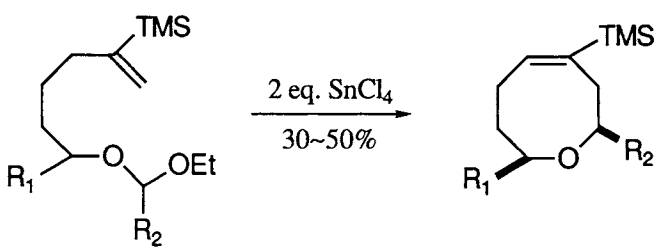
The significant biological activity of natural products containing eight-membered cyclic ethers (1,2,11) as well as the well-known difficulties in forming medium sized rings have accelerated the development of new methodologies for constructing medium cyclic ethers. Prior to the 1980s, few useful procedures existed for preparing eight-membered cyclic ethers. However, during the past decade, a number of important new methods have been reported. These approaches fall into three strategy groups shown in Scheme 1. Representative examples of the each group are shown below.

Group 1. C-O Bond-forming cyclizations (12).



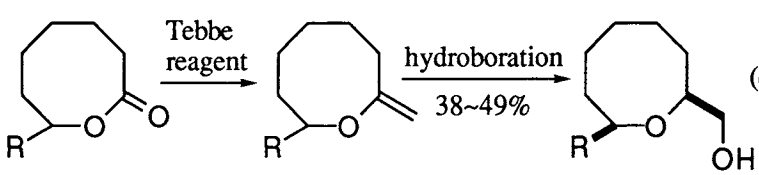
(eq. 1)(12f)

Group 2. C-C Bond-forming cyclizations (13).



(eq. 2)(13d)

Group 3. Transformations of eight-membered lactones (14).

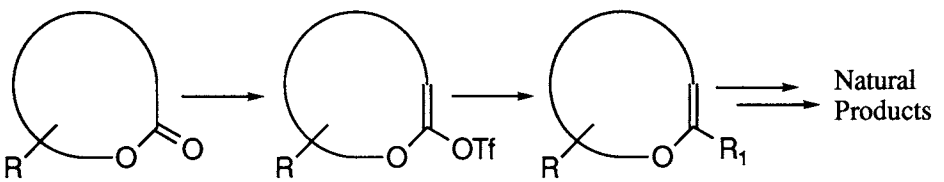


(eq. 3)(14b)

Scheme 1.

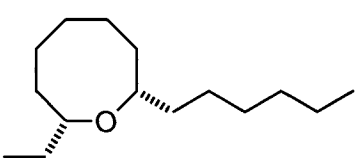
Although oxocenes and oxocanes have been derived through a variety of these new synthetic strategies, none can be categorized as entirely flexible. To date, only an early approach to laurencin (**1**)(3) and a recent synthesis of laurenyne (**3**)(4) have been reported.

The objective of our efforts in this area is the development of a new and general synthetic strategy of substituted cyclic ethers that involves conversion of lactones into substituted cyclic ethers *via* their enol trifluoromethanesulfonates (triflates) as shown below (Scheme 2), and demonstrations of their practicability.



Scheme 2.

Enol triflates derived from ketones or aldehydes have been used and recognized in a broad spectrum of reactions used in synthetic organic chemistry (15-18). Owing to the versatile reactivities of enol triflates, these can be transformed to dienes, conjugated enones, unsaturated esters, and alkylated and/or reduced alkenes (19). While preparation and reactivity of enol triflates derived from aldehyde and ketone have been studied extensively, those from ester or lactone carbonyl have not yet been reported (19). The author describes herein the conversion of lactones into substituted cyclic ethers *via* lactone enol triflates (20) and its application to the synthesis of (+)-lauthisan (**6**)(21) as well as the total synthesis of (+)-laurencin (**1**)(5).



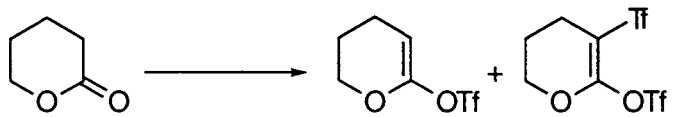
(+)-**6**

2-2 Conversion of Lactone into Substituted Cyclic Ether *via* Lactone Enol Triflate and Its Application to the Synthesis of (+)-Lauthisan

In spite of the versatile utility of enol triflates in organic synthesis, those from ester or lactone carbonyl have not yet been investigated. According to our synthetic strategy involving the conversion of lactones into substituted cyclic ethers *via* their enol triflates, we have initially investigated the preparation of lactone enol triflates.

After many fruitless attempts, we found a new reaction condition for the preparation of 6-trifluoromethanesulfonyloxy-3,4-dihydro-2*H*-pyran (**8**) starting with a lactone (**7**) in a stable state (Table 1). Table 1 indicates that (i) as compared to triflic anhydride (Tf_2O), *N*-phenyl triflimide ($PhNTf_2$) as a triflating reagent gave better results (runs 5 and 6); (ii) lithium hexamethyldisilazide (LiHMDS) (runs 4 and 6) as a base also gave better results rather than lithium diisopropylamide (LDA).

Table 1
Preparation of lactone enol triflate.



7 **8** **9**

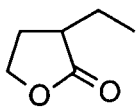
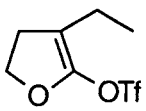
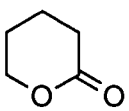
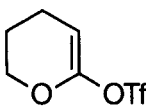
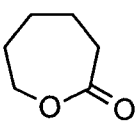
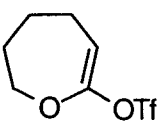
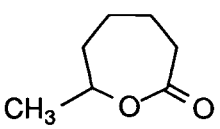
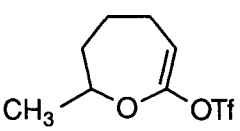
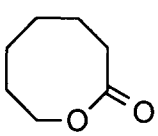
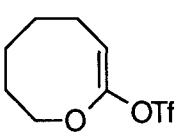
Run	Conditions	Yield (%) of enol triflates **8**	**9**	Recovery of **7** (%)
1	1) LDA/THF → 2) Tf_2O	25	25	50
2	1) LDA/DME → 2) Tf_2O	trace	40	60
3	1) LDA/THF-HMPA → 2) $PhNTf_2$	29	0	71
4	1) LiHMDS/DME → 2) $PhNTf_2$	25	0	75
5	1) LiHMDS/THF-HMPA → 2) Tf_2O	no reaction		
6	1) LiHMDS/THF-HMPA → 2) $PhNTf_2$	75	0	25

The considerable difference between LDA and LiHMDS clearly resulted from the steric hindrance and acidity of the corresponding amines {diisopropylamine (i-Pr_2NH): pKa (THF)=33.1, hexamethyldisilazane [$(Me_3Si)_2NH$]: pKa (THF)=24.7](22)}; (iii) hexamethylphosphoric triamide (HMPA) was essentially needed for the preparation

of enol triflate (O-triflated product) (runs 3 and 6). In the absence of HMPA, the C-triflated product (**9**) was predominant (runs 1 and 2).

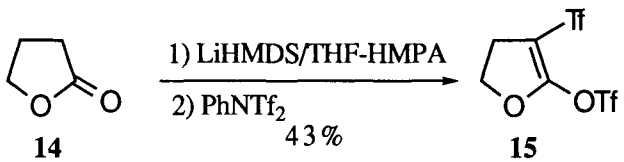
Schreiber et al. have also reported similar results (23). Furthermore, we found that a trace amount of HMPA is indispensable for stocking the enol triflate in a stable state. On the basis of these results, we established the best reaction conditions

Table 2
Conversion of lactones into enol triflates.

Run	Lactone	Enol triflate	Yield (%)[a]
1	 **10**	 **11**	32 (63)[b]
2	 **7**	 **8**	75
3			89
4			95
5	 **12**	 **13**	0

a) Since each product contains a trace amount of HMPA, the yield in the Table is estimated by 400 MHz ^{1}H NMR analysis. b) The value in the parenthesis denotes as the yield based on the recovered starting material.

shown in run 6. These reaction conditions could be applied to several lactones (20). The results are summarized in Table 2. The data indicate that (i) six- or seven-membered lactones could be converted efficiently to the corresponding enol ethers (run 2, 3, and 4); (ii) although the reaction with five-membered lactone (**10**) proceeded completely, the desired enol triflate (**11**) was isolated only in a poor yield because of its labile property (run 1); (iii) eight-membered lactone (**12**) did not lead to production of its enol triflate (**13**) under the present conditions, but resulted in oligomerization (run 5). The reaction of γ-butyrolactone (**14**) with LiHMDS in tetrahydrofuran (THF) and HMPA, followed by $PhNTf_2$ under the same conditions afforded 4-trifluoromethanesulfonyl-5-trifluoromethanesulfonyloxy-2*H*,3*H*-dihydrofuran (**15**) in 43% yield, along with the recovered starting material (**14**, 40%) (Scheme 3). The use of LDA in THF alone under the usual conditions led to almost the same results as above, the desired enol triflate not being detected even in traces.

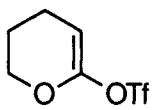
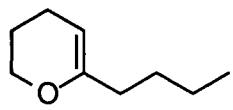
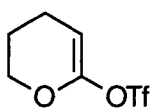
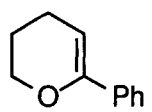
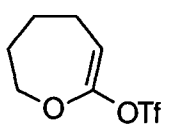
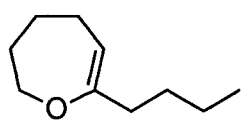
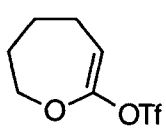
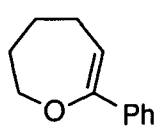
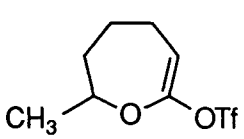
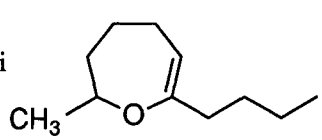
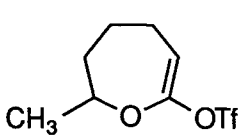
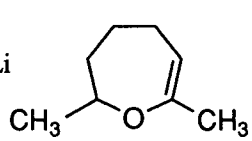


14 **15**

Scheme 3.

Without further purification, the enol triflates thus obtained were smoothly coupled with various lithium dialkylcuprates (R_2CuLi)(24) to give the corresponding α-alkylated cyclic enol ethers in good yields, as summarized in Table 3 (20). The data reveal clearly that the reaction proceeded smoothly as expected in each case. We have attempted the conversion of lactone (**12**) into its enol triflate (**13**) under several conditions. For example, we have investigated the nonnucleophilic bases {such as 9-borabicyclo[3.3.1]nonyl trifluoromethanesulfonate (9-BBNOTf)/ diisopropylethylamine (*i*-Pr_2NEt), dibutylboron triflate (Bu_2BOTf)/*i*-Pr_2NEt, diisopropylaminomagnesium bromide (*i*-Pr_2NMgBr), potassium hexamethyldisilazide (KHMDS), or sodium hexamethyldisilazide (NaHMDS)}, additives [18-crown-6, or 15-crown-5 ethers, HMPA, *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TMEDA)] and solvents (THF, ether, or toluene) for the preparation of the enol triflate (**13**). Unfortunately, all attempts under these conditions only resulted in complex mixtures or in recovery of the starting materials. The failure of the direct conversion of the lactone (**12**) into its enol triflate (**13**) led us to investigate the two step preparation of **13**. The first step involves the transformation of lactone (**12**) into the corresponding cyclic silylketene acetal (**16**). Although silylketene acetals can usually be prepared by using a strong base, e.g., LDA or LiHMDS, with the eight-membered lactone (**12**) it required alternative reaction conditions for the preparation of the silylketene acetal (**16**), because such a strong base was found to cause a

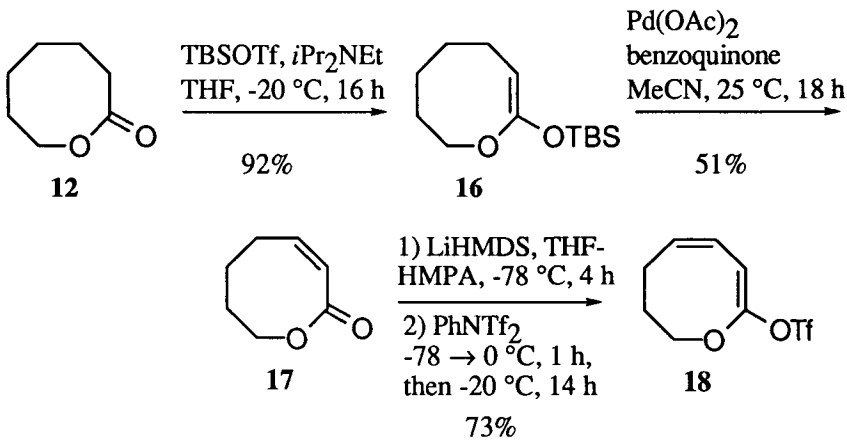
Table 3
Conversion of enol triflates into alkylated cyclic enol ethers.

Run	Enol triflate	R_2CuLi	Product	Yield (%)
1	 **8**	Bu_2CuLi		65
2	 **8**	Ph_2CuLi		40
3		Bu_2CuLi		60
4		Ph_2CuLi		71
5		Bu_2CuLi		72
6		Me_2CuLi		58

transannular reaction instead of the desired enolate anion formation. Eventually, we found that treatment of **12** with *t*-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf) and *i*-Pr_2NEt in THF provided the desired silylketene acetal (**16**) in a quantitative yield (Scheme 4). The course of the reaction depends on the basicity of the amine and the polarity of the solvent [TBSOTf, 2,6-di-*t*-butylpyridine, CH_2Cl_2,

0 °C, no reaction: TBSOTf, *i*-Pr_2NEt, CH_2Cl_2, 0 °C, a trace amount of **16**). For the second step for the conversion of **16** into the desired enol triflate, several reactions were investigated under various conditions [cesium fluoride (CsF), THF; tetrabutylammounium fluoride (TBAF), THF; CsF, dimethyl sulfoxide (DMSO); methyllithium (MeLi), ether]. However, the desired triflate (**13**) was not detected.

In the case of the eight-membered lactone (**12**), we could not transform it into the corresponding enol triflate (**13**), only oligomerization occurring. The results seem to be due to a transannular reaction between the lactone oxygen and the proton at the 4-carbon atom. We expected that the more rigid conformation might suppress such a transannular reaction. Accordingly, we changed the lactone (**12**) to the 2-heptenolide (**17**) as the starting material. The compound (**17**) was easily obtained by Saegusa oxidation reaction (25) starting from **16** in moderate yield (Scheme 4). When the heptenolide (**17**) was then treated under the same consitions as described above, the reaction proceeded as expected to yield the desired dienol triflate (**18**) in a good yield (73%)(21). Such dienol triflates without further purification were smoothly coupled with lithium dialkylcuprates to afford the corresponding α-alkylated oxocenes in good yields (Table 4)(21).

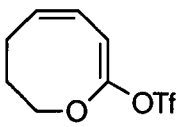
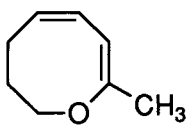
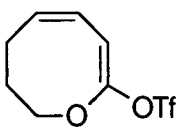
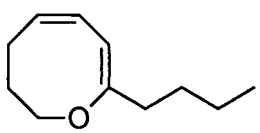
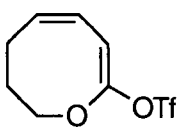
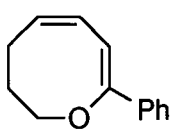
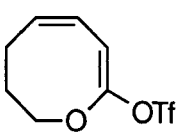
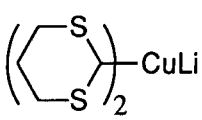
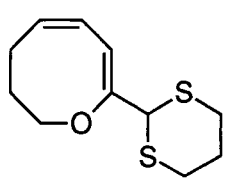
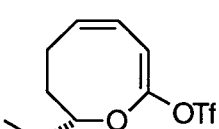
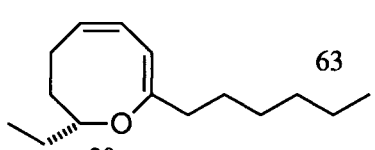


Scheme 4.

We have therefore developed a new and general method for the conversion of lactones into substituted cyclic ethers *via* lactone enol triflates. This methodology would be valuable as it provides a general procedure for the synthesis of various marine natural products including (+)-laurencin (**1**). First, we applied the methodology to the synthesis of (+)-lauthisan (**6**)(Scheme 5)(21). (+)-Lauthisan (**6**) is an artificial compound derived from natural laurencin (+)-**1** by chemical reactions (26). The compound has often served as the testing ground for demostrating the construction

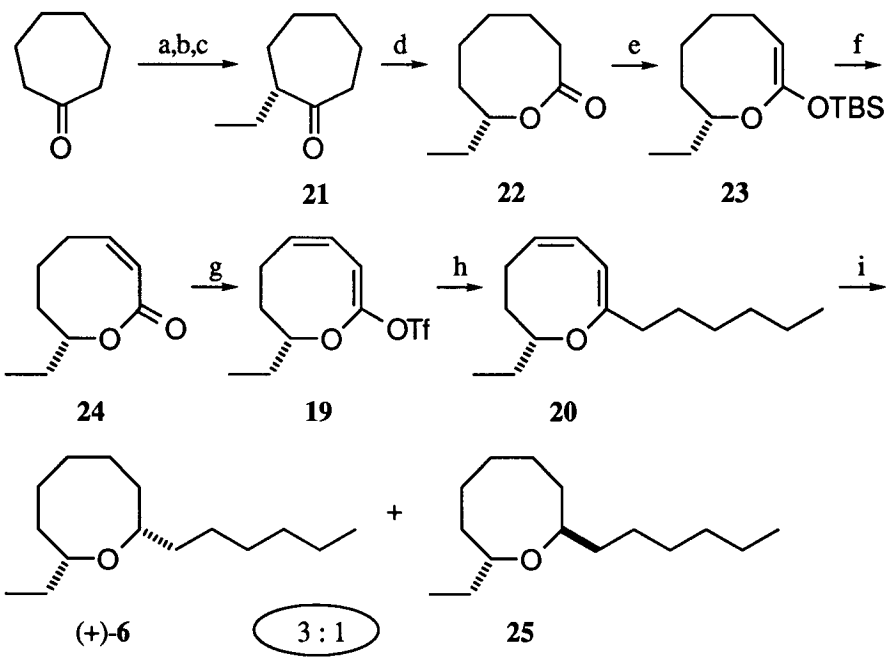
of oxocene framework (14d,27).

Table 4
Conversion of dienol triflates into alkylated dihydrooxocenes.

Run	Dienol triflate	R_2CuLi	Dihydrooxocene	Yield (%)
1	 **18**	Me_2CuLi		60
2	 **18**	Bu_2CuLi		82
3	 **18**	Ph_2CuLi		82
4	 **18**			74
5	 **19**	$(Hex)_2CuLi$	 **20**	63

Our synthesis commenced with the known compound, (*R*)-2-ethylcycloheptanone (**21**)(27a,28). The compound (**21**) was oxidized with *m*-chloroperbenzoic acid (*m*CPBA) to afford (*R*)-7-ethylheptanolide (**22**), $[\alpha]_D^{24}$ -33.8° (c 0.87, $CHCl_3$), in 86% yield. Treatment of **22** with TBSOTf and *i*-Pr_2NEt in THF at -20 °C for 16 h gave the cyclic silylketene acetal (**23**) in quantitative yield. The yield of the oxidation reaction of **23** was much improved by use of the Tsuji procedure (29). Oxidation of **23** with allyl methylcarbonate in the presence of catalytic amount of palladium(II)

acetate in acetonitrile (MeCN) under reflux for 6 h gave the desired (*R*)-7-ethyl-2-heptenolide (**24**), m. p. 53-54 °C, $[\alpha]_D^{25}$ +23.5° (c 1.02, $CHCl_3$), in 83% yield. Compound (**24**) was reacted with LiHMDS in THF-HMPA at -78 °C and then treated with $PhNTf_2$ to afford the crude dienol triflate (**19**) in 60% yield, which was immediately reacted with lithium dihexylcuprate, freshly prepared from hexyllithium and copper(I) iodide, in a 5:1 mixture of THF and ether at -20 °C for 15 h to afford (*R*)-2-ethyl-8-hexyl-3,4-dihydro-2*H*-oxocene (**20**) in 63% yield. Finally, compound (**20**) was hydrogenated over 10% palladium-carbon in THF under 1 atm at room temperature to give a 3:1 epimeric mixture of saturated compounds in 78% yield, which was separated by preparative thin layer chromatography over silica gel into **6** and **25**. The major product (**6**) was identical with an authentic sample of (+)-lauthisan in all respects, while the minor product (**25**) was found to be 8-epi-*trans*-lauthisan



Reagents and conditions: (a) SAMP, 25 °C, 16 h, 95%; (b) LDA/THF, -78 °C, 1 h → 0 °C, 17 h, then EtI, THF, -78 °C → -30 °C, 6 h, 77%; (c) 3M HCl aq., pentane, 25 °C, 16 h, 87%; (d) *m*CPBA, CH_2Cl_2, 20 °C, 2 days, 86%; (e) TBSOTf, *i*-Pr_2NEt, THF, -20 °C, 16 h, 95%; (f) allyl methylcarbonate, $Pd(OAc)_2$, MeCN, reflux, 6 h, 83%; (g) LiHMDS/THF-HMPA, -78 °C, 4 h, then $PhNTf_2$, THF, -78 °C, 0.5 h → 20 °C, 2 h, 60%; (h) Hex_2CuLi, THF-ether, -20 °C, 15 h, 63%; (i) 10% Pd/C, H_2, THF, 25 °C, 4 h, 78% total yield.

Scheme 5. Synthesis of (+)-lauthisan (**6**).

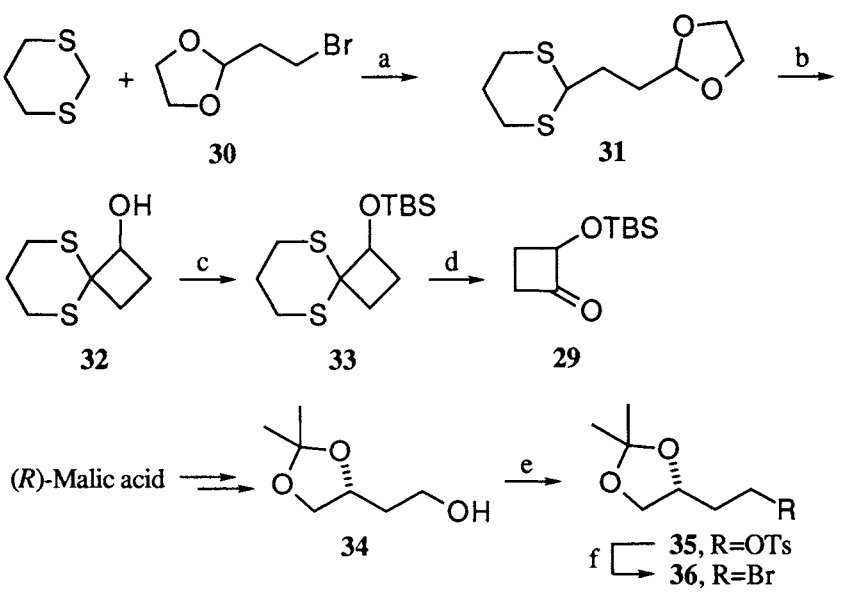
from the spectral data. The present synthesis of (+)-lauthisan involves six steps from the known compound (**21**) with an overall yield of 15%.

The optical purity of compound **22** was determined as follows. The compound (**22**) was treated with 1M sodium methoxide (MeONa)/MeOH solution at 20 °C for 17 h to afford the hydroxy ester (**26**) in 81% yield. Esterification of **26** with (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] in the presence of triethyl-amine (TEA) and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2 at 20 °C for 16 h afforded the corresponding MTPA ester (**27**), whose optical purity was determined to 91% e.e. by ^1H NMR analysis.

We thus demonstrated that our new methodology is practical enough to synthesize the basic eight-membered cyclic ether framework. Our next target was the total synthesis of (+)-laurencin (**1**).

2-3 Total Synthesis of (+)-Laurencin

The title compound (**1**) has been isolated from *Laurencia gladulifera* Kutzing (2). The structure of **1** was initially suggested on the basis of extensive spectroscopic



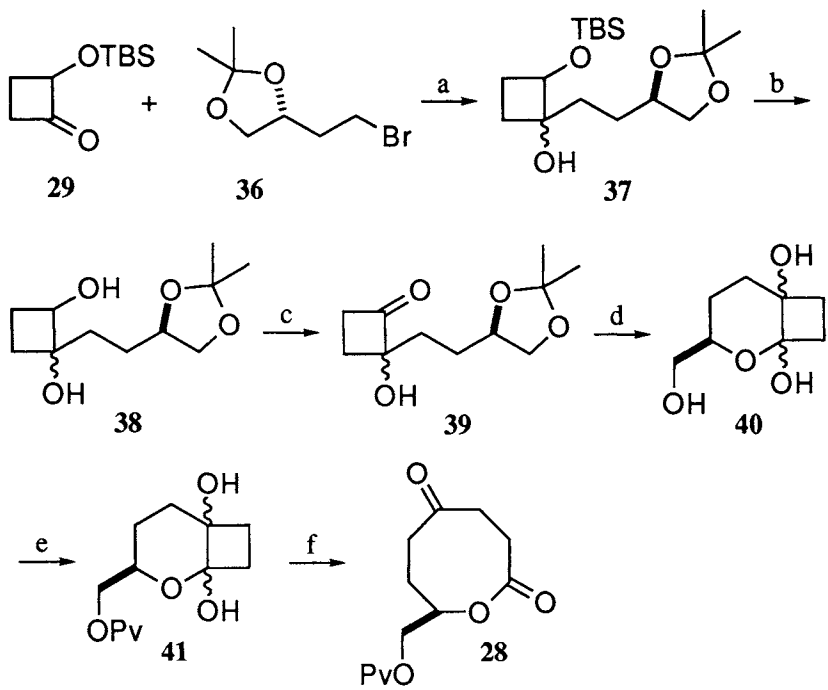
Reagents and conditions: (a) BuLi, THF-HMPA, -20 °C, 12 h → 20 °C, 3 h, 75%; (b) BuLi (2 eq.), THF, -50 °C, 2 h → -20 °C, 16 h, 93%; (c) TBSCl, imidazole, DMF, 20 °C, 19 h, 100%; (d) MeI (5 eq.), $NaHCO_3$ (2 eq.), 80% aq. acetone, reflux, 4 h, 70%; (e) TsCl, TEA, DMPA, CH_2Cl_2, 5 °C, 4 h → 20 °C, 2 h; (f) LiBr, acetone, 25 °C, 36 h, 76%.

Scheme 6. Preparation of the starting materials (**29** and **36**).

evidence (2) and later confirmed by a X-ray analysis (30). The absolute configuration was assigned by application of Prelog's atrolactic method (31) to a laurencin degradation product (2) and by X-ray crystallography (30) We have succeeded in the first total synthesis of (+)-**1** involving our established conversion of readily available medium-sized lactones to α-alkylated ethers *via* enol triflates as the key synthetic step (5,20,21). The synthesis started with the construction of the 4-ketoheptanolide (**28**)(Schemes 6, 7). By our quite novel method (32), the starting 2-*t*-butyldimethylsilyloxycyclobutanone (**29**) has been prepared (Scheme 6). 2-Lithio-1,3-dithiane prepared from 1,3-dithiane and butyllithium (BuLi) in THF was reacted with commercially available 2-(2'-bromoethyl)-1,3-dioxolane (**30**) in the presence of HMPA (-20 °C, 12 h and 20 °C, 3 h) to afford the compound (**31**) in 75% yield. Treatment of **31** with 2 eq. of BuLi in THF (-50 °C for 2 h, then -20 °C for 16 h) gave the spiro compound (**32**) by a migration reaction of 1,3-dioxolane ring in a high yield (93%). The hydroxy group in **32** was protected by *t*-butylchlorodimethylsilane (TBSCl) and imidazole in DMF (20 °C, 19 h) to give **33** in a quantitative yield. The protected compound (**33**) was hydrolyzed with iodomethane (5 eq.) in 80% aqueous acetone in the presence of sodium bicarbonate ($NaHCO_3$, 2 eq.) under reflux for 4 h to give the desired cyclobutanone derivative (**29**) in 70% yield. On the other hand, (*R*)-4-(2'-bromoethyl)-2,2-dimethyl-1,3-dioxolane (**36**) was derived from unnatural (*R*)-malic acid (33). Thus, (*R*)-4-(2'-hydroxyethyl)-1,3-dioxolane (**34**) was converted into its tosylate (**35**) [*p*-toluenesulfonyl chloride (TsCl), TEA, and a catalytic amount of DMAP, CH_2Cl_2, 5 °C, 4 h, then 20 °C, 2 h]. The crude tosylate (**35**) was treated with anhydrous lithium bromide (LiBr) (25 °C, 36 h, acetone) in 76% combined yield from **34**. Compounds **29** and **36** thus obtained were coupled as follows (Scheme 7). A solution of lithium metal in THF was irradiated with ultrasound at 5 °C for 30 min. To the solution was added dropwise a solution of **29** and **36** in THF at -78 °C for 2 h.. The reaction mixture was stirred at -78 °C for 24 h to afford a coupling product (**37**) in 77% yield. The TBS group of **37** was smoothly removed by using 1M TBAF solution in THF at 0 °C for 5 min to afford the cyclobutanediol (**38**) in 95% yield. Using the Parikh reagent [sulfur trioxide pyridine complex (SO_3•Py)](34), the compound (**38**) was then oxidized in the presence of TEA in the mixed solution of DMSO-CH_2Cl_2 (2:1) at 0 °C to 20 °C for 30 min to give the desired cyclobutanone (**39**) in 86% yield. Acid treatment of the cyclobutanone (**39**) with 4 mmol% of *p*-toluenesulfonic acid (PTS) in 90% aqueous THF solution at 20 °C for 36 h yielded the 2-oxabicyclo[4.2.0]octane triol (**40**) in 93% yield. The triol (**40**) was reacted with pivaloyl chloride (PvCl) and pyridine (Py) in THF at -10 °C for 3 h, then at 20 °C for 24 h to give the desired product (**41**), which was immediately treated with lead(IV) tetraacetate in toluene at 0 °C for 15 min, then at 20 °C for 1.5 h to afford the desired ketolactone (**28**) in high yield (90%) as colorless crystals. The structure of **28** was confirmed by ^1H NMR, IR, EI-MS, and EI-HRMS spectra.

X-ray analysis also supported the structure of **28**.

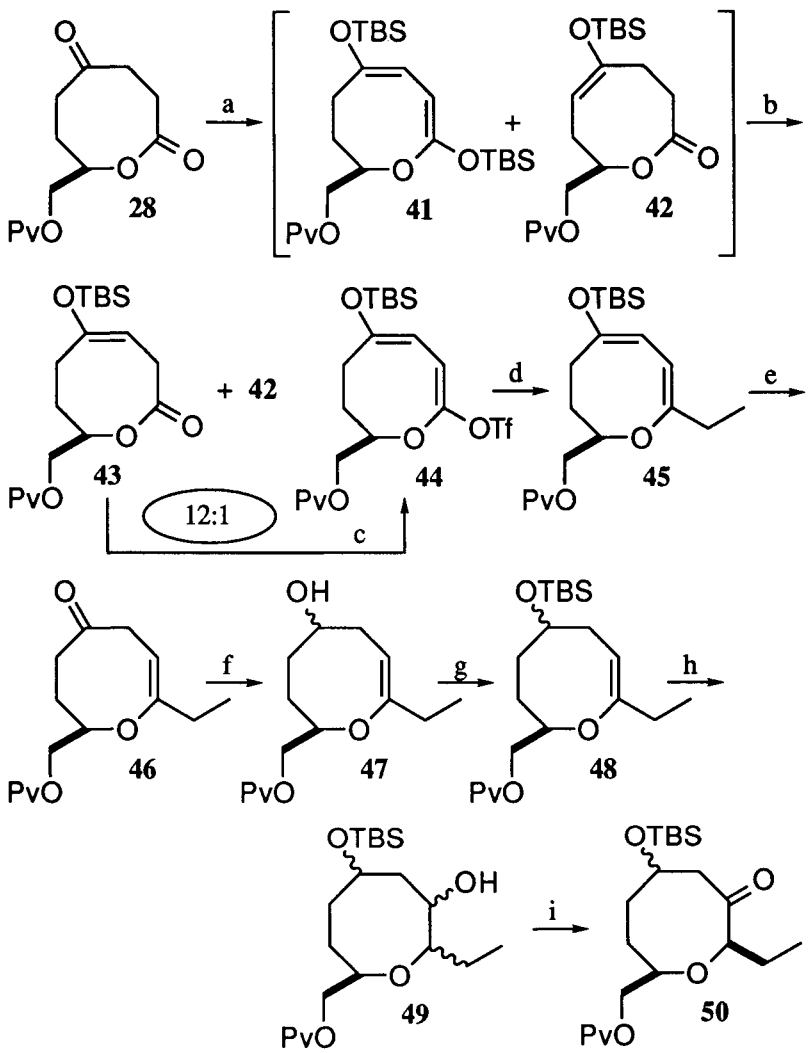
Now, we were ready to transform the lactone (**28**) into the oxocene system in a stereoselective manner *via* the lactone enol triflate. The ketolactone (**28**) was initially transformed into a mixture of dienol silyl ether (**41**) and enol silyl ether (**42**) [TBSOTf, TEA, CH_2Cl_2, -20 °C, 16 h] (Scheme 8). The mixture of **41** and **42** was immediately passed through a silica gel column (elution with CH_2Cl_2) to afford the separable enol ethers (**43** and **42**) in a ratio of 12:1. Next, we directed our efforts to



Reagents and conditions: (a) Li (3 eq.), THF, 5 °C, 30 min (ultrasound), then **29** and **36**, THF, -78 °C, 24 h, 77%; (b) 1M TBAF, THF, 0 °C, 5 min, 95%; (c) SO_3•Py (5 eq.), TEA (10 eq.), DMSO-CH_2Cl_2, 0~20 °C, 30 min, 86%; (d) PTS (4 mmol%), 90% aq. THF, 20 °C, 36 h, 93%; (e) PvCl (1.15 eq.), Py (1.2 eq.), THF, -10 °C, 3 h → 20 °C, 24 h, 86%; (f) $Pb(OAc)_4$, toluene, 0 °C, 15 min → 20 °C, 1.5 h, 92%.

Scheme 7. Preparation of the key intermediate ketolactone (**28**).

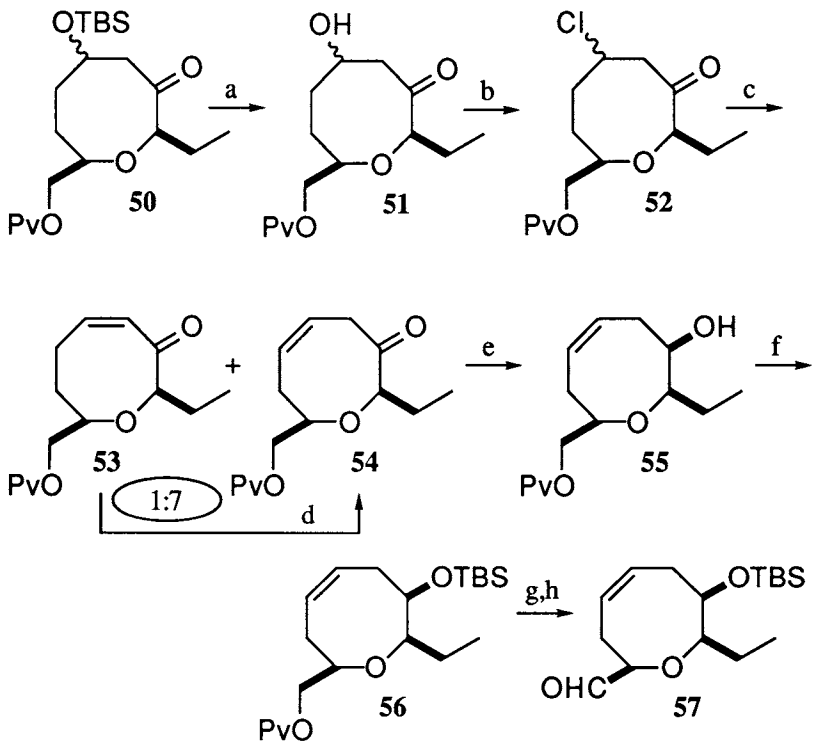
the preparation of the lactone enol triflate, one of the most crucial intermediates in the total synthesis of (+)-laurencin. Treatment of the lactone (**43**) with LiHMDS in THF-HMPA (8 eq.) (-70 °C, 30 min), and then with $PhNTf_2$ afforded the dienol triflate (**44**) in 65% yield. Successive treatment of the triflate (**44**) with lithium diethylcuprate, freshly prepared from ethyl lithium and copper(I) iodide, in a 3:1



Reagents and conditions: (a) TBSOTf (2.5 eq.), TEA (5 eq.), CH_2Cl_2, -20 °C, 16 h; (b) SiO_2, CH_2Cl_2, 77% (**43**+**42**, from **28**); (c) LiHMDS (1.1 eq.), THF-HMPA (8 eq.), -70 °C, 30 min, then $PhNTf_2$ (1.1 eq.), -70 °C, 15 min → 20 °C, 30 min, 65%; (d) Et_2CuLi, THF-ether (3:1), -20 °C, 18 h, 70%; (e) 1M TBAF, THF, -70 °C, 1 h, 80%; (f) $NaBH_4$, THF-EtOH (6:1), -20 °C, 2 h, 97%; (g) TBSOTf (2 eq.), TEA (5 eq.), CH_2Cl_2, -40 °C, 1.5 h, 100%; (h) BH_3•THF, THF, -15 °C, 2 h, then 30% H_2O_2, 10% NaOH, 0 °C, 2 h, 73%; (i) $(COCl)_2$ (2 eq.), DMSO (2.2 eq.), TEA (10 eq.), CH_2Cl_2, -78 °C, 30 min → -78~0 °C, 30 min, 95%.

Scheme 8. Transformation of the ketolactone (**28**) into the β-siloxy ketone (**50**).

mixture of THF and ether at -20 °C for 18 h provided the compound (**45**) in 70% yield. The compound (**45**) was transformed into the β-siloxy ketone (**50**) by the usual five-step sequence in 53% total yield [(i) removal of TBS group of **45** with 1M TBAF in THF, -70 °C, 1 h, 80%; (ii) reduction of **46** with $NaBH_4$ in THF-EtOH (6:1), -20 °C, 2 h, 97%; (iii) protection of **47** with TBSOTf, CH_2Cl_2, -40 °C, 1.5 h, 100%; (iv) hydroboration and oxidation of **48** with BH_3•THF, in THF, -15 °C, 2 h, and then 30% H_2O_2, 10% NaOH, 0 °C, 2 h, 73%; and (v) Swern oxidation of **49** with oxalyl chloride, DMSO, TEA, CH_2Cl_2, -78 °C, 30 min, and then -78~0 °C, 30 min, 95%]. During the last step (**49** → **50**), the ethyl group at the α-position of the ether oxygen was epimerized to the exclusively *cis*-configuration on reaction with TEA (27c). Compound (**50**) was clearly observed only as one pair of the diastereomers

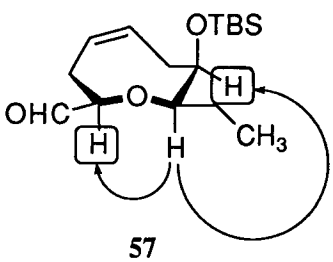


Reagents and conditions: (a) 10% solution of HF in MeCN, 0 °C, 45 min, 96%; (b) Oct_3P (2.5 eq.), CCl_4, 60 °C, 1 h; (c) DBU, toluene, 20 °C, 1 h → 100 °C, 1.25 h, 93% from **51**; (d) DBU, toluene, 100 °C, 5 h, 60%; (e) L-Selectride (1.2 eq.), THF, -70 °C, 1.5 h, 91%; (f) TBSOTf (1.5 eq.), TEA (1.5 eq.), CH_2Cl_2, -45 °C, 3 h, 90%; (g) DIBAH, CH_2Cl_2, -45 °C, 3 h, 100%; (h) $(COCl)_2$ (2 eq.), DMSO (2.2 eq.), CH_2Cl_2, -78 °C, 30 min, then TEA (10 eq.), 0 °C, 30 min, 94%.

Scheme 9. Preparation of the oxocenecarboxaldehyde (**57**).

arising from the C-4 asymmetric carbon atom by ^{1}H NMR analysis.

Further transformation of compound **50** into the target aldehyde (**57**) is described in Scheme 9. We had already achieved the introduction of two stereocenters, which were situated in the 2- and 8-positions on the oxocene ring. At this stage, we faced the following problems to be solved: (i) regioselective introduction of a double bond at the C-4 of the oxocane ring system; and (ii) stereoselective reduction of the C-2 ketone in the molecule (**54**). Compound (**50**) was reacted with hydrogen fluoride (HF) [MeCN, 0 °C, 45 min, 96%] and coverted into a 1:7 mixture of α,β- (**53**) and β,γ-unsaturated ketones (**54**) in a 93% combined yield in a two-step process {(i) chlorination, trioctyl phosphine (Oct_3P) in CCl_4, 60 °C, 1 h, (ii) dehydrochlorination, 1,8-diazabicyclo[4.5.0]undec-7-ene (DBU), toluene, 20 °C, 1 h, and then 100 °C, 1.25 h}. The minor product (**53**) was isomerized to **54** on reaction with DBU in toluene (100 °C, 5 h, 60%). The ketone (**54**), when treated with lithium tri-*sec*-butylborohydride (L-Selectride) in THF (-70 °C, 1.5 h), was transformed into the β-hydroxy derivative (**55**) as the sole product (91%), which was converted in the usual manner [TBSOTf, TEA, CH_2Cl_2, -45 °C, 3 h, 90%] to the protected compound (**56**). Finally, we could convert it to the desired compound (**57**) after reduction with diisobutylaluminium hydride (DIBAH) (-45°C, 3 h, CH_2Cl_2, 100%) and Swern oxidation reaction. The stereochemistry of the aldehyde (**57**) was also confirmed by NOE difference measurements as shown in Figure 1.



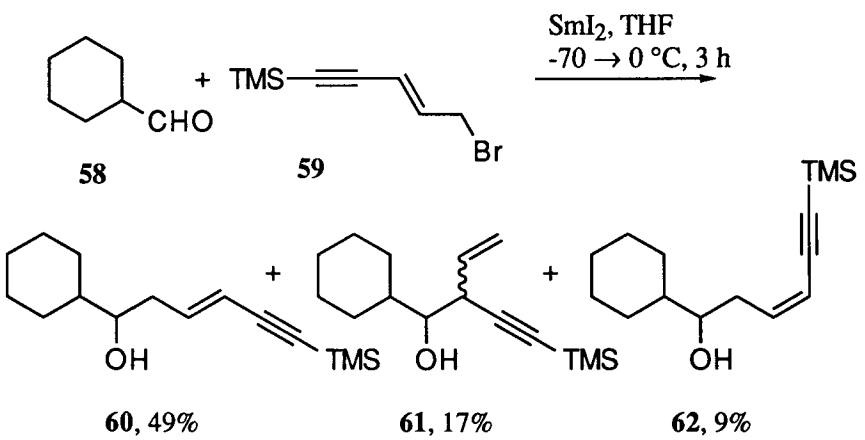
57

Fig. 1. The NOE difference interactions in compound (**57**).

The ketone at C-2 in **50** played an important role in the stereoselective synthesis of the oxocene aldehyde (**57**). Namely, the compound (**49**), which was obtained by the hydroboration oxidation reaction, had four stereocenters on the oxocane ring, and existed as a complex mixture of its diastereomers. However, subsequent oxidation of the secondary alcohol into the ketone (**50**) produced only one pair of diastereomers arising from the C-4 asymmetric carbon atom. Basic conditions during the usual work up (TEA) in the Swern oxidation resulted in epimerization at the 2-substituent to the most stable configuration, in which the two substituents at C-2 and C-8 positions were located *cis* each other and adopted equatorial conformations on the oxocane ring. Moreover, the ketone is also important for regioselective introduction

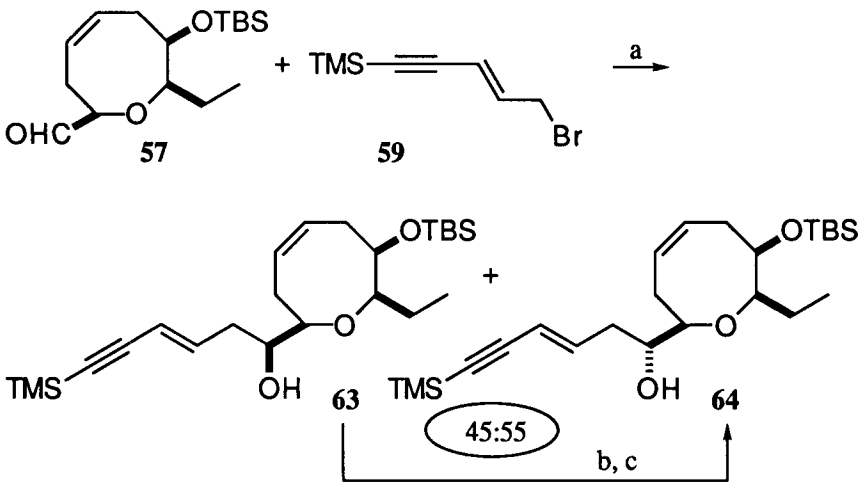
of the double bond on the oxocane ring. The chlorinated compound **52** was treated with DBU to afford the β,γ–unsaturated ketone as the major product. The β,γ-unsaturated ketone is predominantly produced as the thermodynamically more stable substance in comparison to the α,β-unsaturated ketone in the medium ring systems. Finally, the ketone (**54**) was stereoselectively reduced with hydride anion from the sterically less hindered side (α-face) to afford the β-hydroxy compound (**55**) as the sole product. These results clearly reveal that (i) the double bond and substituents were introduced exactly as desired due to the presence of the C-2 ketone and (ii) our retrosynthetic analysis is clearly reasonable. Introduction of the allylic enyne moiety was the next problem to be solved as shown in the following section.

The stage was now set to introduce the whole C-5 enyne unit to the aldehyde (**57**). Although the total syntheses of two natural products including the similar enyne moiety have been reported (4b,9), there have been no examples for the introduction of the whole C-5 enyne unit directly. After many fruitless attempts, we accomplished the model coupling reaction by use of samarium(II) iodide (SmI_2)(35) (Scheme 10). Cyclohexanecarboxaldehyde (**58**), when treated with the enyne bromide (**59**) in the presence of SmI_2 (3 eq.) in THF (-70 → 0 °C, 3 h), was transformed into the α-attack compound (**60**) in 49% yield along with the γ-attack product (**61**, 17%) and the *cis*-isomer (**62**, 9%).



Scheme 10.

In the light of these results, we applied the coupling reaction to the real system, involving the oxocene aldehyde (**57**) and the enyne bromide (**59**) under the same conditions as above (Scheme 11). The reaction proceeded smoothly to afford a 45:55 separable mixture of β-(**63**) and α-enyne alcohols (**64**) in 71% total yield. It was remarkable that even a trace amount of the corresponding γ-attacked product

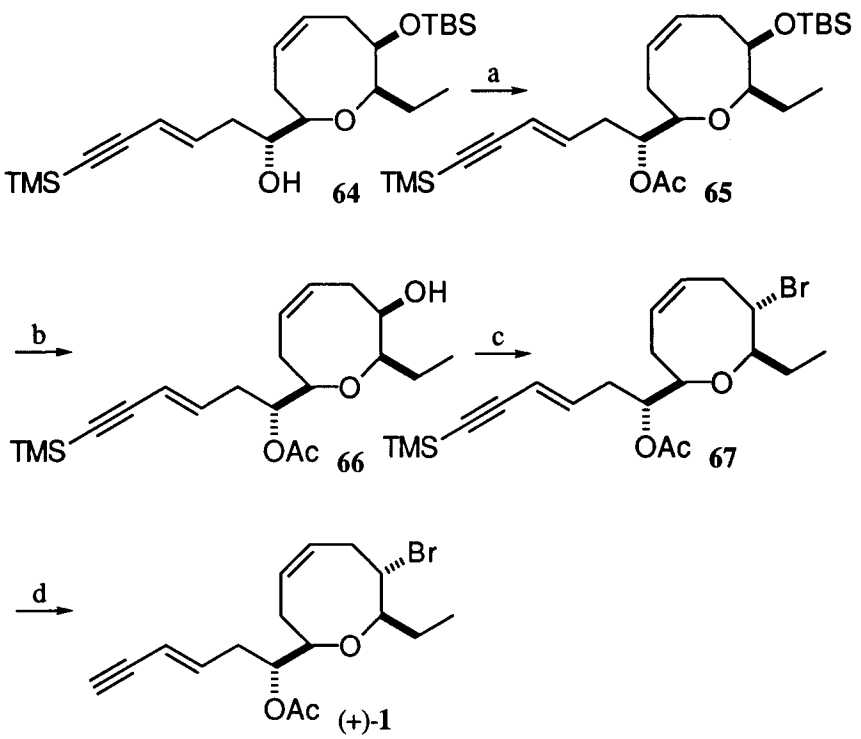


Reagents and conditions: (a) SmI_2 (3 eq.), THF, -70 → 0 °C, 4 h, 71%; (b) excess Jones reagent, acetone, -20 °C, 1 h, 95%; (c) L-Selectride (1.5 eq.), THF, -78 °C, 15 min, 93%.

Scheme 11. Introduction of the enyne bromide (**59**) into the aldehyde (**57**).

could not be detected. The β-alcohol (**63**) was subjected to oxidation (Jones reagent, -20 °C, 1 h, 95%) followed by reduction (L-Selectride, THF, -78 °C, 15 min, 93%) to afford exclusively the desired α-alcohol (**64**). Success of the direct coupling reaction of the whole C-5 enyne unit with oxocene aldehyde **57** prompted us to conquer the final major problem, which was the stereoselective introduction of a bromine atom into the oxocene ring.

In the usual manner, compound (**64**) was treated with acetic anhydride, Py, and a catalytic amount of DMAP in CH_2Cl_2 (20 °C, 16 h) to afford the acetate (**65**) in 96% yield (Scheme 12). In the next step, we had to chemoselectively detach the TBS group of the two silyl groups, that is, the TBS group of the secondary alcohol on the oxocene ring and the trimethylsilyl (TMS) group at the terminal acetylene moiety, because the terminal proton of the acetylene would also be brominated under the subsequent bromination conditions. In fact, it was found that treatment of **65** in 80% aqueous acetic acid (AcOH) at 20 °C for 12 h successfully removed the TBS group to give the alcohol (**66**) in 94% yield. As the final crucial step, when the compound (**66**) was reacted with carbon tetrabromide (CBr_4) in the presence of Oct_3P in toluene at 70 °C for 3.5 h, an inversion reaction occurred smoothly to provide the α-bromo derivative (**67**) in 86% yield. The TMS group of **67**, which protected the terminal acetylene in the side chain, was finally removed [TBAF-HF (20:1), pH 3~4, -15~-10 °C, 15 min, 97%] to complete the total synthesis of (+)-(**1**).



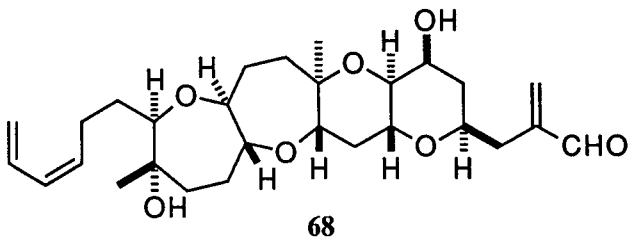
Reagents and conditions: (a) Ac_2O, Py, DMAP (cat.), CH_2Cl_2, 20 °C, 16 h, 96%; (b) 80% AcOH aq., 20 °C, 12 h, 94%; (c) CBr_4, Oct_3P, toluene, 70 °C, 3.5 h, 86%; (d) TBAF-HF (20:1), pH 3~4, -15~-10 °C, 15 min, 97%.

Scheme 12. Total synthesis of (+)-laurencin (**1**).

The synthetic sample thus obtained was identical in every respect {m.p. (36), $[\alpha]_D$ (37), ^1H NMR, IR, and MS} with the natural sample by a direct comparison. The present synthesis involves 27 steps with an overall yield amounting to 2.5% from the starting coupling reaction.

The present work constitutes the conversion of lactones into substituted cyclic ethers *via* lactone enol triflates, and the total synthesis of (+)-lauthisan (**6**) and (+)-laurencin (**1**) as examples of application of this methodology. We have established the general transformation of readily available medium-sized lactones into the corresponding cyclic ethers *via* the enol triflates, and demostrated the wide applicability of our methodology. In the total synthesis of (+)-laurencin (**1**), all asymmetric centers were prepared stereoselectively. Furthermore, two points should be noted; the first is a derivation to the desired chiral cyclic ether (**45**) and the second is an introduction of the whole enyne unit directly to the compound (**57**) at the final

critical stage. The former has been accomplished by a well-designed procedure. The latter has been performed by the new methodology using SmI_2. Considering the novel structure of the target molecule, the whole route is relatively short (27 steps) and the overall yield is rather high (2.5%). These results may be summarized as follows: (i) the lactones were converted effectively into substituted cyclic ethers *via* lactone enol triflates; (ii) total syntheses of (+)-lauthisan and (+)-laurencin were achieved by this methodology; and (iii) these results were valuable as providing a new general procedure for the synthesis of the various medium-sized cyclic ether system. After completion of these works, we have applied this procedure to the construction of the whole mother skeleton in hemibrevetoxin-B (**68**)(38), which is a fused 7,7,6,6-tetracyclic ether isolated from the red tide organism, *Gymnodium breve* (39).



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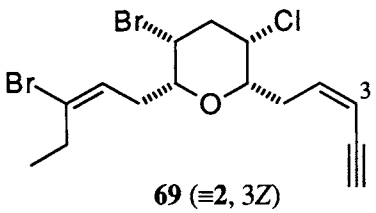
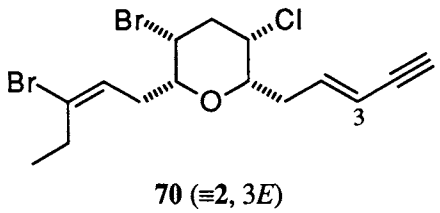
3. TOTAL SYNTHESIS OF (-)-DACTYLYNES

3-1. Regioselective Ring-Opening Reactions of Epoxy Alcohols with Halogeno-Nucleophiles

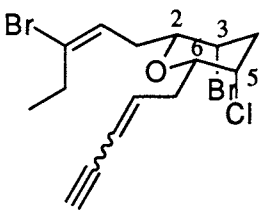
In 1975 and 1976, Schmitz et al. have isolated dactylyne (**69≡2**, 3*Z*)(40) and isodactylyne (**70≡2**, 3*E*)(41) from the digestive tract of the sea hare *Aplysia dactylomela.* The structure of **69** including its absolute stereochemistry has been determined using spectroscopic and X-ray crystallographic techniques (40). The structure of **70** was elucidated on the basis of spectral comparison with **69** (41). The absolute configurations at C-2, C-3, C-5, and C-6 are *R*, *R*, *S*, and *S*, respectively. These products are structurally characterized by unbranched 15 carbon skeletons containing tetrahydropyran rings with all *cis*-oriented four substituents, which consist of (2*E*)-3-bromo-2-pentenyl, and pentenynyl groups at C-2 and C-6, and diaxial bromine and chlorine atoms at C-3 and C-5, respectively. Both of them possess central nervous depressant activities characterized by a potentiation of pentobarbital hypnosis and exhibit nontoxic characters. These unique structures, high biological potencies, and relatively nontoxic natures make these natural products attractive synthetic targets. After many failures in attempts at replacing the hydroxyl groups by halogen atoms, we considered it impossible to construct all *cis*-substituted



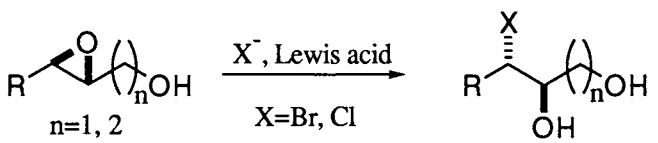
69 (≡**2**, 3*Z*)



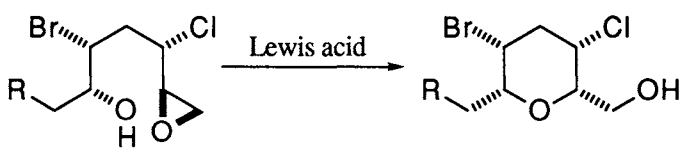
70 (≡**2**, 3*E*)



tetrahydropyran systems with halogen atoms, such as dactylynes (**69** and **70**) and dactomelynes (**3** and **4**), from the corresponding hydroxylic compounds. Our alternate strategy contains two critical reactions: the first is to construct an acyclic framework



n=1, 2 X=Br, Cl (eq. 4)

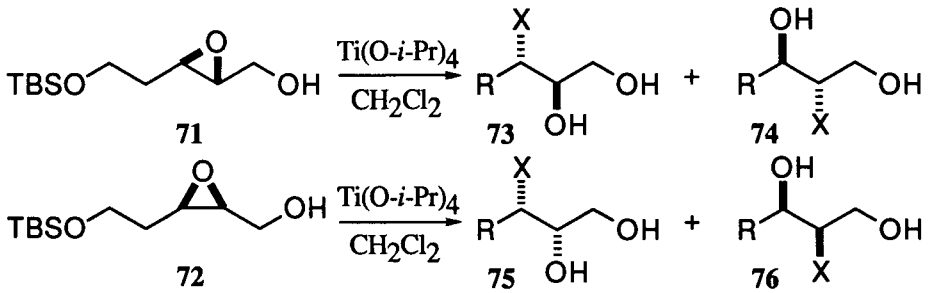


(eq. 5)

containing bromine and chlorine atoms with correct stereochemistries by the ring-opening reactions of epoxy alcohols with halogeno-nucleophiles (eq. 4), and the second is to construct an all *cis*-substituted tetrahydropyran ring *via* intramalecular cyclization of the epoxy alcohol (eq. 5). According to this strategy, we recently accomplished the first total synthesis of dactylynes (6).

Since Sharpless asymmetric epoxidation (42) has been applied extensively, regioselective ring-opening reactions of 2,3-epoxy alcohols with nucleophiles have been utilized frequently in the course of asymmetric syntheses of natural products (43). Recently, two groups have reported that regioselective openings of 2,3-epoxy alcohols with halides (Cl^- or Br^-) at 3-position are effected by use of ammonium halides in the presence of titanium(IV) isopropoxide [Ti(O-*i*-Pr)$_4$](44) or zeolite-CaY (45). However, there is still considerable scope for further developments in this

Table 5
Ring-opening reactions of 2,3-epoxy alcohols (**71** and **72**) with halogeno-nucleophiles.



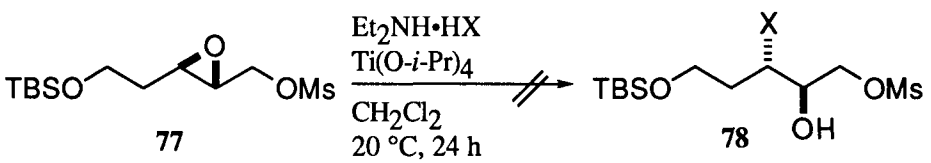
Run	Epoxide	Nucleophile	X	Yield (%) 73+74	Yield (%) 75+76	Ratio of Products 73:74	Ratio of Products 75:76
1	**71**	$EtNH_2$•HCl	Cl	78		78:22	
2	**71**	Et_2NH•HCl	Cl	87		91: 9	
3	**72**	Et_2NH•HCl	Cl		93		89:11
4	**71**	Me_2NH•HCl	Cl	80		90:10	
5	**71**	*i*-Pr_2NH_2•HCl	Cl	75		83:17	
6	**71**	Pyrrolidine•HCl	Cl	78		90:10	
7	**71**	Piperidine•HCl	Cl	71		90:10	
8	**71**	2,2,6,6-Tetramethylpiperidine•HCl	Cl	86		85:15	
9	**71**	Et_3N•HCl	Cl	89		81:19	
10	**72**	Et_3N•HCl	Cl		79		75:25
11	**71**	NH_4Cl	Cl	63		64:36	
12	**71**	Et_4NCl	Cl	0		—	
13	**71**	Pyridine•HCl	Cl	73		77:23	
14	**71**	$EtNH_2$•HBr	Br	77		76:24	
15	**71**	Et_2NH•HBr	Br	87		90:10	
16	**72**	Et_2NH•HBr	Br		94		88:12
17	**71**	Et_3N•HBr	Br	83		80:20	
18	**71**	NH_4Br	Br	72		69:31	
19	**71**	Bu_4NBr	Br	0		—	

field. After many fruitless attempts, we found that the regioselectivity in the ring-opening reactions of epoxy alcohols promoted by Ti(O-*i*-Pr)$_4$ was dependent on the

type of halogeno-nucleophiles and solvents used. At first, we investigated the effects of various nucleophiles on the titanium-mediated reactions with *trans*-2,3-epoxy-1-ol (**71**) and *cis*-2,3-epoxy-1-ol (**72**). The results are summarized in Table 5 (46).

The data indicate that when dialkylamine hydrohalides (R_2NH•HX) were used, the ring openings proceeded in satisfactory practical yields and with the higher selective nucleophilic attack of halides on the 3-position. It is emphasized that (i) diethylamine hydrohalides (Et_2NH•HX) are the most efficient for the openings at the 3-position in both cases of **71** and **72** (runs 2, 3, 15, and 16); (ii) diethylamine hydrochloride (Et_2NH•HCl) is superior to triethylamine hydrochloride, which has been reported recently to be the best chloride nucleophile for the corresponding opening of *cis*-2,3-epoxy alcohols (4b) (compare run 3 with run 10); (iii) dimethylamine hydrochloride (run 4) and cyclic secondary amine hydrochlorides (runs 6 and 7) are available as well; and (iv) tetraalkylammonium halides are inert to the epoxide rings (runs 12 and 19). These results suggest that R_2NH•HX might exist as some complexs with $Ti(O\text{-}i\text{-}Pr)_4$, though its detailed mechanism is not yet clarified.

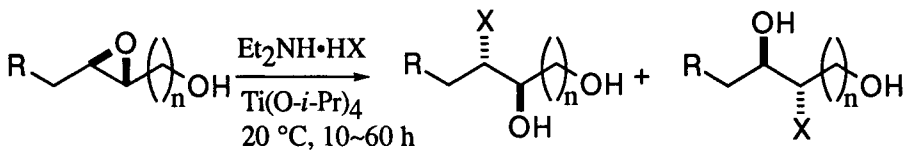
It is worth noticing that the methanesulfonate derivative (**77**) of the corresponding *trans*-2,3-epoxy alcohol is inert to nucleophilic attacks of halides giving **78** under the same conditions (eq. 6). This indicates that the free hydroxyl group present in the starting epoxide is indispensable for coordination to $Ti(O\text{-}i\text{-}Pr)_4$.



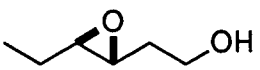
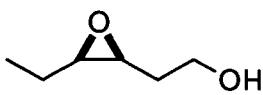
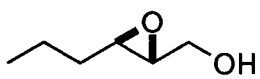
(eq. 6)

While ring-openings reactions of 2,3-epoxy alcohols have been examined extensively (44,45) and used in the synthesis of a natural product (4b), the reactivity and the regioselectivity in ring openings of 3,4-epoxy-1-ols have not yet been reported (47). The ring opening of 3,4-epoxy alcohols by halogeno-nucleophiles would be necessary in order to synthesize dactylynes according to our synthetic analysis. As a result, we extended our new reactions to 3,4-epoxy alcohols. The results were summarized in Table 6 (48). The data indicate that, (i) the ring-opening reactions proceed smoothly in good yields and halogeno-nucleophiles attack selectively on the 4-position of 3,4-epoxy alcohols in S_N2 manners; (ii) CH_2Cl_2 is a much more effective solvent than benzene for regioselective opening at the 4-position; but (iii) in the case of 2,3-epoxy-1-ols, benzene is better than CH_2Cl_2 in the regioselectivity of the openings at 3-position. Conclusively, we found that the titanium-mediated ring openings of 2,3-epoxy-1-ols at the 3-position were performed more efficiently

Table 6
Ring-opening reactions of 3,4- and 2,3-epoxy alcohols with $Et_2NH•HX$ and $Ti(O\text{-}i\text{-}Pr)_4$.



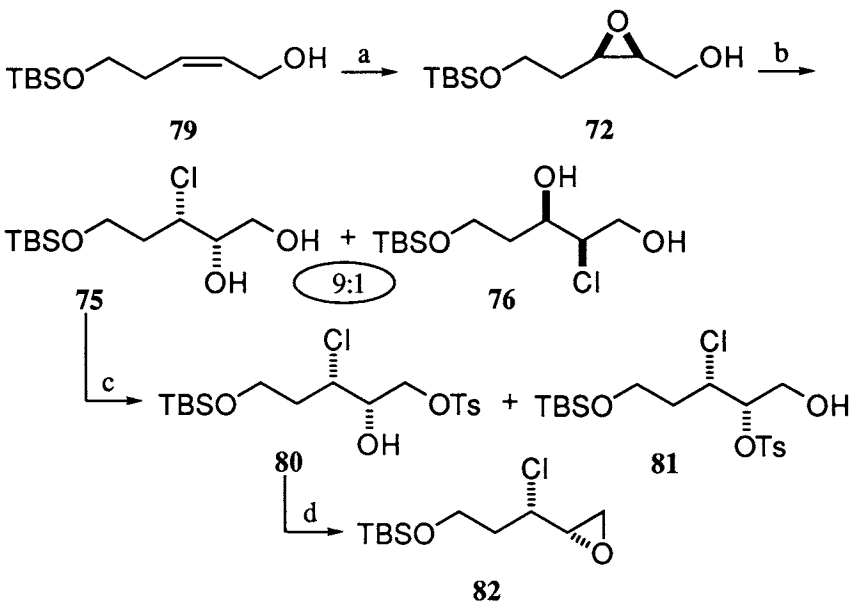
n=1 C-3 opening >> C-2 opening
n=2 C-4 opening >> C-3 opening

Epoxy alcohol	$Et_2NH•HX$	Solvent	Yield %	Regioselectivity C-4:C-3	C-3:C-2
	X=Cl	CH_2Cl_2	84	91: 9	
		PhH	52	72:28	
	X=Br	CH_2Cl_2	71	97: 3	
		PhH	62	83:17	
	X=Cl	CH_2Cl_2	71	79:21	
		PhH	55	70:30	
	X=Br	CH_2Cl_2	77	95: 5	
		PhH	65	86:14	
	X=Cl	CH_2Cl_2	90		94: 6
		PhH	91		99: 1
	X=Br	CH_2Cl_2	88		88:12
		PhH	93		93: 7
	X=Cl	CH_2Cl_2	85		92: 8
		PhH	78		95: 5
	X=Br	CH_2Cl_2	75		88:12
		PhH	83		91: 9

with use of $Et_2NH•HX$ as sources of halide nucleophiles. Furthermore, we observed that ring-opening reactions of 3,4-epoxy-1-ols at 4-position proceeded smoothly with higher regioselectivity in CH_2Cl_2, while the requisite openings of 2,3-epoxy-1-ols at the 3-position in benzene.

3-2 Highly Regioselective Ring-Opening Reactions of 2,3-Epoxy Alcohol Derivatives

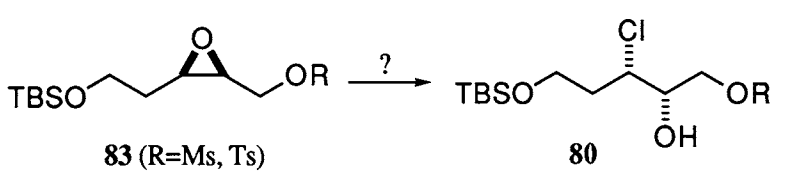
In the process of our synthetic studies on dactylynes (**69** and **70**), the preparation



Reagents and conditions: (a) L-(+)-DET, Ti(O-*i*-Pr)$_4$, TBHP, 4Å-MS, CH_2Cl_2, -20 °C, 16 h, 97%, 87% ee; (b) Et_2NH•HCl (3.0 eq.), Ti(O-*i*-Pr)$_4$ (1.5 eq.), CH_2Cl_2, 20 °C, 2.5 h, 72%, **75**:**76**=90:10; (c) TsCl, Py, 0 °C, 18 h, 92%, **80**:**81**=93:7; (d) K_2CO_3, MeOH, 0 °C, 1 h, 91%.

Scheme 13. Preparation of chiral C-5 unit (**82**).

of a chiral five carbons unit, (2*S*,3*S*)-5-*t*-butyldimethylsilyloxy-3-chloro-1,2-epoxy-pentane (**82**) was necessitated. At first, it was prepared from the readily available **79** (49) according to the procedure described above (Scheme 13). Thus, treatment of the *cis*-epoxy alcohol **72**, derived from the allyl alcohol **79** by Sharpless asymmetric epoxidation (42), with Et_2NH•HCl and Ti(O-*i*-Pr)$_4$ in CH_2Cl_2 at 20 °C for 2.5 h afforded a mixture of C-3 (**75**) and C-2 opening products (**76**) in a ratio of 9:1. When the separated main compound (**75**) was treated with TsCl in Py at 0 °C for 18 h, the desired *p*-toluenesulfonate (**80**) was obtained in 92% yield, which was easily



83 (R=Ms, Ts) **80** (eq. 7)

converted to the C-5 fragment (**82**) in 91% yield on reaction with potassium carbonate (K_2CO_3) in methanol at 0 °C for 1 h. In this process, a thorny problem encounted

was that the mixtures of **75** and its regioisomer **76**, as well as **80** and its regioisomer **81** could not be separated easily by flash chromatography. To overcome this difficulty, we required a more effective method so that *cis*-2,3-epoxy-1-ol sulfonyl esters (**83**) could be directly converted to the corresponding chlorohydrins (**80**) (eq. 7).

As described in 3-1, the epoxy alcohol methanesulfonate (**77**) was inert to the reaction with Et_2NH•HX and Ti(O-*i*-Pr)$_4$. This result encouraged us to search for a more active ring-opening promoter using readily available epoxy alcohols as model compounds. As a result, we found that diethylaluminium chloride (Et_2AlCl) and a mixture of Et_2AlCl-diethylamine hydrobromide (Et_2NH•HBr) are efficient reagents for converting the epoxy alcohol sulfonates to the corresponding chloro- and bromohydrins, respectively, with high regioselectivities. The preliminary results are summarized in Table 7 (50). The data reveal that, (i) every reaction proceeded with very high regioselectivity of the C-3 opening in an S_N2 fashion; (ii) the sulfonyl esters resulted in preferable openings at the C-3 position compared to the original alcohols; (iii) the TBS ether also proceeded similarly with a high C-3 opening selectivity; (iv) the secondary alkyl group at C-4 did not exert any influence on the reaction course; and (v) the reaction conditions were milder than that using Ti(O-*i*-Pr)$_4$, and the yields of all reactions were very high.

The scope and limitation of these new reactions were then checked by using the methanesulfonates of 2,3-epoxy-1-ols as shown in Scheme 14. When compound **84** was used as the starting material, method A gave rise to the 92:8 ratio of inversion (**86**) and retention products (**87**) opening at the C-3 position, while method B afforded the 98:2 ratio of the requisite opening products (**88** and **89**). The formation of retention products revealed that the reaction would proceed partially with an S_N1 pathway because the cation generated in the reaction course at 3-position was stabilized by the phenyl group to some extent. On the other hand, when compound **85** was reacted by method A, the yield of the desired chlorohydrin (**90**) decreased to 56% with the formation of the rearranged product (**91**). On treatment of **85** with the method B, the desired bromohydrin (**92**) was obtained in only 24% yield, contaminated with C-2 opening products (93 and 94) and the unreacted starting material. These

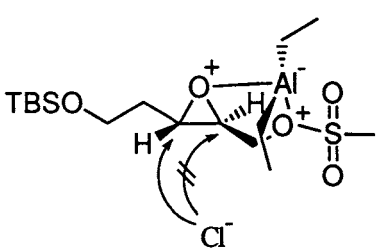
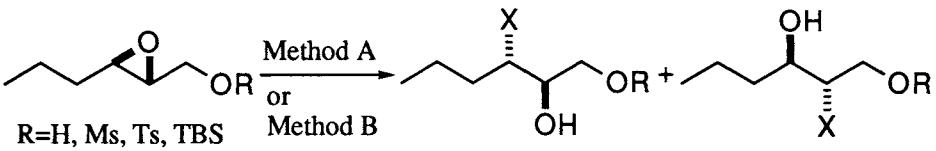
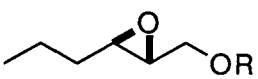
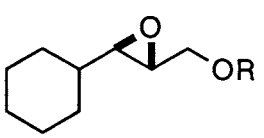


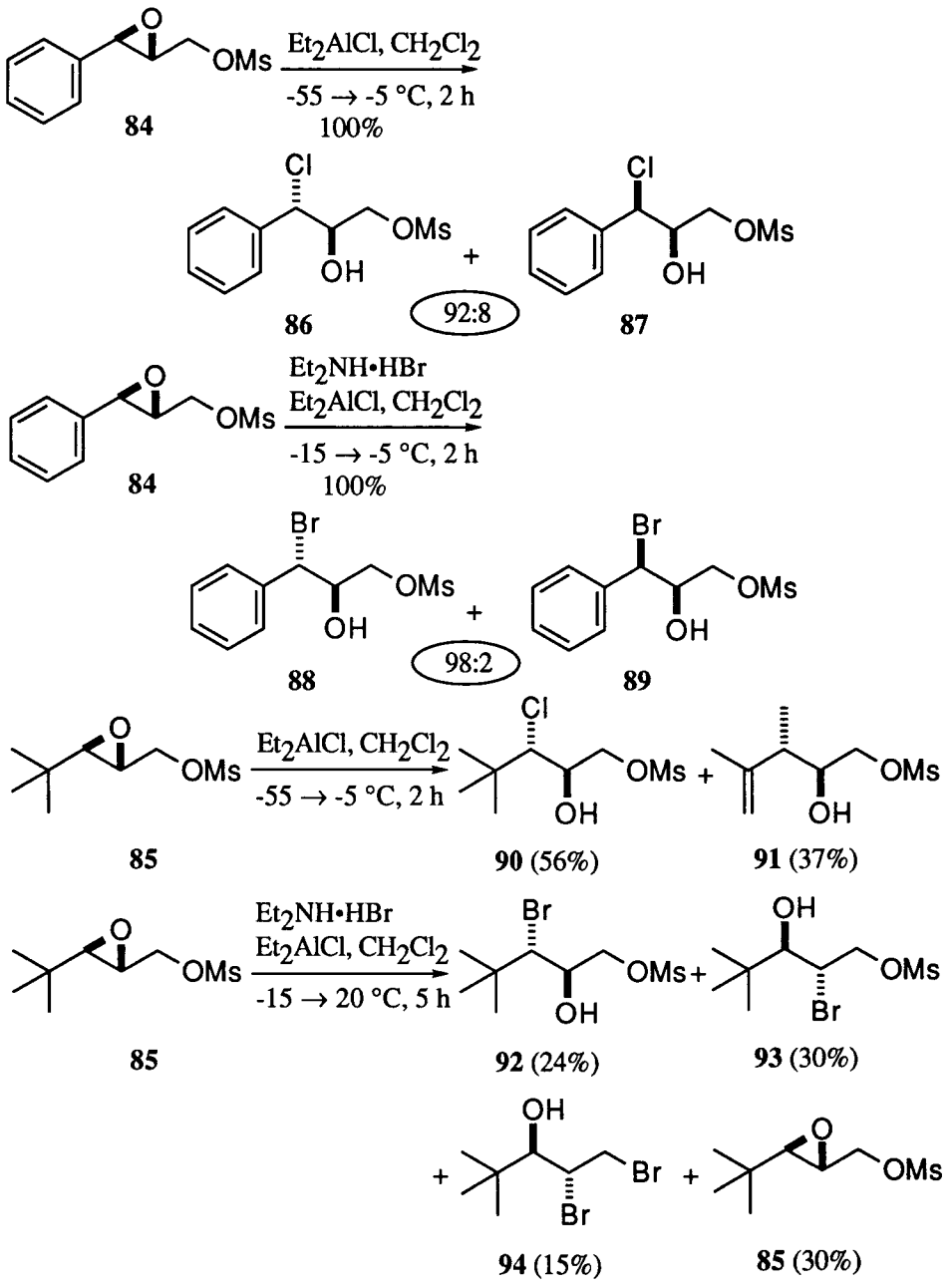
Figure 2.

Table 7
Ring-opening reactions of 2,3-epoxy-1-ols and their derivatives.



Method A (X=Cl): Et_2AlCl (1.5 eq.) in CH_2Cl_2
Method B (X=Br): Et_2AlCl (1.5 eq.)-$Et_2NH{\cdot}HBr$ (3.0 eq.) in CH_2Cl_2

Epoxy alcohol or its derivatives	Method	Temp. °C	Time h	Yield %	Regioselectivity C-3:C-2
					
R=H	A	-55 → 0	3	98	160: 1
R=H	B	-15 → -5	2	95	96: 4
R=Ms	A	-55 → -5	2	99	200: 1
R=Ms	B	-15 → -5	2	100	120: 1
R=Ts	A	-55 → -5	2	99	210: 1
R=Ts	B	-15 → -5	2	100	160: 1
R=TBS	A	-55 → 5	4	86	135: 1
R=TBS	B	-55 → 5	4	95	160: 1
					
R=H	A	-55 → 0	3	85	89:11
R=H	B	-15 → 0	3	88	89:11
R=Ms	A	-55 → 0	3	99	140: 1
R=Ms	B	-15 → -5	2	100	98: 2
					
R=Ms	A	-55 → 5	4	100	100: 0
R=Ms	B	-15 → 5	4	100	97: 3



Scheme 14.

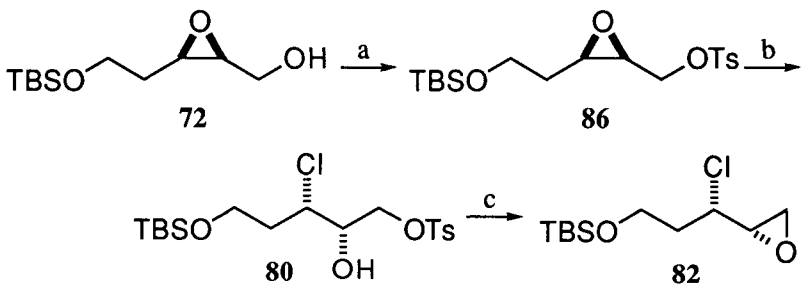
results indicate that the bulkiness at C-4 position would tend to decrease the desired C-3 opening due to steric hindrance when the halogeno-nucleophile attacked the

oxirane ring from the backside.

The high selectivity for the openings of general epoxy sulfonate derivatives may be rationalized as shown in Figure 2. The aluminium atom of the reagent would tightly combine with both oxirane and sulfonyloxy oxygen atoms to form a rigid five-membered ring. Steric repulsion between the two ethyl groups and the sulfonyl oxygen atoms would constrain the ethyl groups to adopt an orientation near to the C-2 position of oxirane, leading to the nucleophile attacks on the C-3 position preferentially.

In method B, the active species might be diethylaluminium bromide. Since it is generally difficult to prepare and handle the reagent experimentally (51), this procedure would provide the *in situ* preparation of the reagent.

Finally, these results were applied to preparation of the chiral C-5 segment **82** for the synthesis of dactylynes as shown in Scheme 15. Thus, the readily available (2*S*,3*R*)-4-*t*-butyldimethylsilyloxy-2,3-epoxypentan-1-ol (**72**) was tosylated in the usual manner and treated with Et_2AlCl in CH_2Cl_2 at -20 °C to -10 °C for 5 h to afford exclusively (2*S*,3*S*)-4-*t*-butyldimethylsilyloxy-3-chloropentane-1,2-diol 1-tosylate (**80**). Compound **80** was reacted with K_2CO_3 in methanol at 0 °C for 1 h to give **82** in 91% yield.



Reagents and conditions: (a) TsCl, TEA, DMAP, CH_2Cl_2, 0 °C, 5 h, 100%; (b) Et_2AlCl, CH_2Cl_2, -20 → -10 °C, 5 h, 100%; (c) K_2CO_3, MeOH, 0 °C, 1 h, 91%.

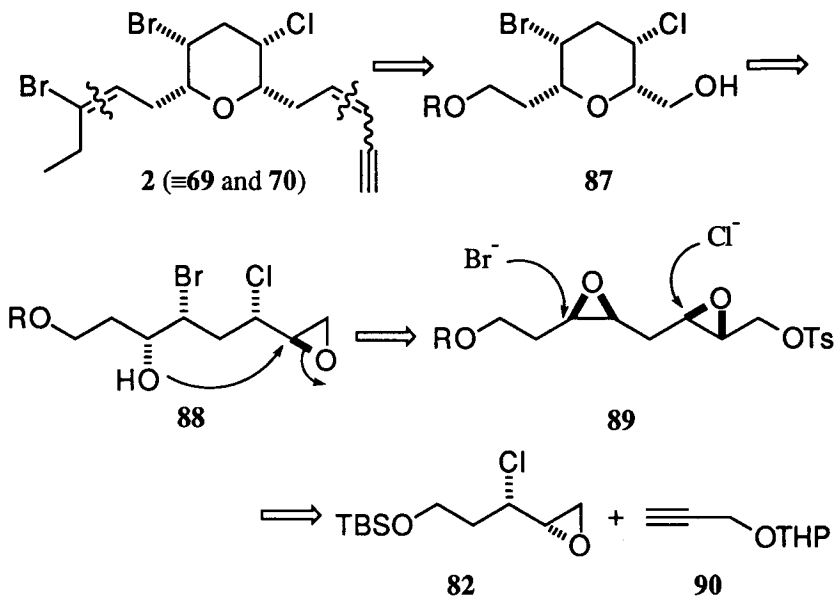
Scheme 15. Facile preparation of chiral C-5 unit (**82**).

According to this new procedure, the segment (**82**) was easily prepared from the epoxy alcohol **72** in 91% total yield. These results reveal that this new reaction procedure is easy to conduct and allows a highly efficient and general entry to halohydrins with high regioselectivity under mild conditioins.

3-3 Synthetic Study on the Halogenated Tetrahydropyran Framework of (-)-Dactylynes

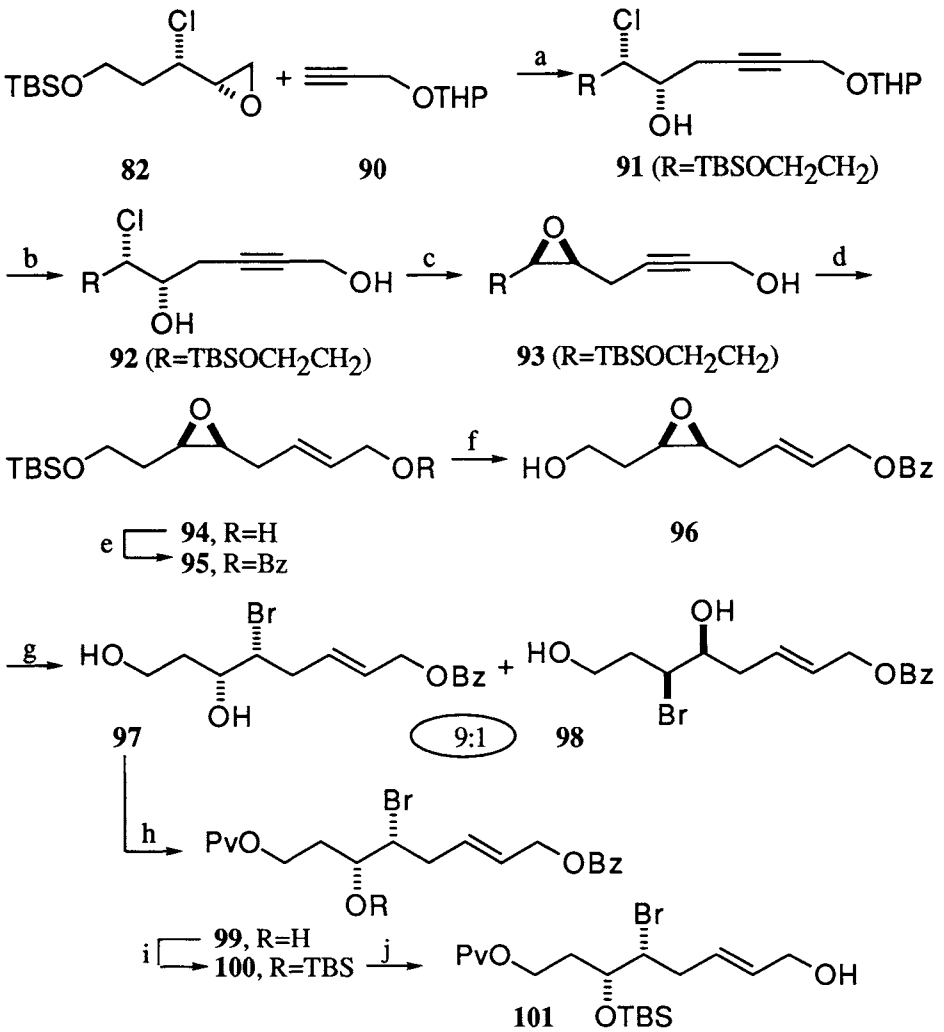
We chose the retrosynthetic route for dactylynes (**2**≡**69** and **70**) as shown in Scheme 16. Compounds **69** and **70** were disconnected to an all *cis*-substituted

tetrahydropyran framework (**87**) as indicated in Scheme 16 by employing double Wittig reactions and simple functional group manipulations. Then, disconnection of the C-O bond in **87** led to an acyclic halogenated epoxy alcohol (**88**), which could be derived from an equivalent of the bisepoxide (**89**) by use of the epoxy-opening methods developed by us. The epoxide (**89**) was further disconnected to a chiral C-5 unit (**82**) and propargyl alcohol tetrahydropyranyl (THP)-ether (**90**) on the basis of the coupling reaction of the acetylide to a terminal epoxide.



Scheme 16. Synthetic plan of dactylynes.

The C-5 unit (**82**) has been prepared on a large scale in a few steps from the readily available (2*Z*)-5-*t*-butyldimethylsilyloxy-2-penten-1-ol (**79**)(49) (Schemes 13 and 15). With the chiral terminal epoxide (**82**) in hand, our synthesis was commenced from the coupling reaction of **82** with propargyl alcohol THP-ether (**90**) according to the Yamaguchi method (52) (Scheme 17). Thus, the latter ether was treated with BuLi and boron trifluoride diethyl etherate (BF_3•OEt_2) to afford the alkynyl borane complex, which was treated rapidly *in situ* with **82** at -78 °C for 45 min to give the β-hydroxyacetylene (**91**) in 98% yield based on the recovered **82** (19%). The THP group in **91** was detached with Et_2AlCl in CH_2Cl_2 (53) at 0 °C to 25 °C for 45 min to furnish the propargyl alcohol (**92**), which was converted to the *cis*-epoxide (**93**) on treatment with K_2CO_3 in MeOH at 25 °C for 3 h in 97% yield over two steps. The triple bond in **93** was reduced with sodium bis(2-methoxyethoxy)aluminium hydride (Red-Al®)(54) in ether at 0 °C to 25 °C for 5 h

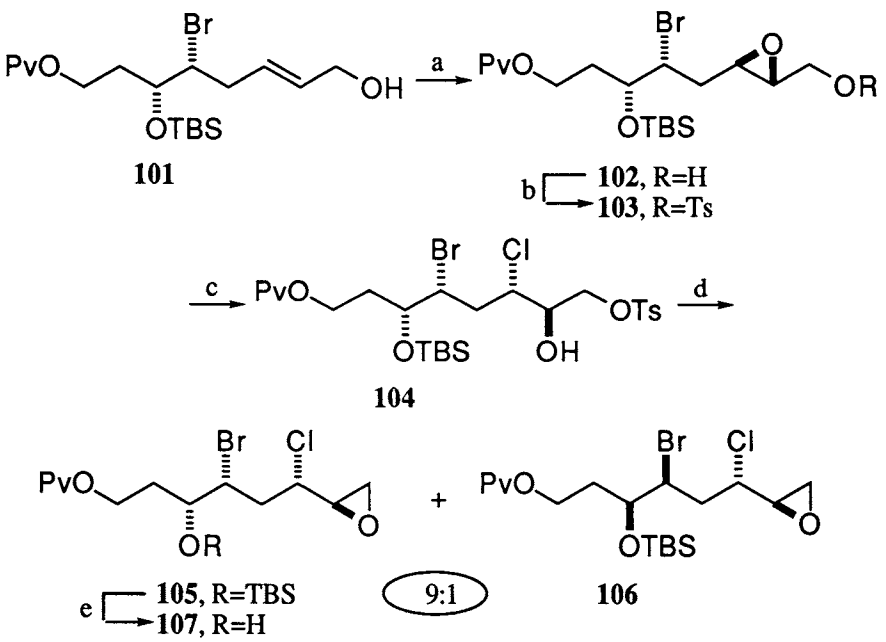


Reagents and conditions: (a) BuLi, BF_3•OEt_2, THF, -78 °C, 45 min, 79%; (b) Et_2AlCl, CH_2Cl_2, 0 → 25 °C, 4 h, 100%; (c) K_2CO_3, MeOH, 25 °C, 3 h, 97%; (d) Red-Al®, ether, 0 → 25 °C, 5 h, 98%; (e) PhCOCl, TEA, DMAP, CH_2Cl_2, -40 °C, 2 h, 97%; (f) TBAF, THF, 0 °C, 4 h → 20 °C, 2 h, 90%; (g) Ti(O-*i*-Pr)$_4$ (1.5 eq.), Et_2NH•HBr (4.0 eq.), CH_2Cl_2, 25 °C, 12 h, 76%; (h) PvCl, Py, -20 °C, 2 h → -10 °C, 12 h, 70%; (i) TBSOTf, 2,6-lutidine, CH_2Cl_2, -20 °C, 4 h, 96%; (j) K_2CO_3, MeOH, 20 °C, 80 min, 96%.

Scheme 17.

to the *trans*-olefin (**94**) in 98% yield. The hydroxyl group of **94** was protected with

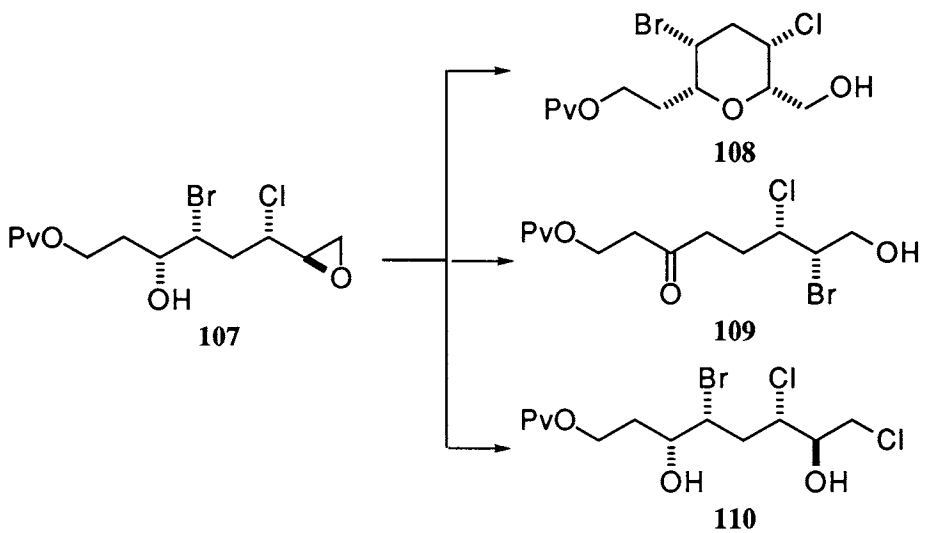
benzoyl chloride (PhCOCl) in the usual manner, followed by detaching the TBS group with TBAF in THF at 0 °C for 4 h and then at 20 °C for 2 h to afford the 3,4-epoxy alcohol (**96**) *via* **95** in 86% combined yield from **93**. Further transformation of **96** into the bromohydrin (**97**) was performed efficiently by a titanium-mediated epoxy-opening process. Thus, when compound **96** was allowed to react with $Et_2NH{\cdot}HBr$ in the presence of $Ti(O\text{-}i\text{-}Pr)_4$ in CH_2Cl_2 at 25 °C for 12 h, the ring-opening reaction of the oxirane proceeded regioselectively leading to a 9:1 separable mixture of the C-4 (**97**) and C-3 opening products (**98**) in 76% combined yield. The separated diol (**97**) was converted to **100** by selective esterification of the primary hydroxyl group with PvCl in Py at -20 °C for 2 h and then at -10 °C for 12 h followed by protection of the secondary hydroxyl group in **99** as the TBS ether in the usual manner. The benzoyl group of **100** was then detached by methanolysis with K_2CO_3 in anhydrous MeOH at 0 °C for 80 min, with no effect on the co-existing Pv group, giving **101**.



Reagents and conditions: (a) D-(-)-DET, $Ti(O\text{-}i\text{-}Pr)_4$, TBHP, 4Å-MS, CH_2Cl_2, -20 °C, 10 h, 99%; (b) TsCl, TEA, DMAP, CH_2Cl_2, 0 °C, 12 h, 100%; (c) Et_2AlCl, CH_2Cl_2, 20 °C, 13 h, 97%; (d) K_2CO_3, MeOH, 20 °C, 1 h, 93%; (e) TBAF-HF (pH≈3), THF, 20 °C, 20 h, 97%.

Scheme 18.

The allyl alcohol **101** was submitted to Sharpless asymmetric epoxidation (42) with D-(-)-diethyl tartrate (DET) as an auxiliary to afford the desired product (**102**) as the sole product (Scheme 18). Toluenesulfonylation of **102** by standard method gave the corresponding epoxy sulfonate (**103**). When **103** was treated with Et_2AlCl in CH_2Cl_2 at 20 °C for 13 h (50), the ring-opening reaction proceeded smoothly to provide exclusively the desired product (**104**). Exposure of **104** to K_2CO_3 in MeOH at 20 °C for 1 h then resulted in regioselective oxirane formation to afford the terminal epoxides (**105** and **106**) in 93% yield. The diastereoisomer **106**, arising from the contaminated antipode of the C-5 unit (**82**) used in the first step, was easily removed by flash chromatography. Attempted desilylation of **105** with aqueous 5% HF in MeCN resulted in the decomposition of the epoxide to give a mixture. When only TBAF was used as the desilylating agent, debromination occurred preferentially to afford a bisepoxide in 92% yield. Deprotection of the TBS group of **105** to the 6-hydroxy-1,2-epoxide (**107**) was performed with a mixture of TBAF-HF (pH≈3) in THF, prepared from TBAF (1.0 M solution in THF) and 38% HF in water in a ratio of 12:1, at 20 °C for 20 h in 97% yield.



Scheme 19.

With the intermediate (**107**) at hand, attention was then focused on the construction of the all *cis*-substituted tetrahydropyran ring (**108**) *via* an intramolecular cyclization of **107** (Scheme 19). When protic acids, such as pyridinium *p*-toluenesulfonate (PPTS), PTS, camphorsulfonic acid (CSA), and trifluoroacetic acid (TFA), were used as cyclization activators, the reaction did not occur with either recovery or decomposition of the starting **107**, and the cyclized product could not be

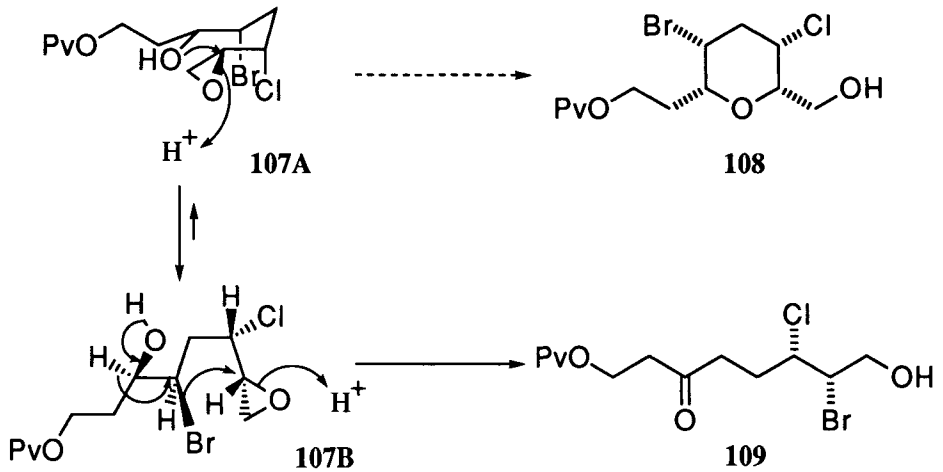
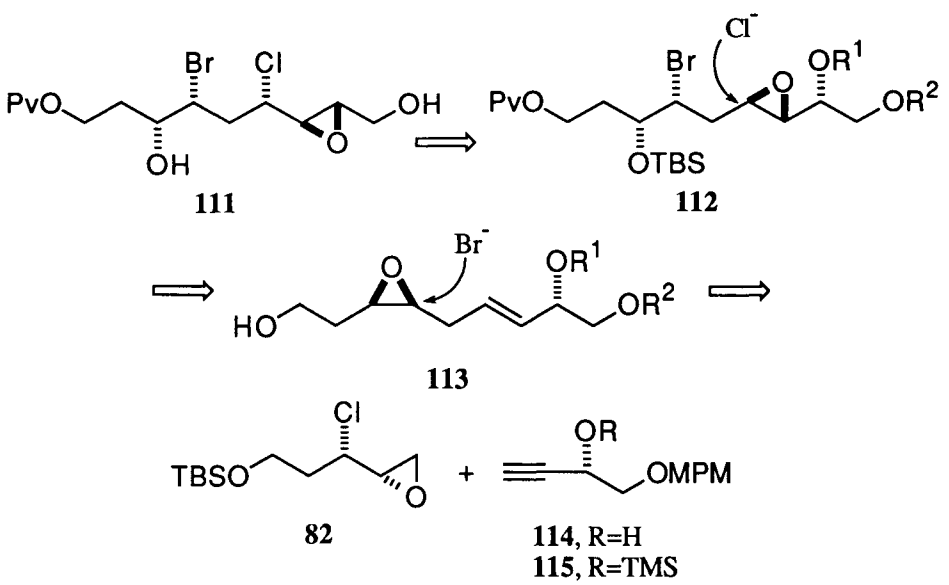


Figure 3. Why didn't **107** lead to formation of the cyclized compound (**108**)?

detected, even in traces. Lewis acids were then examined. Unfortunately, **107** could not be induced to cyclize under various attempted conditions. Interestingly, when **107** was treated with BF_3•OEt_2 (2 eq) in CH_2Cl_2 at -20 °C for 30 min or Ti(O-*i*-Pr)$_4$ (2 eq) in benzene at 80 °C for 24 h, the rearrangement reaction proceeded preferentially to give the ketone (**109**) in 70% or 65% yield, while **107** was transformed into dichlorohydrin **110** on reaction with $TiCl_4$ (2 eq) in CH_2Cl_2 at -20 °C for 1 h. These

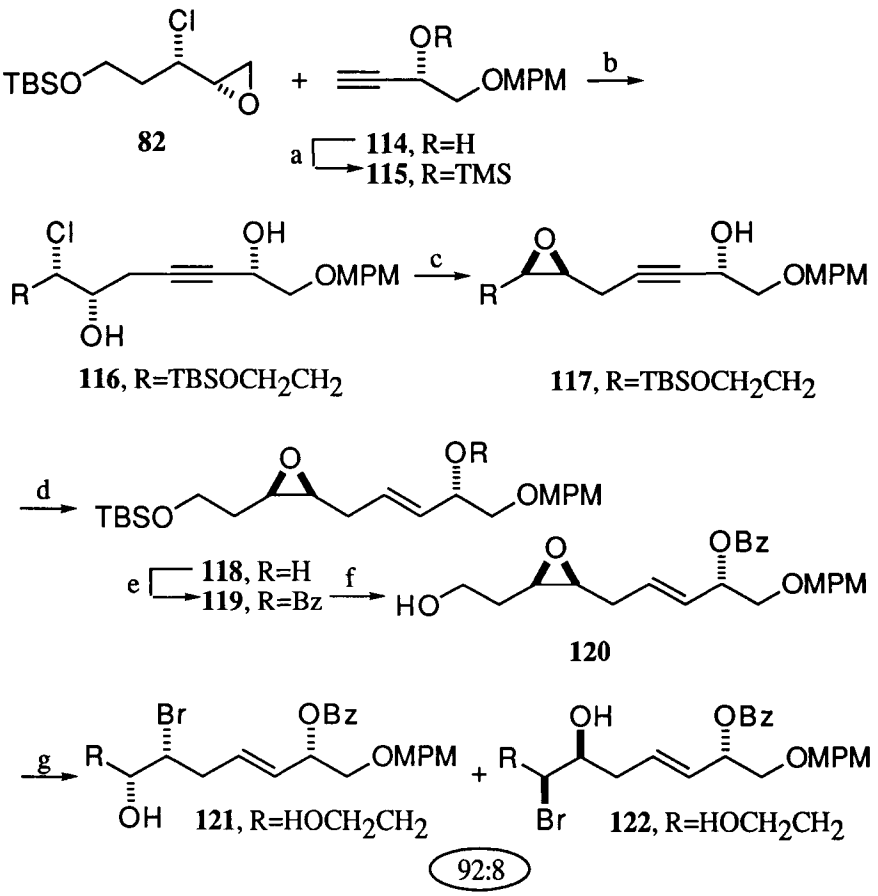


Scheme 20.

results might reflect that **107** would exist mainly as the conformer **107B** in the solution, instead of **107A**, because there would be a serious diaxial repulsive interaction between two halogen atoms in the latter (Figure 3). Thus, **110** would be formed from the preferentially existed conformer (**107B**). The structure of **109** was determined by ^{13}C- and ^{1}H NMR, FAB-MS, and elemental analysis. The stereochemistry at C-2 in **109** was assigned to be *S* on the assumption that the bromine atom would be rearranged in an S_N2 fashion *via* a five-membered ring transition state. These results led us to revise our strategy so as to employ the isolated terminal epoxide to 2,3-epoxy-1-ol type, expecting regulation of regioselective opening at C-3 position, because the intramolecular cyclization would be favored in the 2,3-epoxy-1-ol due to the stronger coordination to some Lewis acids (55). Accordingly, we anticipated that compound **111** would be a more efficient synthetic precursor for **69** and **70** (Scheme 20). The compound could be prepared from the reaction of epoxy alcohol derivative **112** with some chloro-nucleophile according to our procedure (48). Its precursor (**113**) would be formed by coupling of **82** with the chiral acetylene (**115**). Detached **114** has been prepared from D-(-)-tartaric acid by Yadav et al. (56) Therefore, the synthetic study of **69** and **70** started from the coupling reaction of these two compounds.

Protection of the OH group in **114** with chlorotrimethylsilane (TMSCl) in CH_2Cl_2 at -15 °C for 30 min gave the unstable silyl ether (**115**), which was directly used in the next step without purification (Scheme 21). According to the Yamaguchi method (52), compound **115** was treated with BuLi and BF_3•OEt_2 in THF to afford an alkynyl borane complex, which reacted *in situ* with the epoxide (**112**) at -78 °C for 1.5 h to yield, after an AcOH work-up, the chlorohydrin alcohol (**116**) in 98% yield. Exposure of **116** to K_2CO_3 in MeOH at 23 °C for 6 h led to the epoxide (**117**) in 98% yield. The triple bond of **117** was reduced with Red-Al® (54) in ether at 0 °C for 1 h affording the allylic alcohol **118** in 98% yield. The alcohol (**118**) was converted to the benzoyl ester (**119**), which was treated with TBAF in THF to furnish the *cis*-3,4-epoxy alcohol (**120**) in 95% yield. When compound **120** was treated with Et_2NH•HBr and Ti(O-*i*-Pr)$_4$ in CH_2Cl_2 at 25 °C for 12 h, the ring-opening reaction of the oxirane proceeded regioselectively to yield a 92:8 separable mixture of C-4 opening (**121**) and C-3 opening products (**122**) in a 78% combined yield. Elaboration of **121** to the requisite 2,3-epoxy alcohol methanesulfonate (**127**) was accomplished in five steps (Scheme 22). Thus, the primary hydroxyl group of **121** was esterified with PvCl and Py in CH_2Cl_2 at 0 °C for 12 h to give the pivaloate (**123**), of which the secondary alcohol was protected as a TBS ether to afford **124**. The benzoyl group of **124** was saponified with K_2CO_3 in a mixture of MeOH-water (20:1) to yield the allylic alcohol (**125**) in 91% yield. Compound **125** was submitted to Sharpless asymmetric epoxidation (42) using D-(-)-diisopropyl tartrate (DIPT) in the presence of Ti(O-*i*-Pr)$_4$, *t*-butyl hydroperoxide (TBHP), and 4Å-MS in CH_2Cl_2

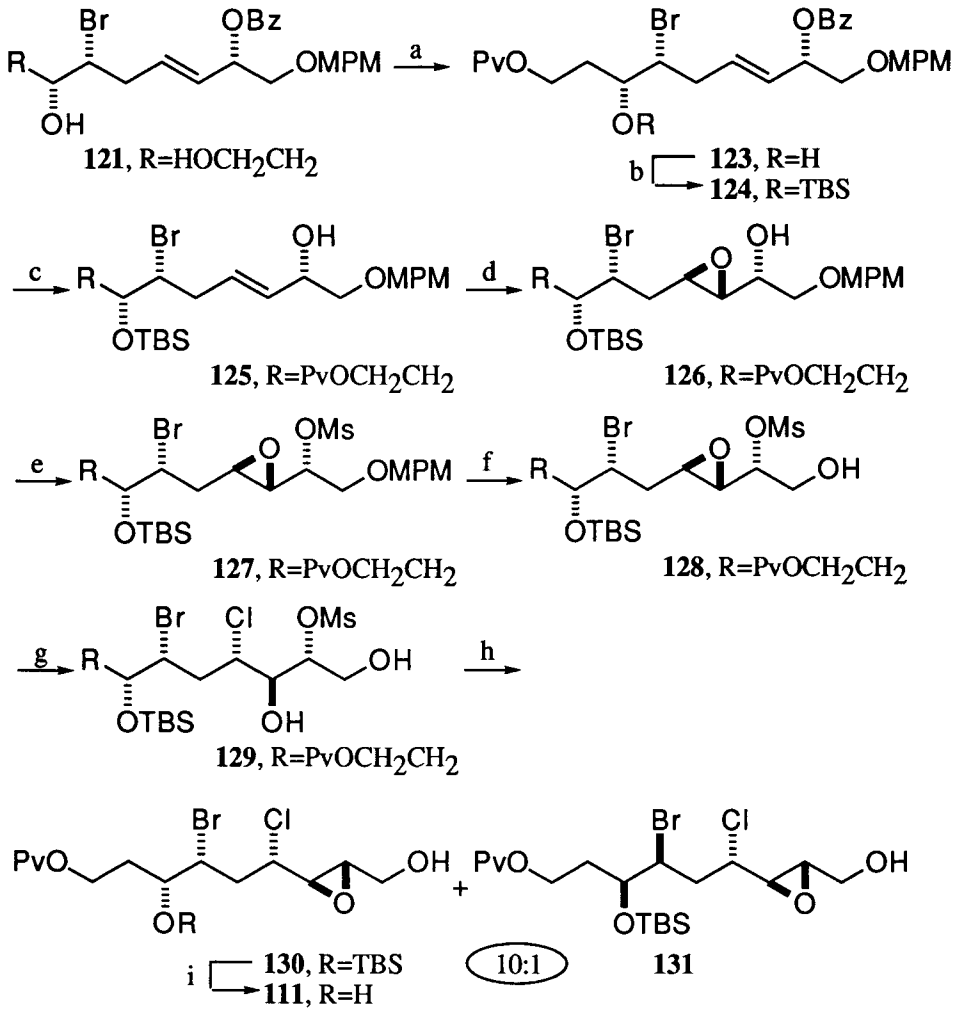
at -20 °C for 72 h to give β-epoxide **126** as the sole product in 97% yield. Methanesulfonylation of **126** produced the sulfonate (**127**) quantitatively, which was detached with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) to give an alcohol (**128**) in 94% yield. When **128** was treated with 3.0 eq of Et_2AlCl in CH_2Cl_2-hexane (3:1) at 0 °C for 3 h, the ring-opening reaction of the oxirane proceeded smoothly to provide exclusively the desired chlorohydrin (**129**) in 90-100% yield. Then, exposure



Reagents and conditions: (a) TMSCl, TEA, CH_2Cl_2, -15 °C, 30 min, 95%; (b) BuLi, BF_3•OEt_2, THF, -78 °C, 1.5 h; 20% AcOH, -78 → 20 °C, 2 h, 98%; (c) K_2CO_3, MeOH, 23 °C, 6 h, 98%; (d) Red-Al®, ether, 0 °C, 1 h, 98%; (e) PhCOCl, TEA, DMAP, CH_2Cl_2, 0 °C, 3 h, 98%; (f) TBAF, THF, -10 °C, 3 h, 97%; (g) Ti(O-*i*-Pr)$_4$, Et_2NH•HBr, CH_2Cl_2, 25 °C, 12 h, 78%.

Scheme 21.

of **129** to K_2CO_3 in MeOH at 20 °C for 1 h resulted in regioselective oxirane formation to furnish the 2,3-epoxy alcohol (**130**, 87% and **131**, 8.7%) without the loss of the chlorine atom. Compound **131**, which arose from the antipode of C-5

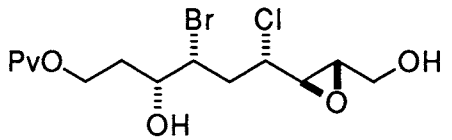


Reagents and conditions: (a) PvCl, Py, CH_2Cl_2, 0 °C, 12 h, 97%; (b) TBSOTf, TEA, DMAP, CH_2Cl_2, -20 °C, 4 h, 97%; (c) K_2CO_3, MeOH-H_2O (20:1), 20 °C, 3 h, 91%; (d) D-(-)-DIPT, Ti(O-*i*-Pr)$_4$, TBHP, 4Å-MS, CH_2Cl_2, -20 °C, 72 h, 97%; (e) MsCl, TEA, DMAP, CH_2Cl_2, -40 °C, 1 h, 100%; (f) DDQ, CH_2Cl_2-H_2O (18:1), 23 °C, 4 h, 94%; (g) Et_2AlCl (3.0 eq.), CH_2Cl_2-hexane (3:1), 0 °C, 3 h, 90~100%; (h) K_2CO_3, MeOH, 20 °C, 1 h, 96%; (i) TBAF-HF, THF, 20 °C, 28 h, 89%.

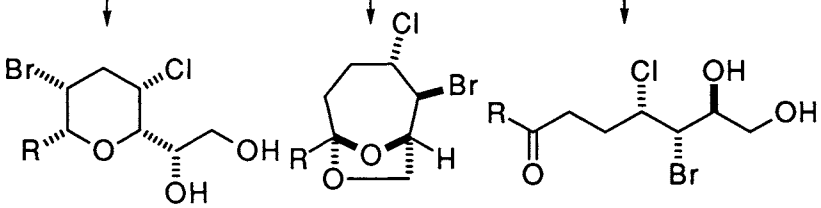
Scheme 22.

segment **82**, could be easily removed from the main product by flash chromatography. Detachment of TBS group from **130** under mild conditions (TBAF-HF, THF, 20 °C, 28 h) provided the requisite 7-hydroxy-2,3-epoxy alcohol (**111**) quantitatively.

Table 8
Intramolecular cyclization of the epoxy alcohol (**111**).



111



132, R=PvOCH$_2$CH$_2$ **133**, R=PvOCH$_2$CH$_2$ **134**, R=PvOCH$_2$CH$_2$

Ti(O-i-Pr)$_4$ (0.25 eq.) toluene, 4Å-MS, 105 °C, 3 h	16%	—	—
Sn(OTf)$_2$ (1.0 eq.) CH_2Cl_2, 20 °C, 2 h	26%	50%	—
Sn(OTf)$_2$ (1.1 eq.) PhH, 20 °C, 2 h	34%	30%	24%
Sn(OTf)$_2$ (1.1 eq.) toluene, 20 °C, 6 h	30%	18%	32%
Zn(OTf)$_2$ (1.1 eq.) PhH, reflux, 8 h	13%	42%	—
Zn(OTf)$_2$ (1.1 eq.) septum (Aldrich) PhH, reflux, 15 h	38% (74%)	—	—

The intramolecular cyclization reaction of the 2,3-epoxy alcohol (**111**) was

then examined. Table 7 summarizes some of the attempted conditions and results. Initial reaction of **111** with $Ti(O\text{-}i\text{-}Pr)_4$ (0.25 eq.) and 4Å-MS in toluene at 105 °C for 3 h gave rise to the desired cyclized product (**132**), though in a low yield (16%). Next, employment of a soft Lewis acid, Tin(II) triflate [$Sn(OTf)_2$], in CH_2Cl_2 at 20 °C led to **132** in 26% yield along with 50% of a bicyclic product **133**. However, when rather non-polar solvents, such as benzene or toluene, were used, the other by-product (**134**) was produced besides **133**. Next, treatment of **111** with zinc triflate [$Zn(OTf)_2$] in refluxed benzene yielded **132** in 13% yield, along with 42% of **133**. Fortunately, when a two-necked flask equipped with a reflux condenser and a septum (Aldrich, Z10, 076-5) (not a glass stopper) was used, the reaction with $Zn(OTf)_2$ in benzene under reflux for 15 h gave rise to only the desired product (**132**) in a 74% corrected yield based on the recovered starting material. The details of the effect of the septum on the reaction is not yet clear. The structure of **133** including its stereochemistry was established by ^1H NMR and HR-MS spectroscopic analysis combined with an NOE experiment (Fig. 3). The structure of the ketone (**134**) was confirmed from its ^1H NMR spectrum and finally by the chemical transformation into **133** with $Sn(OTf)_2$ (1.0 eq.) in CH_2Cl_2 at 20 °C for 12 h (72% yield).

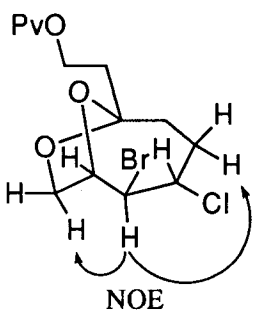
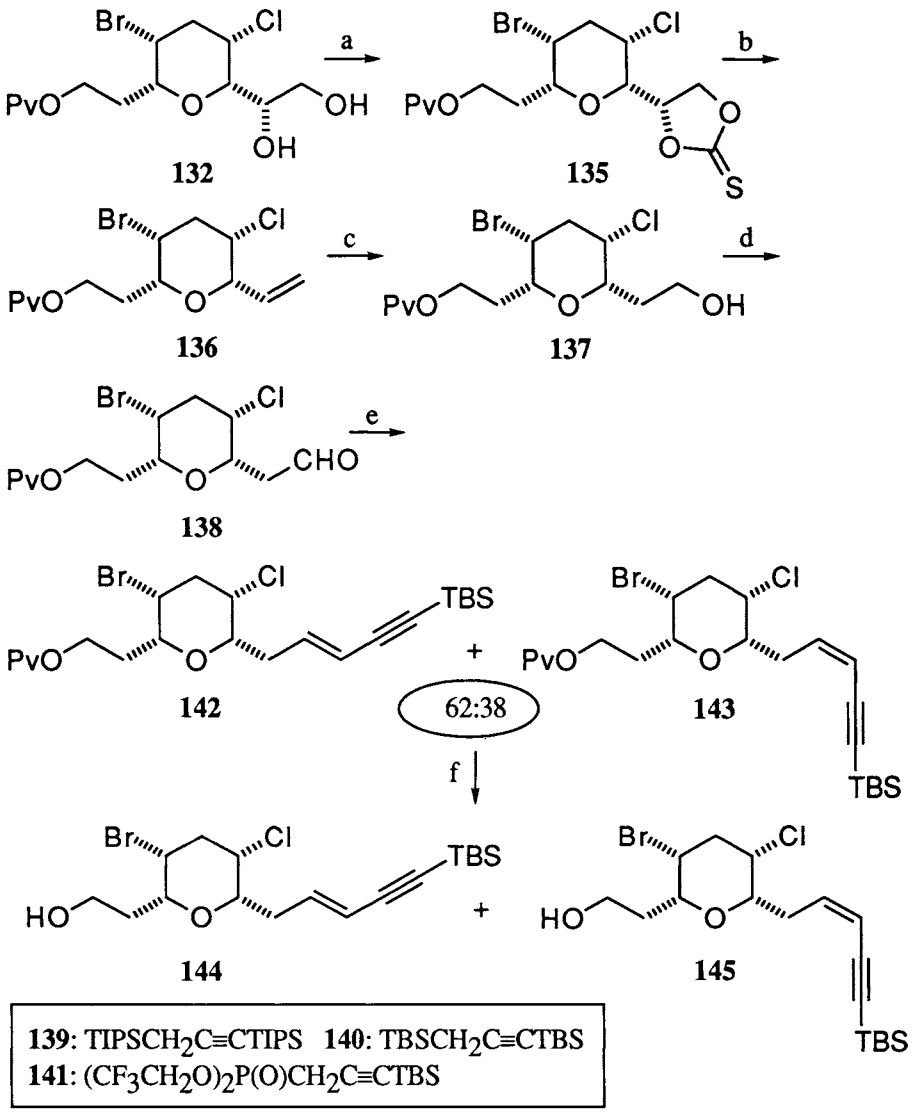


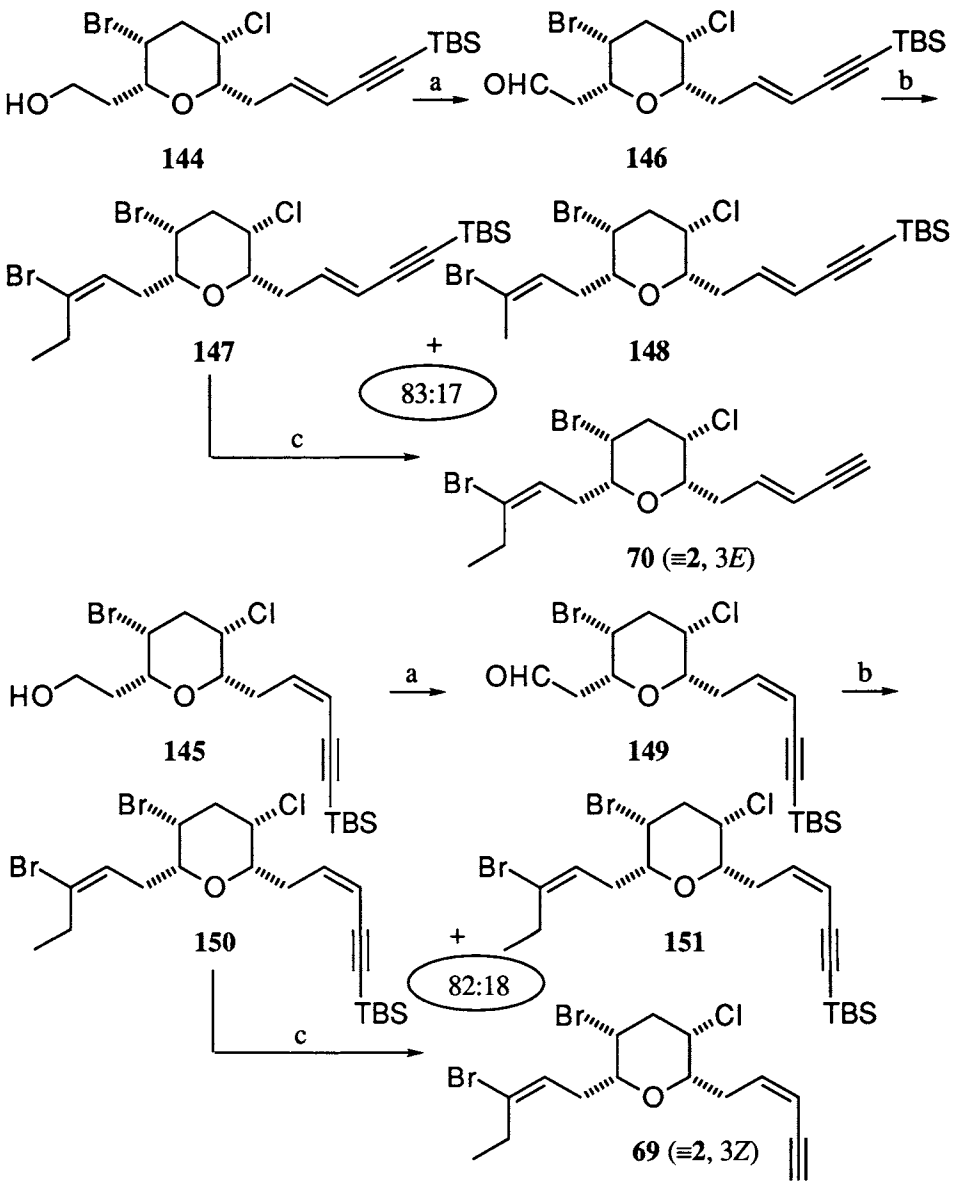
Figure 4. The NOE interactions in **133.**

With the ring closed product at hand, attention was then paid to elaborate the enyne functionality, so that **132** was transformed into the aldehyde **138** in four steps (Scheme 23). Firstly, the vicinal diol part of **132** was converted to the vinyl derivative (**136**) by the modified Corey procedure (57). Conversion of the diol to the corresponding thiocarbonate (**135**) was accomplished with thiophosgene ($CSCl_2$) in the presence of DMAP in CH_2Cl_2 at 0 °C for 1 h in 82% yield. Then, treatment of **135** with 1,3-dimethyl-2-phenyl-1,3-diazaphospholidine (58) in dry toluene at 20 °C for 2 h afforded the terminal olefin (**136**) in 97% yield. Hydroboration of the vinyl function in **136** with 9-borabicyclo[3.3.1]nonane (9-BBN) in THF, followed by oxidative work-up, produced the alcohol (**137**) in 96% yield. Finally, Swern



Reagents and conditions: (a) $CSCl_2$, DMAP, CH_2Cl_2, 0 °C, 1 h, 82%; (b) 1,3-dimethyl-2-phenyl-1,3-diazaphospholidine, toluene, 20 °C, 2 h, 97%; (c) 9-BBN, THF, 20 °C, 2 h; 5% NaOH, H_2O_2, 20 °C, 1 h, 96%; (d) $(COCl)_2$, DMSO, TEA, -78 → 20 °C, 1.5 h, 89%; (e) **141** (1.5 eq.), $KN(TMS)_2$ (1.2 eq.), 18-crown-6 (1.4 eq.), THF, -78 °C, 45 min, 85%; (f) DIBAH, CH_2Cl_2, -78 → -40 °C, 1 h, 61% (**144**) and 38% (**145**).

Scheme 23.



Reagents and conditions: (a) $(COCl)_2$, DMSO, TEA, CH_2Cl_2, -78 → 20 °C, 1.5 h, 100% (**146**) or 95% (**149**); (b) $Ph_3P^+CBr_2EtBr^-$ (1.5 eq.), BuLi (1.5 eq.), THF-DMSO (5:1), -78 °C, 45 min, 87% (**147+148**) or 67% (**150+151**); (c) TBAF-HF (pH≈4.5, THF, -5 °C, 1 h and -5 → 0 °C, 1 h, 79% (**70**) or 82% (**69**).

Scheme 24.

oxidation of **137** furnished the aldehyde (**138**) in 89% yield. For the next sequence, rather mild conditions were needed to elongate the enyne unit to the aldehyde (**138**), since the compound would be unstable under basic conditions due to the liable elimination of the axially situated halogen atoms, particularly the bromine atom. The initial Wittig reaction with the Corey reagent (**139**)(59) gave an unknown mixture, which showed no enyne protons (δ5.5-6.2 ppm) on the ^{1}H NMR spectrum of the crude mixture. Next, modification of the Corey method using compound **140** led to the formation of a 1:1 mixture of (*E*)- (**142**) and (*Z*)-olefins (**143**) in only a 19% combined yield. Therefore, a Horner-Wittig-type reagent (**141**) was newly prepared for this transformation, because **141** was expected to show a higher reactivity rather than the Corey reagent. The new Horner-Wittig-type reagent (**141**) was prepared as follows: the readily available propargyl alcohol THP-ether was converted to 3-*t*-butyldimethylsilyl-2-propyn-1-ol in a two-step process [(i) BuLi, TBSCl, THF, 20 °C, 2 h; (ii) PTS, MeOH, 20 °C, 1 h] in 90% total yield. The alcohol was treated with *N*-bromosuccinimide (NBS) and dimethyl sulfide (Me_2S) in CH_2Cl_2 at 20 °C for 7 h to yield the corresponding bromo derivative in 86% yield, which gave rise to **141** in 11% yield on reaction with tris(2,2,2-trifluoroethyl)phosphite at 130 °C (neat) for 48 h. Indeed, the aldehyde (**138**), when treated with the potassium-salt of **141** in the presence of 18-crown-6 in THF at -78 °C for 45 min, gave rise to a 62:38 mixture of (*E*)- and (*Z*)-enynes (**142** and **143**) in an 85% combined yield. The mixture was then converted to the corresponding alcohols (**144** and **145**) on DIBAH reduction (CH_2Cl_2, -78 → -40 °C, 1 h) in 99% yield, which were easily separated by flash chromatography.

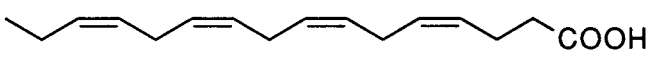
The final critical stage of the synthesis was the efficient construction of the vinylic bromide, (-)-**70**. The synthesis of (-)-**70** was completed in three steps as revealed in Scheme 24. Thus, Swern oxidation of the (*E*)-olefin alcohol (**144**) afforded the aldehyde (**146**) in a quantitative yield, which, on reaction with the Smithers reagent ($Ph_3P^+CBr_2EtBr^-$](60) in the presence of BuLi in THF, afforded a 1:1 mixture of the (*E*)-olefin (**147**) and its (*Z*)-isomer (**148**) in 23% yield. After many invalid attempts, we found that addition of DMSO raised greatly the yield and enhanced the geometrical selectivity. Treatment of **146** with the above Wittig reagent and BuLi in a mixture of THF-DMSO (5:1) at -78 °C for 45 min provided an 83:17 mixture of **147** and **148** in 87% combined yield. The pure (*E*)-olefin (**147**) was obtained by separating the mixture with HPLC. Finally, **147** was treated with a mixture of TBAF-HF in THF at -5 °C for 1 h and then at 0 °C for 1 h to give a colorless oil in 79% yield. This material was identical with the natural isodactylyne (-)-(**70**) in all respects (TLC behavior, specific rotation, IR, ^{1}H NMR, and MS). On the other hand, the (-)-dactylyne synthesis was accomplished from the (*Z*)-olefin alcohol (**145**) in the same manner as that mentioned above. Thus, the alcohol (**145**) was oxidized to the aldehyde (**149**) by the Swern method in 95% yield, which was

transformed into a 82:18 mixture of the (*E*)-vinyl bromide (**150**) and its (*Z*)-isomer (**151**) in 67% total yield. Similarly, desilylation of the separated compound (**150**) gave rise to white crystals, mp 62.5-63.2 °C, which was identical with (-)-dactylyne (**69**) by comparison with their mps, specific rotations, as well as IR, ^{1}H NMR, and MS spectra. In this way, the first total synthesis of (-)-dactylyne (**69**) and (-)-isodactylyne (**70**) was achieved from **82** *via* the key intermediate (**132**) in 25 steps. The overall yields toward **69** and **70** amounted to 14% and 15%, respectively, from the initial coupling reaction.

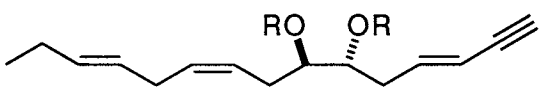
4. ENZYMATIC ONE-STEP SYNTHESIS OF DEACETYLLAURENCIN

4.1 Proposed Biogenetic Routes to Cyclic Bromo Ethers

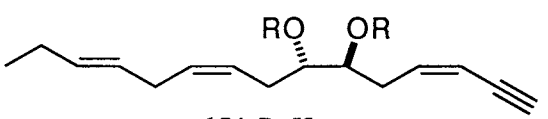
Sea water contains high concentrations of halide ions (Cl^-, 19000 mg/l; Br^-, 65; F^-, 1.3; I^-, 0.06), and it would not be surprising, therefore, if halogen atoms were incorporated into algal metabolites and marine products. Since the pioneering isolation



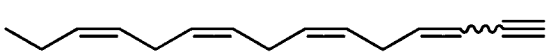
152



153, R=H
155, R=Ac



154, R=H
156, R=Ac



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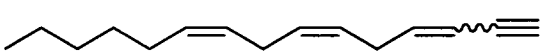
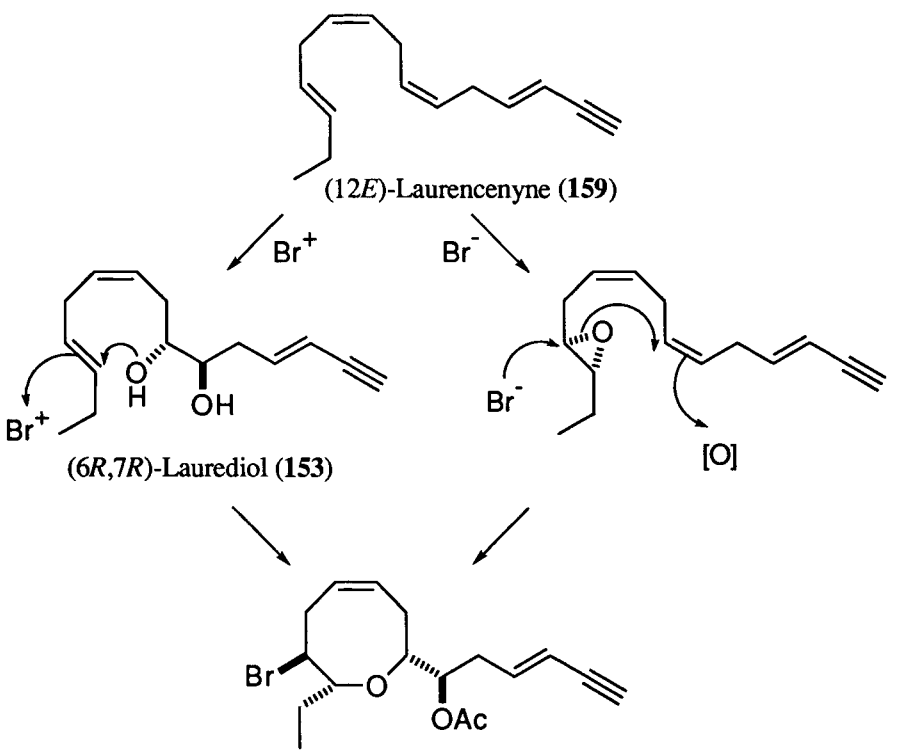


158

of laurencin (**1**) in 1965 (2), many researchers have sought new acetogenins related to **1** in *Laurencia* sp. It has generally been accepted, though with no experimental evidence, that the medium sized cyclic halo ethers represented by **1** arise from C_{15}

linear hydroxy-, halohydroxy-, or epoxypolyenynes, which in turn trace their origin to hexadeca-4,7,10,13-tetraenoic acid (**152**)(61). The isolation of the lauredіols (**153** and **154**) and their acetates (**155** and **156**) from *L. nipponica* by Irie's group in 1972 (62) provides some support for this postulate. Yamada's group has also succeeded in the isolation of laurencenynes (**157**) and neolaurencenynes (**158**) from *L. okamurai* (63).

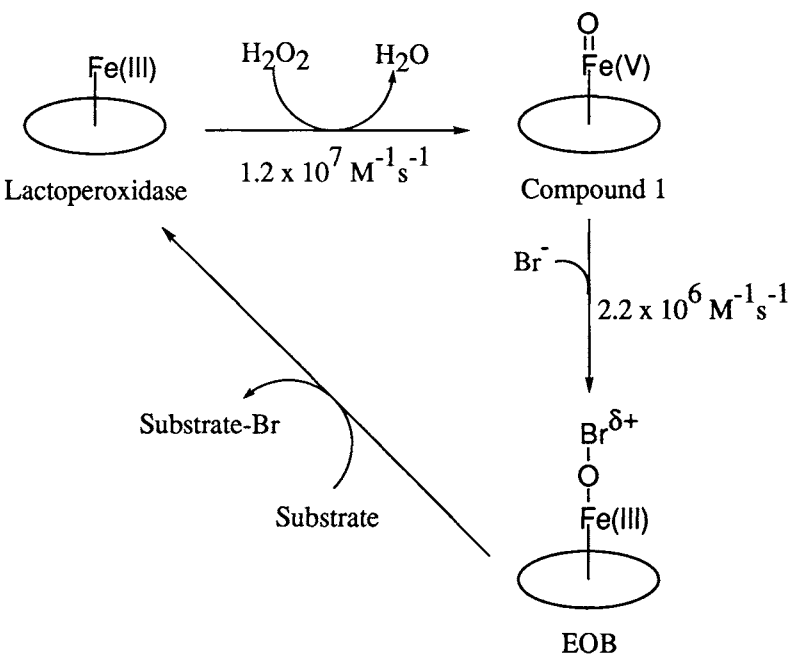
Two routes have been proposed for the introduction of a bromine atom into a straight-chain C_{15} skeleton. In 1975, Gonzalez et al. proposed that the ring closure of a polyne system could proceed with participation of a positively charged bromine atom in the biogenetic pathway of bromine-containing tepenoids (64). On the other hand, in 1979, Kurosawa proposed that the starting polyene could be oxidized biosynthetically to give the terminal epoxide. The epoxide could be attacked by a negatively charged bromine atom to afford the corresponding bromohydrin, which would be cyclized internally with acid to provide the brominated ring (65). Although no attempt has yet been made to establish lauredіols or their original polyenes as the



Scheme 25. Proposed biogenetic pathways for laurencin (**1**).

real biosynthetic precursors, two biogenetic routes would be possible starting from the putative (12*E*)-laurencenyne (**159**), if these proposals are applied to laurencin (**1**) (Scheme 25). The question arises, which path is more plausible in nature? Is the bromine atom introduced into polyenynes as positively or negatively charged bromine? We have proposed that construction of cyclic bromo-ether systems in algae might be attributable to the reaction of laurediols (**153** and **154**) with enzyme-bound bromonium ion (66) *in vivo*. Bromoperoxidase (abbreviated as BPO)(66) would be a plausible enzyme for bromo-etherification, because peroxidases could generate positively charged bromine from hydrogen peroxide (H_2O_2) and bromide.

Bromoperoxidase (BPO) had not been isolated from *Laurencia* sp. when we started to study such enzymatic reactions. Accordingly, we chose to use commercially available lactoperoxidase (abbreviated as LPO) as an enzyme related in character to BPO. LPO can be prepared from cow's milk, and has been well characterized. Its molecular weight is 78,500, and it contains an iron-porphyrin thiol as the prosthetic group. In 1987, the structure of LPO heme was clarified by Australian biochemists (67), who proposed that the unusual strength of binding of the prosthetic group to the apoprotein is due to the formation of a disulfide bond from a cysteine residue to the porphyrin thiol. As shown in Scheme 26, the iron atom (oxidation value of



Scheme 26. General mechanism for the reaction of halogen accepter substrates with lactoperoxidase.

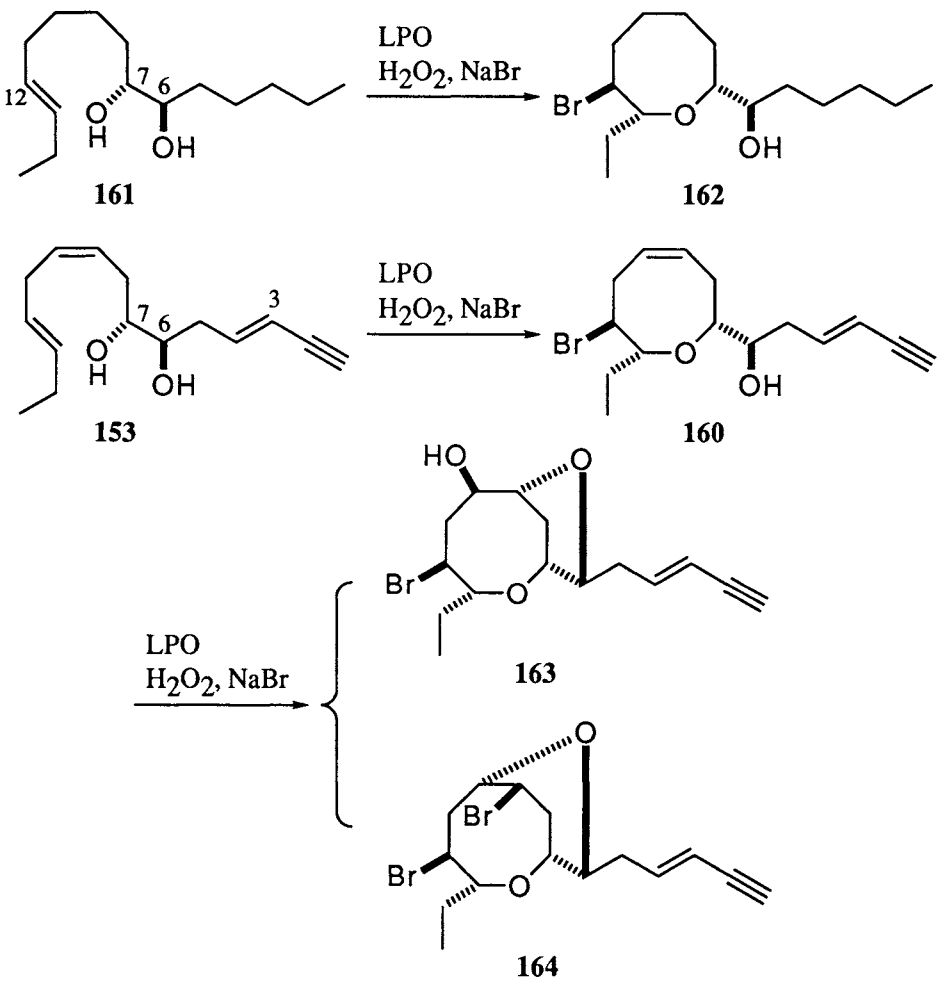
Fe=3) in the porphyrin skeleton can be oxidized in contact with H_2O_2 to give compound 1 (oxidation value of Fe changes to 5). The conversion of the native enzyme to compound 1 is very fast, as shown by the rate constant (1.2×10^7 $M^{-1}s^{-1}$). Compound 1 reacts with bromide ion to afford enzyme-bound bromonium ion (abbreviated as EOB) with an estimated rate constant of 2.2×10^6 $M^{-1}s^{-1}$. The results revealed that the bromide ion was oxidized by the enzyme by way of a two-electron transfer (not *via* a bromine radical). Reaction species generated by iron porphyrin in LPO have been established to be enzyme-bound bromonium ion [porphyrin-Fe(III)-O-$Br^{\delta+}$, *i.e.*, EOB). The bromine atom of EOB would attack the substrate as a positively charged bromine. After completion of the reaction, the ferric form would revert to the native enzyme.

4.2 Enzymatic Total Synthesis of Deacetyllaurencin, Laurefucin, and Laureoxanyne

On the basis of several preliminary experiments, we attempted the corresponding enzymatic reactions with the highly unstable laurediol (**153**), aiming at the enzymatic synthesis of laurencin-type compound. Herein the enzymatic synthesis of deacetyllaurencin (**160**) starting from (3*E*,6*R*,7*R*)-laurediol (**153**) is described (Scheme 27)(68).

As a model experiment, (6*R*,7*R*,12*E*)-12-pentadecene-6,7-diol (**161**) was treated with H_2O_2 and LPO in the presence of sodium bromide (NaBr) in phosphate buffer (pH 5.5) at 23 °C for 24 h to afford the desired eight-membered bromo-ether (**162**, 1.4% yield) and bromohydrins (17.5%) as major products, together with the recovered starting material (54.0%). Compound **162** was identical with deacetylocta-hydrolaurencin prepared from natural laurencin (**1**). Next, a 20:1 (3*E*/3*Z*)-mixture of (6*R*,7*R*)-laurediol (**153**) was prepared as the substrate from natural laurencin (**1**). The compound (**153**, 73.8 mg) was highly labile and was immediately subjected to the enzymatic reaction. A solution of **153** in a trace amount of DMSO was injected into phosphate buffer (pH 5.5) containing NaBr, and the vessel was fitted with an argon balloon and kept at 5 °C. Solutions of H_2O_2 and LPO were added in 12 portions during 2 h. The mixture was stirred at 5 °C for 24 h to give crude deacetyllaurencin (**160**, 1.0 mg), an unknown cyclic ether (0.9 mg), an undetermined mixture of bromohydrins (29.2 mg), and the recovered starting material (38.4 mg). The impure sample of **160** was acetylated under the usual conditions and separated over silica gel to afford a pure sample of laurencin (**1**, 0.8 mg, 0.73%). Since the enatiomer of the starting laurediol (**153**) has been totally synthesized (69), the reaction results represent a the formal total synthesis of **1**. The results provide strong support for the hypothesis that laurediols are the real biosynthetic precursors of **1** and its related cyclic ether compounds of marine origin. This experimental result would strongly suggest that the bromine atom in biosynthetic bromo-etherification in the algae could be incorporated as positively charged bromine, and not as negatively

charged bromine. Furthermore, we examined the action of LPO on **160**, because the compound still includes an olefin alcohol moiety (70). Deacetyllaurencin (**160**, 205 mg) dissolved in DMSO was added to phosphate buffer (pH 5.5) containing NaBr. Solution of LPO and H_2O_2 were added in 9 portions to the reaction mixture at 10 min intervals in the dark at room temperature. After having been stirred for 24 h, the mixture gave laurefucin (**163**, 13.9 mg), laureoxanyne (**164**, 8.2 mg), bromohydrins (13.7 mg), and the recovered substrate (124.6 mg). Compounds (**163**) and (**164**) are also natural products isolated from *L. nipponica*. Accordingly, this enzymatic reaction also constitutes the formal total synthesis of these compounds.



Scheme 27. Enzymatic Syntheses of deacetyllaurencin (**160**), laurefucin (**163**), and laureoxanyne (**164**).

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Oxidative Ring Transformation of 2-Furylcarbinols in Natural Product Syntheses

Toshio Honda

Summary: The stereoselective synthesis of variuos types of natural poducts including steroids, alkaloids, and antibiotics, was successfully achieved in optically active forms by utilization of the oxidative ring transformation of 2-furylcarbinols to the corresponding pyranones as the key step. The strategy for the synthesis of optically active 2-furylcarbinols was also reviewed.

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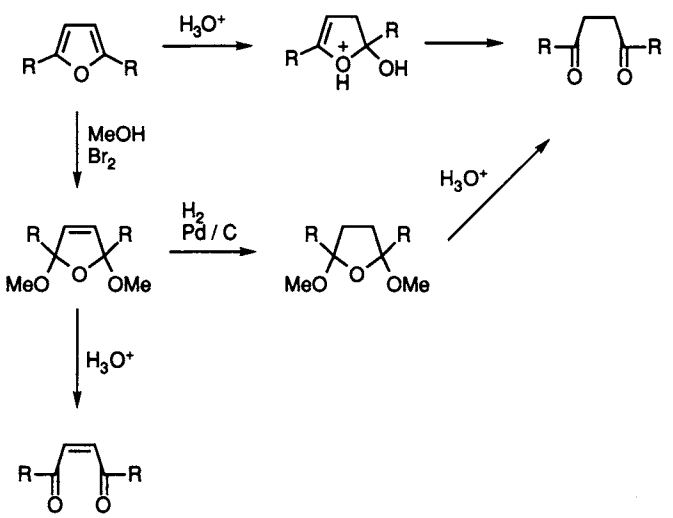
I. Introduction

Furan and substituted furans were the most frequently exploited monocyclic heteroaromatic compounds in natural products syntheses. For example, the intra- and intermolecular Diels-Alder reactions of substituted furans emerged as useful implements for the syntheses of structurally complicated natural products or their key intermediates.

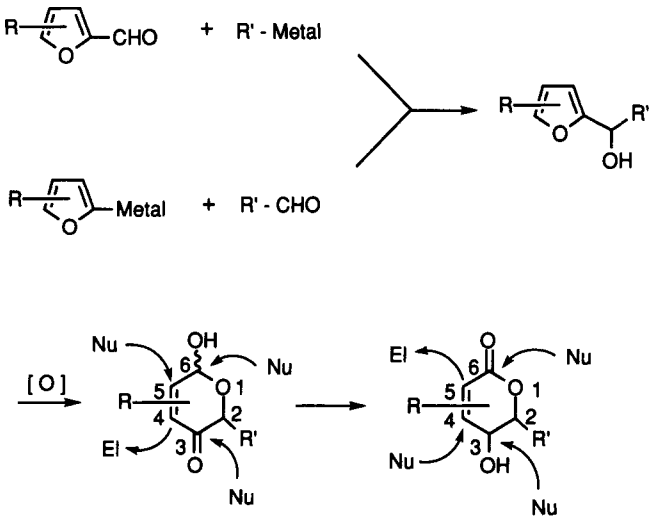
Furan nucleus is also recognized as a masked 1,4-dicarbonyl compound (1-3) (Scheme 1). In fact, exposure of furan derivatives to mineral acids afforded the corresponding ring opened 1,4-dicarbonyl compounds and a number of natural products have been synthesized by employing this sequence (4).



Scheme 1.

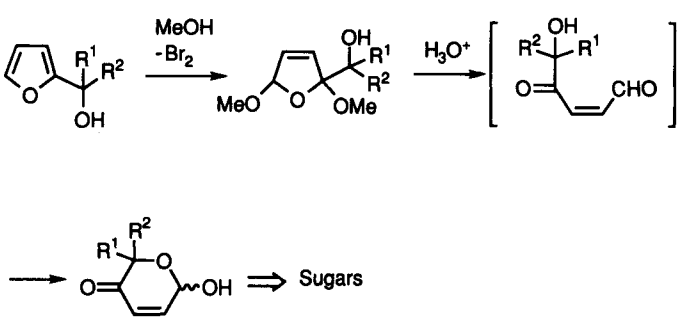
Among the various kind of valuable ring transformations of furan derivatives so far reported, the conversion of 2-furylcarbinols leading to the corresponding pyranones by oxidative treatment provided one of the most useful synthetic pathway to polyoxygenated natural products, since the

functionalizations of such pyranones with either nucleophiles or electrophiles would easily be elaborated in stereo- and regioselective manners (Scheme 2).



Scheme 2.

This type of ring transformation has successfully been utilized by Achmatowicz (5) in the synthesis of saccharides, in which revarsal of the formation of furfural in the course of heating sugars with mineral acid, firstly observed by Döbereiner in 1832, (6) was involved as a key step (Scheme 3).



Scheme 3.

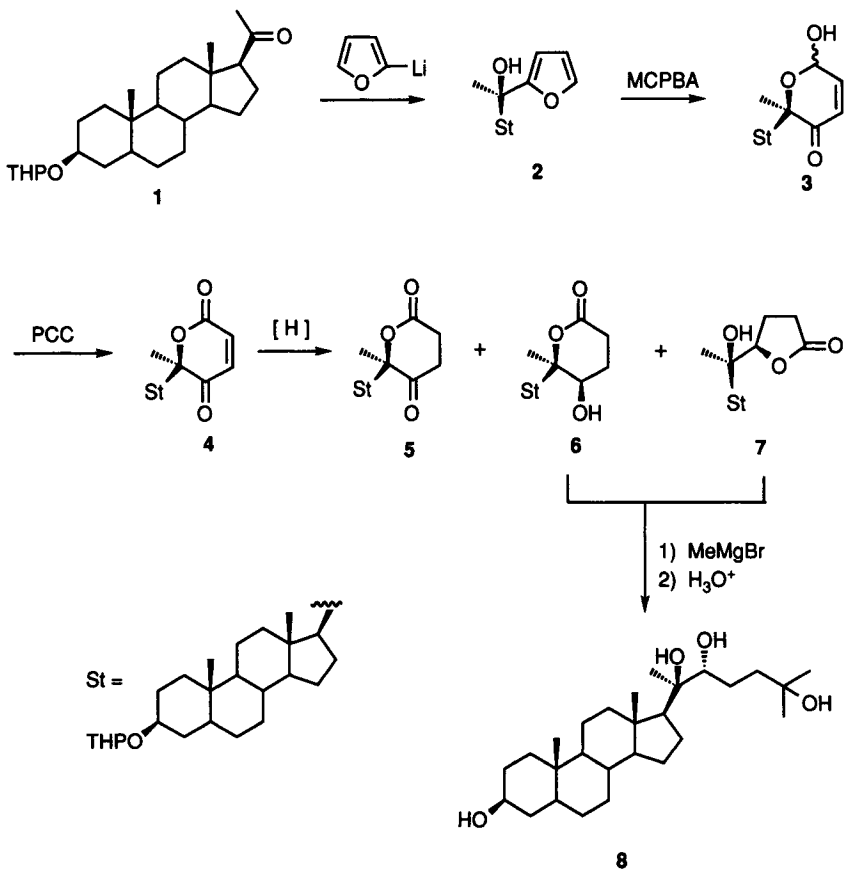
In this review, the utilization of the pyranone derivatives generated by oxidation of 2-furylcarbinols in the chiral syntheses of highly oxygenated natural products developed in our group will be summarized.

II. Syntheses of Polyoxygenated Steroids

Naturally occurring steroids, with their wide range of structural and stereochemical features, continue to provide challenging synthetic targets, and the development of effective synthetic methods for polyoxygenated steroids, such as crustecdysone, withanolide, and brassinolide has long been investigated owing to their interesting physiological activities. It is also well known that the biological activities of these steroids are dependent on their stereochemistry of the side chains (7), therefore, much efforts have been devoted to the stereocontrolled construction of the side chains. We have utilized the methodology involving an oxidation of a 2-furylcarbinol in the stereoselective synthesis of the steroidal side chains as follows.

1. Crustecdysone

20-Hydroxyecdysone (crustecdysone) is known as a crustacean molting hormone. Since the ecdysteroids possess the same configuration of the hydroxyl group at the 22-position on their side chains, its stereocontrolled introduction is the main objective in the synthesis of these steroids. The synthetic methodology for 20-hydroxyecdysone developed by us (8) is outlined in Scheme 4, where the oxidation of the 2-furylcarbinol affording the pyranone followed by its stereoselective reduction played important roles to introduce the 22- and 25-hydroxyl groups. The stereoselectivity of the reduction of the pyranone (**4**), derived from **1** and 2-lithiofuran followed by the oxidation of **2** with *m*-chloroperbenzoic acid, was investigated with various kinds of reducing agents and the results obtained are summarized in Table 1. As can be seen in Table 1, reduction of the pyranone (**4**) over platinum oxide in ethyl acetate under an atmospheric pressure of hydrogen provided the lactones (**6** and **7**) in high yield, both of which on treatment with methylmagnesium bromide afforded the same desired product (**8**). (9) The observed stereoselectivity in the reduction of the pyranone (**4**) can be rationalized by assuming that the attack of hydrogen occurred exclusively from the less hindered side of the carbonyl group, because the steroidal nucleus was large enough to prevent the attack of hydrogen from the other side. Since the simple and efficient method for introducing the 20-hydroxyecdysone-type side chain into 20-oxopregnane was thus achieved (10), this procedure was applied to the synthesis of 2-deoxycrustecdysone (11) and related compounds.



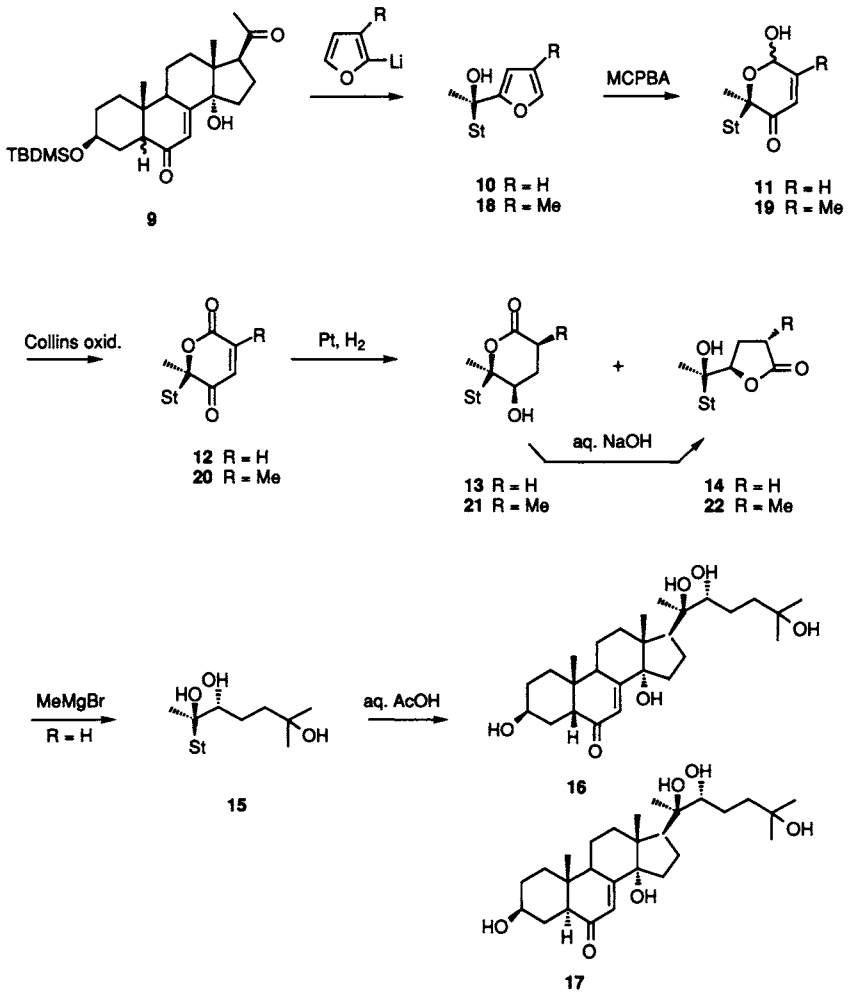
Scheme 4.

	Products (%)		
Reaction conditions	5	6	7
10% Pd / C, H_2, benzene	97.6		
Pt, H_2, benzene	17.4	trace	80.6
Pt, H_2, AcOEt		80.1	19.8
$NaBH_4$, MeOH - CH_2Cl_2		4.9	78.3

Table 1. Reduction of Compound **4**.

Introduction of 2-lithiofuran (12) into the 20-oxosteroid (**9**) gave the tertiary alcohol (**10**), which on exposure to *m*-chloroperbenzoic acid (13) resulted in ring enlargement and gave the lactol (**11**). Collins oxidation of **11** gave keto lactone (**12**) which was then hydrogenated over platinum oxide to afford δ-lactone (**13**) and γ-lactone (**14**) in a ratio of 3:2. The crude mixture could be

converted into the thermodynamically more stable isomer (**14**) by treatment with aqueous sodium hydroxide.



Scheme 5.

It should be again anticipated that the hydrogenation of the pyranone (**12**) proceeded in a stereoselective manner to give the 22*R* hydroxyl group as the sole stereoisomer (Figure 1). The lactone (**14**) was reacted with methylmagnesium bromide (14) to afford **15**. Removal of the silyl ether furnished 2-deoxycrustecdysone (**16**) and its epimer (**17**) in moderate yield (Scheme 5). This synthetic strategy was further employed in the synthesis of 2-deoxymakisterone A (15). The 20-

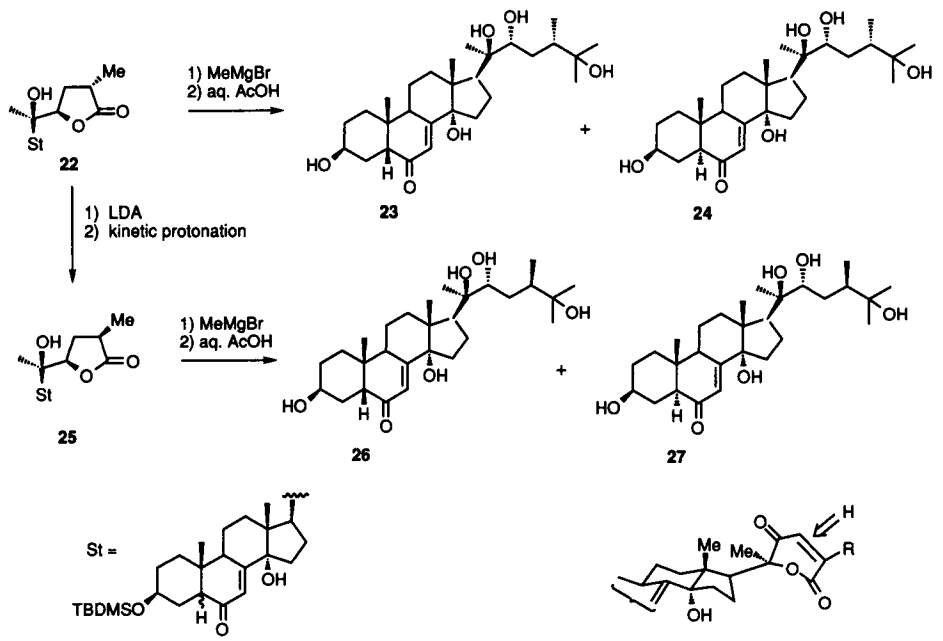


Figure 1.

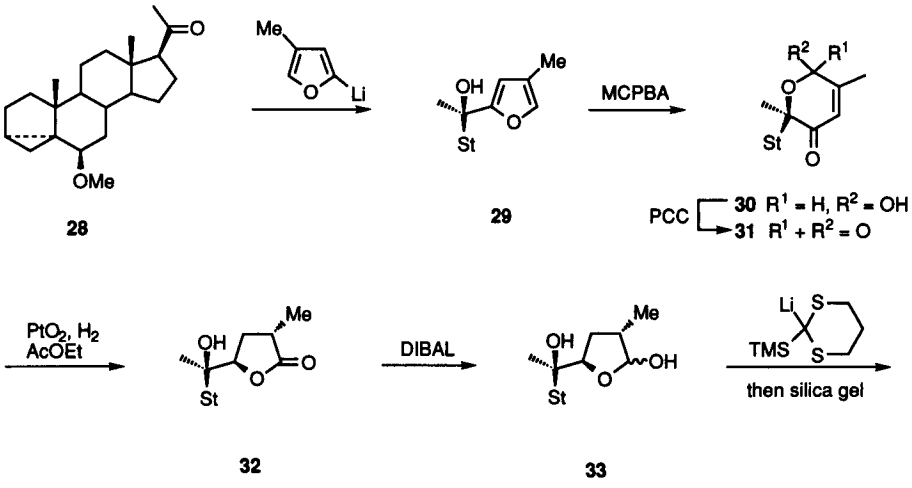
Scheme 6.

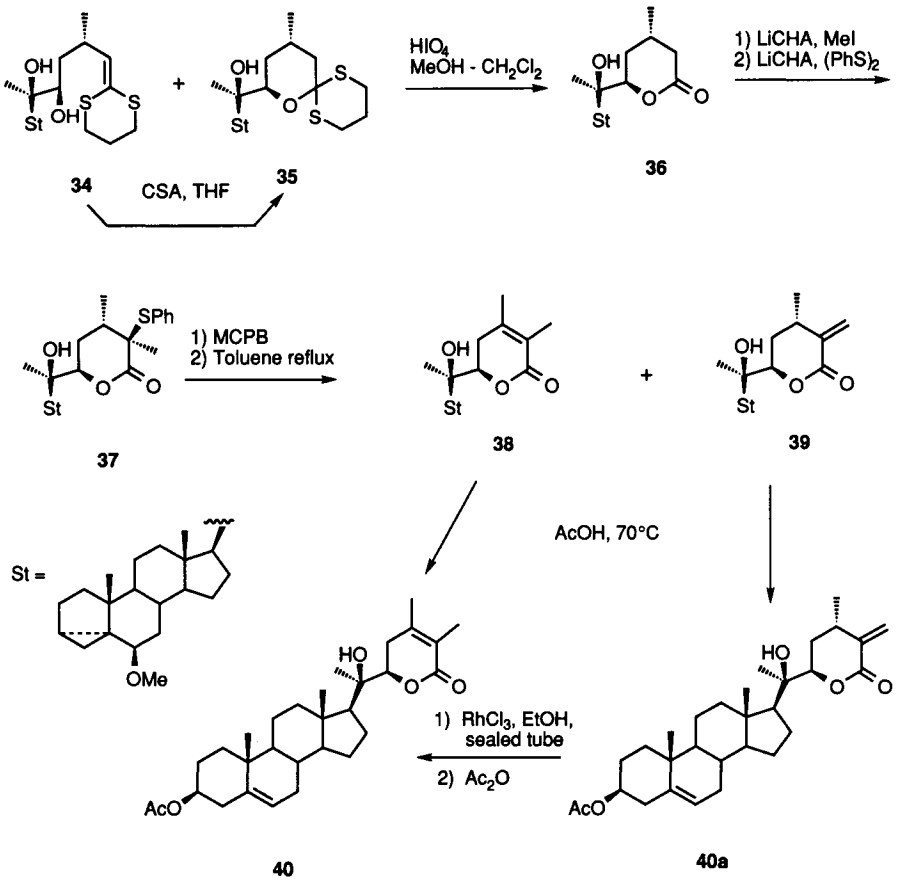
oxosteroid (**9**) was treated with 2-lithio-4-methylfuran (16) to give the adduct (**18**) which was converted *via* the lactol (**19**) to the lactone (**20**) as described above. Hydrogenation of the lactone (**20**) over platinum oxide in tetrahydrofuran and recyclization of the intermediate (**21**) provided the γ-lactone (**22**) in quantitative yield. The stereochemistry of the the lactone was assumed to have 20*R*, 22*R*, 24*S* on the basis of its NMR spectrum. Methylation of the lactone with methylmagnesium bromide, followed by removal of the silyl group gave 24-*epi*-2-deoxymakisterone A (**23**) and its epimer (**24**). In order to synthesize the makisterone A-type side chain, the inversion of the stereochemistry at the 24-position was required. Stereoselective inversion of the 24-configuration in **22** was accomplished by kinetic protonation (17) of the γ-butyrolactone providing the lactone (**25**). The ratio of the 24*R* and 24*S* epimers in **25** was 4:1, and the ratio of the 5α and 5β epimers was 7:1. Treatment of the lactone (**25**) with methylmagnesium bromide, followed by cleavage of the silyl ether afforded 5-*epi*-2-deoxymakisterone A (**26**) and its stereoisomer (**27**) (Scheme 6). The stereocontrolled construction of the steroidal side chain described above involved the utilization of the 1,4-dicarbonyl functionality of the furan ring and the stereoselective reduction of the carbonyl group(s) of the pyranone generated by oxidative treatment of the 2-furylcarbinol, was only employed as the key step in these syntheses.

2. Withanolide

Withanolides, a group of highly oxygenated ergostane type steroids possessing a δ-lactone in the side chain, have been isolated from the plants of *Solanaceae* family (18) and also from marine organisms. (19) Because of their novel structural feature and remarkable biological activities (20), such as antitumor and insect antifeedant properties, much attention has focused on the development of their effective synthesis. It was reasonably assumed that the formation of the δ-lactone ring could be achieved by oxidation of the corresponding 2-furylcarbinol.

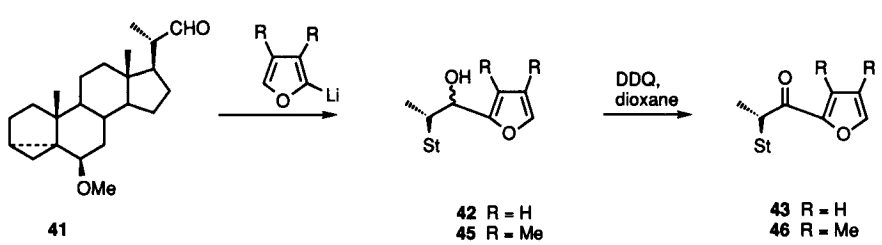
Thus, the stereoselective construction of withanolide D type side chain, having 20-hydroxy group, was first investigated. The readily accessible 2-furylcarbinol (**29**) by addition of 2-lithio-4-methylfuran (16) to aldehyde (**28**), was subjected to ring transformation reaction with *m*-chloroperbenzoic acid in dichloromethane to give the lactol (**30**), which on oxidation with PCC provided the keto-lactone (**31**). Catalytic reduction of **31** over platinum oxide in ethyl acetate afforded the γ-lactone (**32**) stereoselectively. Ring enlargement of the γ-lactone to δ-lactone was accomplished by application of ketene thioacetal chemistry (21). Reduction of the γ-lactone with DIBAL in THF gave the lactol (**33**), which in turn on treatment with 2-lithio-2-trimethylsilyl-1,3-dithiane (22) in THF at 0°C afforded the thioacetal (**34**). Acid catalyzed cyclization (23) of **34**, followed by deprotection of the thioacetal (**35**) with periodic acid (24) in dichloromethane-methanol provided the δ-lactone (**36**). Successive methylation and phenylsulfinylation of **36** yielded the sulfide (**37**), whose oxidative elimination gave the α,β-unsaturated lactones (**38** and **39**) in a ratio of 1:4.2. Both lactones were subjected to a ring opening reaction of the cyclopropane rings with acetic acid to afford the acetates (**40** and **40a**), respectively. The major isomer (**40a**) was finally converted to **40** (25) by isomerization of the double bond with rhodium chloride (26) in a sealed tube at 100°C and subsequent acetylation (Scheme 7). Since the construction of withanolide D type side chain was thus achieved (27) stereoselectively, the synthesis of the most simple withanolide family of compound, minabeolide-3 (19), isolated from a soft coral *Minabea* species, was then investigated.

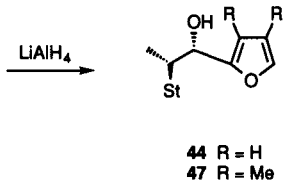




Scheme 7.

We note in advance that the addition reaction of 2-lithiofuran (12) to the 20-carboxaldehyde (**41**) (28) provided mainly the 22*R*-isomer (**42**), a Cram product.





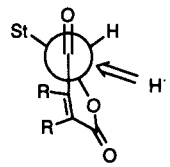
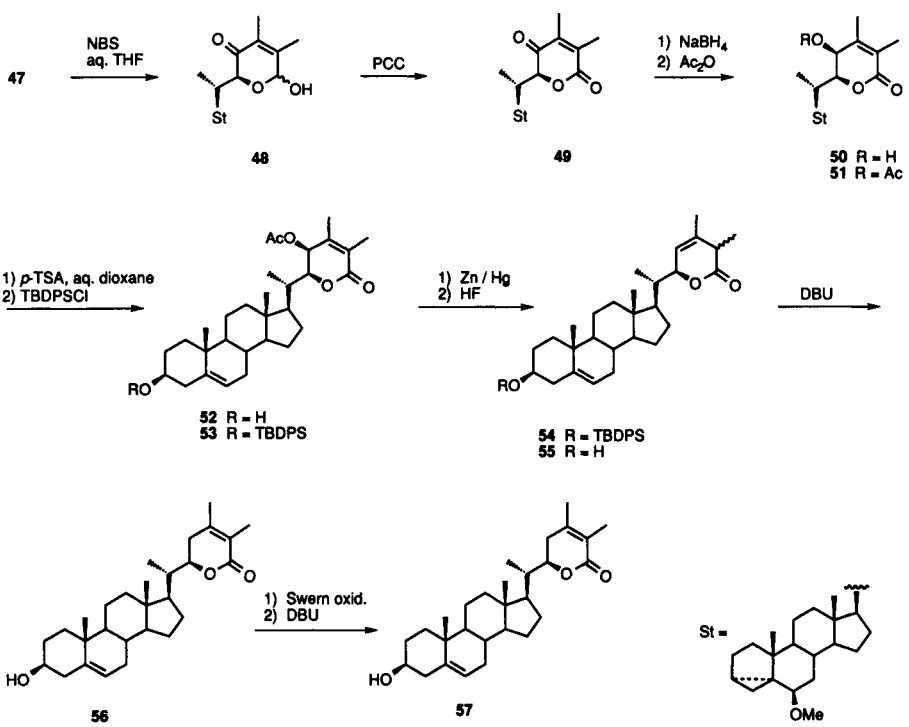


Figure 2.

Table 2. Reduction of **43** with Reducing Agents

reducing agent	ratio of products **42** : **44**	% yield
DIBAL	1 : 1.8	91.2
$Zn(BH_4)_2$	1 : 2.2	86.9
L-Selectride	1 : 3.2	88.8
$LiAlH_4$	1 : 3.6	91.1
$NaBH_4$	1 : 3.7	90.0
Red-Al	1 : 3.8	93.1



Scheme 8.

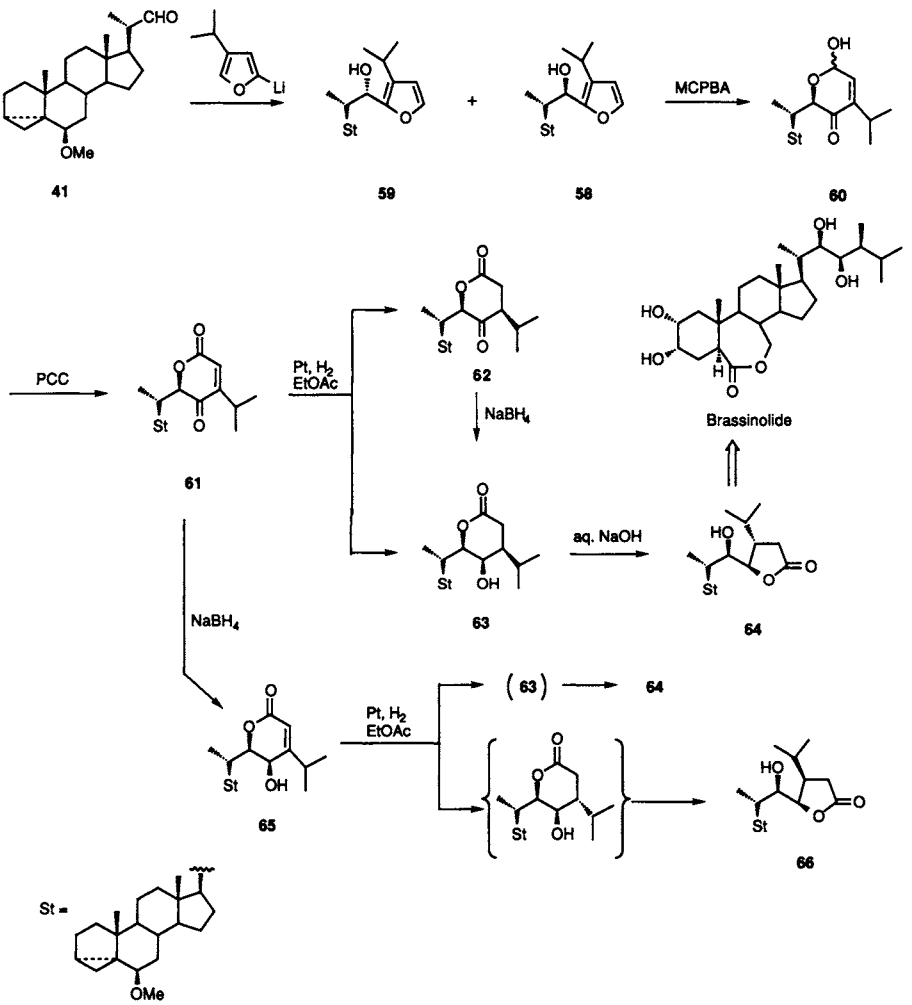
For the synthesis of withanolide side chains, it is necessary to prepare the anti-Cram product at 22-position. Although the well established Yamamoto's procedure (29) giving the anti-Cram adduct predominantly was attempted for this addition reaction using methylaluminum bis(2,4,6-tri-*tert*-butylphenoxide) as a catalyst, the major isomer was again the 22*R*-compound. The stereoselective reduction of the ketone (**43**), readily derived from the alcohol (**42**), was therefore attempted to prepare the 22*S*-isomer. The results of the reduction of **43** with a variety of reducing agents are summarized in Table 2. Interestingly, the reduction proceeded with moderate Cram selectivity to afford the isomer (**44**) predominantly in a ratio of 1.8-3.8:1, although reduction of 22-oxosteroids having saturated side chains provides mainly the anti-Cram products (7). This selectivity agrees with the previous observation in the reduction of 22-oxo-$^{\Delta}$23-steroids with L-Selectride (30). Based on these results, the desired (22*S*)-isomer was synthesized as follows. Addition of 2-lithio-3,4-dimethylfuran (31) to the aldehyde (**41**) gave the mixture of the 22*R*- and 22*S*-isomers (**45**), which on oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (32) in dioxane afforded the 22-oxosteroid (**46**). Reduction of **46** with LAH in THF proceeded with high diastereoselectivity to afford the desired 22*S*-furylcarbinol (**47**) together with its isomer in a ratio of 16:1. The increased stereoselectivity observed in this reduction was presumably owing to the presence of the bulky dimethyl group on the furan ring. Ring transformation of the 2-furylcarbinol (**47**) with aqueous NBS (33), followed by PCC oxidation of the lactol (**48**) afforded the relatively unstable δ-lactone (**49**), which, without purification, was reduced with sodium borohydride to give the alcohol (**50**), withanolide R-type side chain (34), as the sole product (Figure 2). Ring opening of the cyclopropane was carried out prior to further transformation of the side chain moiety. Acid treatment (35) of the acetate (**51**) with *p*-toluenesulfonic acid in aqueous dioxane afforded the olefinic alcohol (**52**), which was then protected as the silyl ether (**53**). Reductive deoxygenation of the acetate with zinc-amalgam in ethereal hydrogen chloride gave the olefin (**54**), whose deprotection of the silyl group with hydrogen fluoride (36) and subsequent olefin isomerization with DBU provided the α,β-unsaturated lactone (**55**). Finally, Swern oxidation of **55** gave the ketone (**56**), which was treated with oxalic acid in ethanol to afford minabeolide-3 (**57**) (37) (Scheme 8).

The above syntheses clearly indicated that the oxidation of 2-furylcarbinol derivatives can play an important role in the construction of δ-lactone moieties on the steroid side chains stereoselectively. This synthetic strategy should be applicable to the synthesis of other types of naturally occurring withanolides.

3. Brassinosteroid

Since the first isolation of brassinolide (38) from rape pollen (*Brassica napus* L.) as a plant growth regulating steroid in 1979, much effort has been devoted in recent years to develop synthetic routes to brassinolide and its congeners because of its remarkable biological activity and structural novelty (39). We have investigated a synthetic strategy involving an oxidation of 2-furylcarbinol on the construction of brassinosteroid side chain where the contiguous four chiral centers are presented.

The key feature of our first synthetic approach is based on a stereoselective reduction of the unsaturated lactone (**61**) to incorporate the required stereochemistry at the 23- and 24 positions. The lactone (**61**) was easily prepared as follows. Addition of 3-isopropyl-2-lithiofuran (40) to the 20*S*-carbaldehyde (**41**) furnished 22*R*- and 22*S*-furylcarbinols (**58** and **59**) in 81.1 and 17.9% yields, respectively (Scheme 9).



Scheme 9.

The preferred stereochemistry at the 22-position is predicted by the Felkin-Anh models for the transition state (41). Ring transformation of the 2-furylcarbinol (**58**) with *m*-chloroperbenzoic acid,

followed by further oxidation of the resulting lactol (**60**) with PCC afforded the desired lactone (**61**). Catalytic reduction of the lactone (**61**) with platinum oxide in ethyl acetate gave the saturated lactone (**62**) in 34% yield together with a mixture of the hydroxy lactones (**63** and **64**). Base treatment of the δ-hydroxy lactone (**63**) yielded the thermodynamically stable γ-hydroxy lactone (**64**) as the sole product. Sodium borohydride reduction of the saturated lactone (**62**) in methanol and dichloromethane again provided the lactone (**63**), which on isomerization gave **64**. Since compound **64** was already converted into brassinolide by several groups (39, 42), this synthesis constitutes a formal synthesis of brassinolide (43). When sodium borohydride reduction was applied to the keto lactone (**61**), the allyl alcohol (**65**) was isolated stereoselectively, whose hydrogenation and subsequent isomerization gave **64** and **66** in 72.5 and 24.9% yields respectively. Stereoselectivity in the reduction of compounds **61**, **62**, and **65** may be rationalized by assuming that reaction occurs from the less hindered side of the lactone ring as depicted in Figure 3.

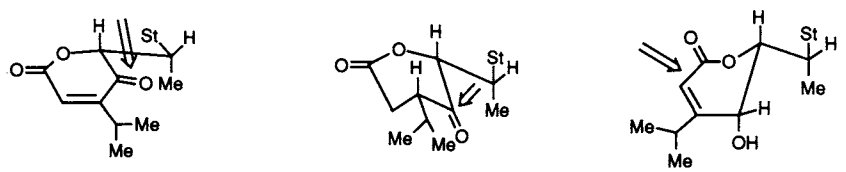
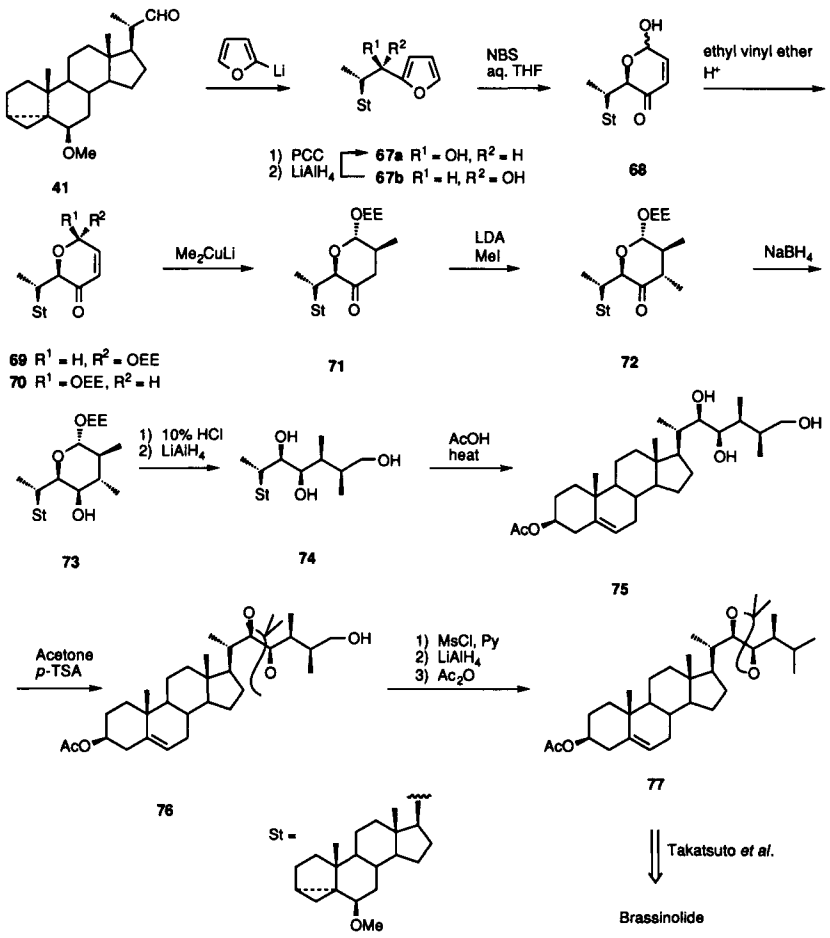


Figure 3.

The second approach which we have developed (44) involved the nucleophilic conjugate addition of the 26-methyl group to the pyranone derivative (**70**) stereoselectively (Scheme 10).

The requisite pyranone (**68**) was prepared by addition of 2-lithiofuran to the 20*S*-carboxaldehyde (**41**) giving the 2-furylcarbinols (**67a** and **67b**) in a ratio of 7:3, respectively, followed by oxidation of the resulting major 2-furylcarbinol (**67a**) on treatment with *N*-bromosuccinimide in aqueous THF. The minor alcohol (**67b**) could be converted to **67a** by oxidation, followed by reduction with lithium aluminum hydride. The lactol group of **68** was then protected as the ethoxyethyl group to give the α-anomer (**69**) as the major product together with the β-isomer (**70**), in a ratio of 3:1, owing to the anomeric effect. Conjugate addition of lithium dimethylcuprate to the pyranone (**69**) afforded the ketone (**71**), which on alkylation with methyl iodide in the presence of LDA furnished the bis-methylated compound (**72**) in 84% yield. These reactions would occur smoothly from the opposite side of the adjacent groups as expected on the basis of literature procedure (45). The 23-hydroxy group was stereoselectively introduced by reduction of the ketone (**72**) with sodium borohydride to give the alcohol (**73**). Deprotection of the ethoxyethyl group with 10% HCl and subsequent reduction with LAH gave the triol (**74**). Heating of **74** in acetic acid afforded the acetate (**75**), which in turn was converted into the acetonide in a usual manner. Finally the alcohol (**76**) was transformed to the known compound (**77**) (39), the key intermediate for the synthesis of brassinolide in three steps involving mesylation, LAH reduction and acetylation.

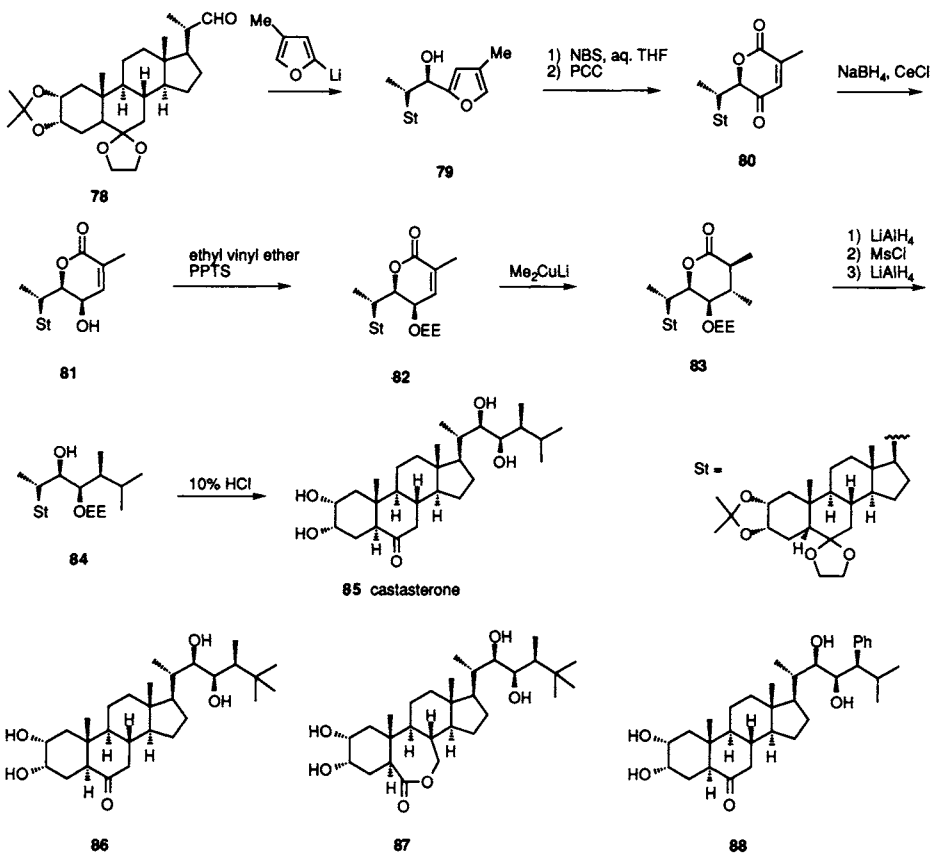


Scheme 10.

In a modification of this synthetic strategy where the nucleophile and electrophile were employed in a reverse mode, a number of brassinosteroids and their congeners were synthesized as follows (46). The key feature of this synthesis is based on the conjugate addition of the 24-methyl function to the α,β-unsaturated lactone, which was again obtained by applying the oxidation procedure to the 2-furylcarbinol as a key step. Thus, addition of 2-lithio-4-methylfuran to the known aldehyde (**78**) gave the Cram adduct (**79**) as a major isomer, which on sequential oxidation with NBS in aqueous THF and with PCC afforded the lactone (**80**). Reduction of the ketone (**80**) with sodium borohydride in the presence of cerium(III) chloride in methanol (47) provided the alcohol (**81**) as a sole product. This result again indicated that the reduction proceeded in a stereoselective manner from the less hindered side of the lactone. After protection of the hydroxyl group as a

ethoxyethyl ether, 1,4-conjugate addition of lithium dimethylcuprate to the α,β-unsaturated lactone (**82**) was carried out at -10°C in ether to give the adduct (**83**) as a single stereoisomer at the 24 and 25 positions. Since the conjugate addition is considered to proceed in an anti-sense with respect to the adjacent ether group at the 23 position, the product should have the 24*S* configuration. Moreover, the stereochemistry at the 25 position was assumed to be *S* configuration because the all-trans conformation for the δ-lactone seemed to be thermodynamically stable. The lactone (**83**) bearing four contiguous chiral centers was further converted to the brassinolide side chain by LAH reduction, mesylation of the primary alcohol, and again LAH reduction. Finally, cleavage of the protecting groups of the compound (**84**) on treatment with 10% hydrochloric acid furnished castasterone (**85**) (48). Conversion of castasterone into brassinolide is already achieved by several groups (39).

Using the lactone (**82**) as the starting material, the synthesis of 25-methylcastasterone (**86**), 25-methylbrassinolide (**87**) and 24*R*-phenylbrassinolide (**88**) was also accomplished (46) (Scheme 11).

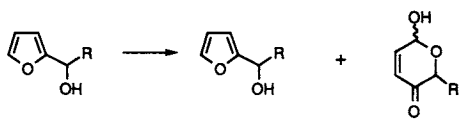


Scheme 11.

III. Kinetic Resolution of Secondary 2-Furylcarbinols under the Sharpless Epoxidation Condition

Since it is recognized that 2-furylcarbinols serve as versatile building blocks in the synthesis of highly oxygenated natural products, a number of their chiral syntheses, such as chemical transformation of sugars (49), optical resolution of racemic 2-furylcarbinols (50), chemoenzymatic technique (51), and aymmetric syntheses (52) have been reported to date. Some of them, however, suffer from the limitation of the presence of other possible functions on the furylcarbinol nucleus. We have also investigated (53) approaches to developping a general method for the synthesis of optically active 2-furylcarbinols employing kinetic resolution of the racemate under the Sharpless epoxidation condition (54), since 2-furylcarbinol was considered to be an extended secondary allyl alcohol system.

Table 3. Kinetic Resolution of 2-Furylcarbinols[a]



Entry	Racemic Starting Material (a - f) R	temp. (°C)	time (h)	optically active 2-furylcarbinols (a - f) [b] yield (%) [c]	% ee [d]	$[\alpha]_D$ (c, $CHCl_3$)	abs. config.	pyranones yield (%) [c]
1	a methyl	-20	5	36	80	+17.4 (1.78)	R	38
2	b ethyl	-20	3.5	32	95	+12.6 (2.09)	R	42
3	c butyl	-35	6	43	94[f]	+ 9.2 (1.07)	R	46
4	d propenyl	-30	7	32	82[e]	-40.4 (1.95)	R	52
5	e cyclohexyl	-25	7	44	>98	+20.0 (1.19)	R	52
6	f decyl	-25	4	44	>98	- 9.6 (2.49)	S [g]	51

[a] All reactions were carried out with 10% $Ti(O^iPr)_4$, 15% L-(+)-DIPT, and 0.7 equiv. of TBHP / isooctane in the presence of 3A molecular sieves, except as noted. [b] All recovered 2-furylcarbinols had the depicted (R) configuration except 1f. [c] Isolated yields. [d] Determined by 400 MHz ^{1}H-NMR analysis of the corresponding MTPA esters. [e] >98% ee indicates that the other wnantiomer was not detectable by NMR. [f] Determined by 400 MHz ^{1}H-NMR analysis of the MTPA ester of 2-furylcarbinol after hydrogenation of the optically active 1d because of the instability of the product on reaction with MTPA chloride. [g] D-(-)-DIPT was employed.

Based on the extensive studies on kinetic resolution of racemic secondary alcohols by Sharpless (54), it is reasonably assumed that the faster reacting 2-furylcarbinol would be oxidized preferentially to give the corresponding pyranone and the unchanged isomer would be recovered in an optically active form with an opposite optical rotation to the pyranone. With this consideration in mind, the kinetic resolution of 2-furylcarbinols was examined as follows. The reactions were performed with catalytic amounts of titanium tetraisopropoxide [Ti(OiPr)4], L-(+)-diisopropyl tartrate (L-DIPT) and *tert*-butyl hydroperoxide (TBHP) in the presence of molecular sieves 3 Å, except for entry 6. The results obtained are summarized in Table 3. (*R*)-2-Furylcarbinols were obtained employing L-DIPT, whereas the use of D-DIPT led to the formation of (*S*)-2-furylcarbinols with high enantiomeric

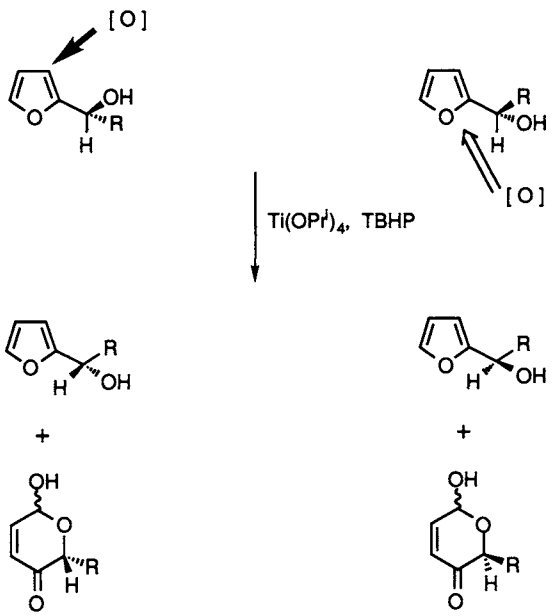


Figure 4.

purity (80-98% ee) (Figure 4). When this reaction was applied to the 2-furylcarbinol having an alkenyl moiety on the side chain, the corresponding pyranone and (*R*)-furylcarbinol were formed, respectively. This result suggests that the oxidation of furyl double bond is faster than that of the alkenyl moiety on the side chain under this reaction condition. The absolute configuration of the resolved furylcarbinols was deduced either from a comparison of their optical rotations with authentic samples or their conversion into known compounds.

One of the reasons for making this reaction usefull is that the absolute configuration of the kinetically resolved 2-furylcarbinols could be predicted by the chirality of the tartrate used. Sato and his coworkers have also reported the preparation of optically active 2-furylcarbinols, 3-furylcarbinols, and 2-thienylcarbinols using the essentially same strategy, independently (52).

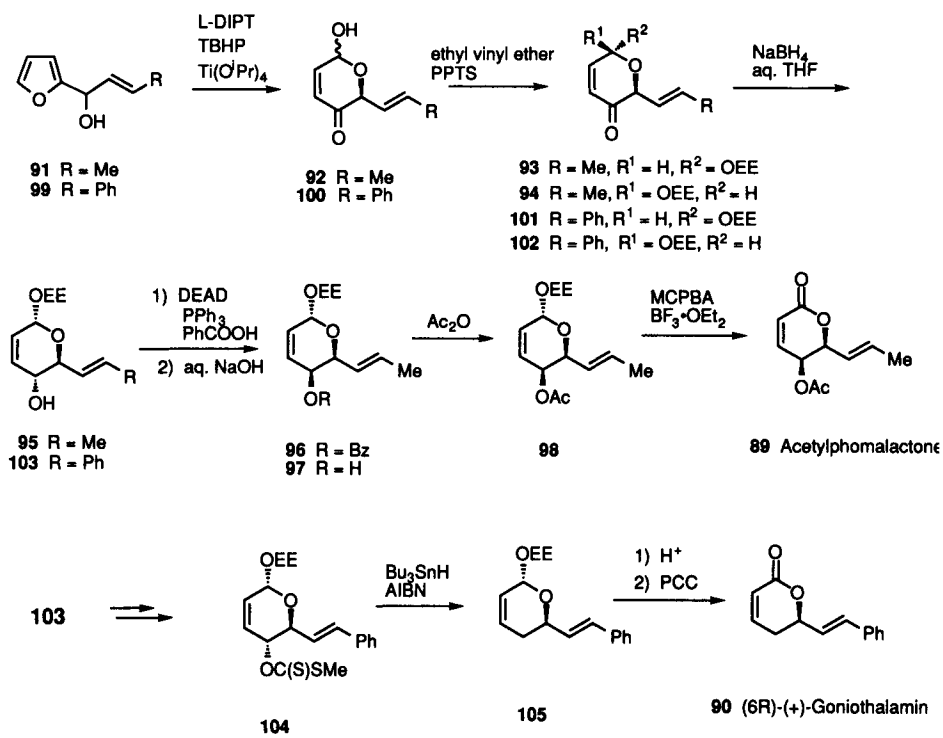
IV. Application of the Sharpless Oxidation of 2-Furylcarbinols to the Synthesis of Natural Products

1. (+)-Acetylphomalactone and (6*R*)-(+)-Goniothalamin

(+)-Acetylphomalactone (**89**) and (6*R*)-(+)-goniothalemin (**90**) have 6-substituted 5,6-dihydro-α-pyrone skeleton as a common structural feature and approximately 65 compounds of this class were isolated from both plants and fungi. These compounds also exhibit interesting physiological activities, such as plant growth inhibitory, insect antifeedant, antifungal, and antitumor activities. The formation of the basic skeleton of these antibiotics is easily thought to be derived by successive

oxidation of the corresponding 2-furylcarbinol and the resulting δ-lactol to the δ-lactone. The problem in the synthesis of these compounds is essentially the induction of the desired chirality for the starting 2-furylcarbinol. This ploblem was solved by using the kinetic resolution of the racemic 2-furylcarbinol under the Sharpless oxidation condition as described above.

Given these considerations, the synthesis of (+)-acetylphomalactone (55) isolated from *Aspergillus caespitosus* as an antimicrobial metabolite having antitumor activity, was attempted (56). Kinetic resolution of the 2-furylcarbinol (**91**) was carried out with *tert*-butyl hydroperoxide (0.5 equiv.), catalytic amounts of L-(+)-diisopropyl tartrate (15 mol%) and titanium tetraisopropoxide (10 mol%) in dichloromethane in the presence of molecular sieves 3Å at -30 °C for 6 hours to give the pyranone (**92**) in 32.7% yield. After protection of the lactol (**92**) with ethyl vinyl ether, the resulting major α-anomer (**93**) was subjected to sodium borohydride reduction in aqueous THF to afford the allyl alcohol (**95**) in 94.1% yield. Although the stereochemistry of the hydroxy group in **95** could not be determined at this stage, it was assumed to be 3*R* based on the similar works by Sammes (57). The Mitsunobu reaction of **95** with triphenylphosphine, diethyl azodicarboxylate, and benzoic acid, followed by hydrolysis of the resulting benzoate (**96**) provided the *syn*-alcohol (**97**) in 84% overall yield. Acetylation of the *syn*-alcohol (**97**) gave the acetate (**98**), which in turn on oxidation with *m*-chloroperbenzoic acid and boron trifluoride etherate (58) furnished (+)-acetylphomalactone (**89**) (Scheme 12).



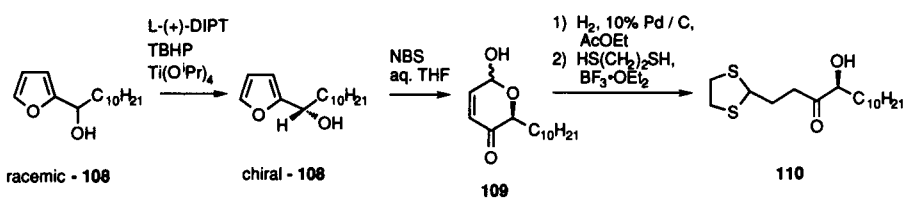
Scheme 12.

The specific optical rotation value of the synthetic product is in agreement with that of the natural product. These results indicate that the stereochemistry of the hydroxy group of compound (**97**) was unambiguously 3*S* and the asymmetric induction in the Sharpless oxidation for 2-furylcarbinol (**91**) proceeded with high optical purity.

This synthetic strategy was also applied to the synthesis of (6*R*)-(+)-goniothalamin (**90**) (59), isolated from the dried bark of *Cryptocarya caloneura*. The furylcarbinol (**99**), prepared from (*E*)-cinnamaldehyde and 2-lithiofuran in 96% yield, was subjected to catalytic kinetic resolution as above to give the pyranone (**100**) in 19.1% yield. The attempted reaction using a stoichiometric amount of the titanium-tartrate complex could not improve the conversion yield due to the instability of the styryl moiety. After conversion of the lactol (**100**) to the corresponding ethoxyethyl ethers, the major α-anomeric ether (**101**) was reduced with sodium borohydride to afford the alcohol (**103**). Deoxygenation reaction of the dithiocarbonate (**104**) derived from **103** using tributyltin hydride in the presence of AIBN in refluxing toluene provided the desired compound (**105**). Finally, deprotection of the ethoxyethyl group of **105**, followed by oxidation with PCC produced (6*R*)-(+)-goniothalamin (**90**) (60). The optical purity of the synthetic compound was estimated to be ca.85% by calculation of the optical rotation. Since no evidence of any racemization could be detected throughout this operation, it may be assumed that the optical purity of compound (**100**) refers to the initial kinetic resolution of the alcohol (**99**).

2. (5*R*,6*S*)-6-Acetoxyhexadecan-5-olide and (+)-Disparlure

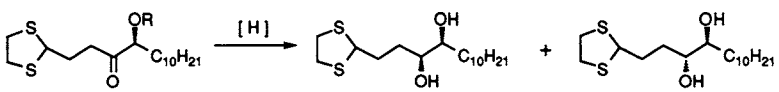
In the synthesis of acetylphomalactone and goniothalamin, the faster reacting isomer of the racemic 2-furylcarbinol (affording the corresponding pyranone) was employed as the starting material. The recovered furylcarbinol could also be an important chiral building block in the synthesis of natural products. In this respect, the syntheses of (5*R*,6*R*)-6-acetoxyhexadecan-5-olide (**106**) (61), an oviposition attractant pheromone of the mosquito *Celux pipens fatigans*, and (+)-disparlure (**107**) (62), a pheromone of the gypsy moth (*Porthetria dispar*) were investigated. Kinetic resolution of the 2-furylcarbinol (**108**) gave the chiral alcohol in an optically pure form, which on oxidation with NBS in aqueous THF provided the lactol (**109**). Catalytic hydrogenation of **109**, followed by acetalization with ethanedithiol in the presence of boron trifluoride afforded the thioacetal (**110**) (Scheme 13).



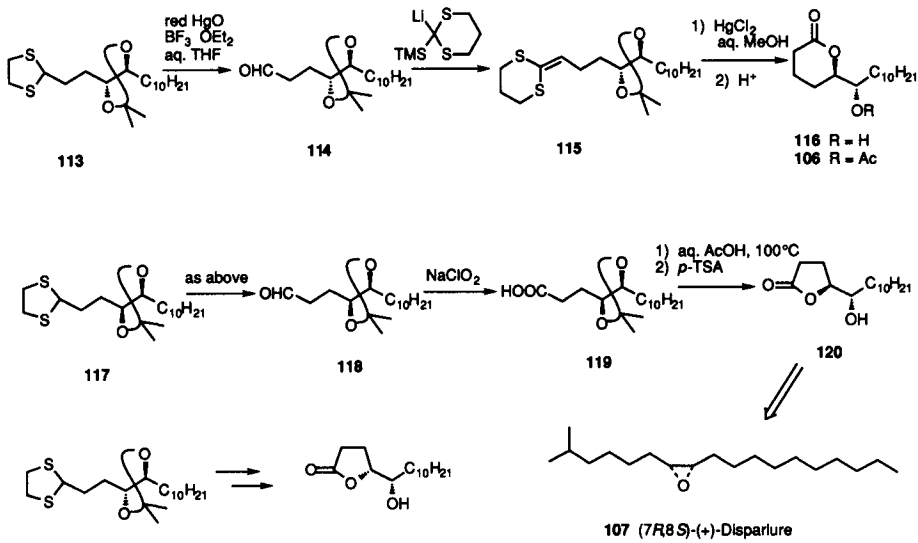
Scheme 13.

For the syntheses of the title compounds, the stereoselective construction of 1,2-*syn* and -*anti* diol functionalities may be expected to be the crucial step. The *anti*-diol (**112**) was stereoselectively obtained by using zinc borohydride (63) as reducing agent where the Cram's chelation model would be involved as a transition state. When the *tert*-butyldiphenylsilyl ether was reduced with 'ate' complexes such as Red-Al (63) and L-Selectride, (64) diverse selectivity occurred, preferentially affording the Cram product. The results in the stereoselective reduction of α-alkoxyketones are summarized in Table 4. After protection of the *anti*-diol (**112**) as the acetonide (**113**), the thioacetal group was removed on treatment with mercury(II) oxide and boron trifluoride (65) to give the aldehyde (**114**). Reaction of **114** with 2-lithio-2-trimethylsilyl-1,3-dithiane gave the ketene thioacetal (**115**) whose treatment with mercury(II) chloride in aqueous methanol furnished a mixture of the dihydroxy carboxylic acid and the hydroxy lactone. This mixture was subjected to lactonization without separation to give the lactone (**116**), whose optical rotation was in agreement with literature values, confirming the absolute configuration of the kinetically resolved furylcarbinol. Acetylation of the alcohol (**116**) yielded (5*R*,6*R*)-6-acetoxyhexadecan-5-olide (**106**) (66). The *syn*-diol (**111**) was also utilized in the synthesis of (+)-disparlure (**107**) as follows (66). Acetonide (**117**), prepared from the *syn*-diol (**111**), was converted into the carboxylic acid (**119**) by deprotection of the thioacetal, and subsequent oxidation of the resulting aldehyde (**118**) with sodium chlorite (67).

Table 4. Stereoselective Reduction of 2-Alkylketones



R	Reaction conditions			syn / anti (ratio)	Yield (%)
	[H]	Solvent	Temp. (°C)		
H	$NaBH_4$	MeOH	0	75 / 25	86
			-30	84 / 16	82
		MeOH - CH_2Cl_2	-78	77 / 23	99
	$Zn(BH_4)_2$	Et_2O	0	19 / 81	92
			-30	18 / 82	87
	$LiAlH_4$	Et_2O	-30	26 / 74	83
$SiPh_2{}^tBu$	$LiAlH_4$	THF	-35	87 / 13	60
	Red - Al	Toluene	-78	88 / 12	75
	$^iBu_3Al$	Pentane	18	42 / 58	36
	L-Selectride	THF	-78	93 / 7	42
CH_2OCH_2Ph	$LiAlH_4$	Et_2O	-10	41 / 59	99
	$Zn(BH_4)_2$	Et_2O	-30	<2 / >98	99



Scheme 14.

Deprotection of the acetonide (**119**) in aqueous acetic acid, followed by ring closure reaction of the hydroxy acid with *p*-toluenesulfonic acid gave the lactone (**120**), whose optical rotation agreed with the literature value.

The enantiomeric excess of the lactone (**120**) (68) was determined on the basis of its NMR analysis for the corresponding Mosher's (MTPA) ester, indicating the optical purity of the lactone to be >98%. Since the lactone (**120**) was already transformed to (+)-disparlure (**107**), this constitutes its formal synthesis (Scheme 14).

These syntheses suggested that the absolute configuration of the kinetically resolved 2-furylcarbinols could be predicted by the chirality of the tartrate used.

3. Canadensolide

In order to extend the applicability of the above synthetic strategy to the synthesis of other types of polyoxygenated natural products, kinetic resolution of 2-furylcarbinol bearing substituent(s) on a furan ring was investigated. The results for the Sharpless kinetic resolution of the substituted 2-furylcarbinols are summarized in Table 5 which presents the isolated yields and enantiomeric excess.

These results clearly showed that the Sharpless kinetic resolution could be applicable to the various types of furylcarbinol derivatives, however, the presence of a bulky substituent at the 3-position of 2,3,4-trisubstituted furan derivatives decreases their optical purities in this kinetic resolution probably owing to steric hindrance. Using the 3-substituted 2-furylcarbinol (**123**) as the

starting material, the synthesis of (-)-canadensolide (**121**) (69), an antibiotic isolated from *Penicillium canadense*, was successfully carried out.

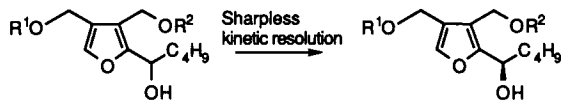
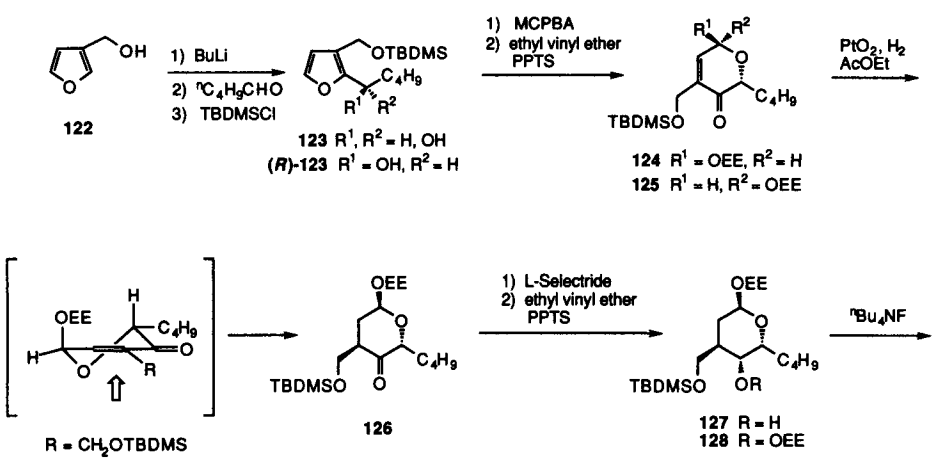


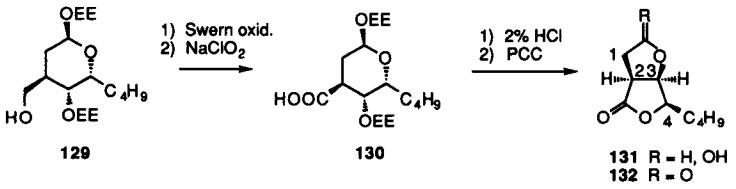
Table 5. Kinetic Resolution of Substituted 2-Furylmethanols Using TBHP, $Ti(OiPr)_4$, and L-(+)-DIPT [a]

	substrate		TBHP (equiv.)	slow-reacting enantiomer	
	R^1	R^2		yield (%)[b]	e.e.(%)[c]
a	MOM	TBS	2.0	32	55
b	MOM	SEM	3.0	54	31
c	TBS	Ac	0.7	44	62

[a] The reaction was carried out by using TBHP, $Ti(O^iPr)_4$ (1.0 equiv.), and L-(+)-DIPT (1.2 equiv.) in CH_2Cl_2 at -25°C in the presence of 3A molecular sieves. [b] Isolated yields based on racemic 5. [c] Determined by ^1H NMR analysis of the corresponding MTPA ester.

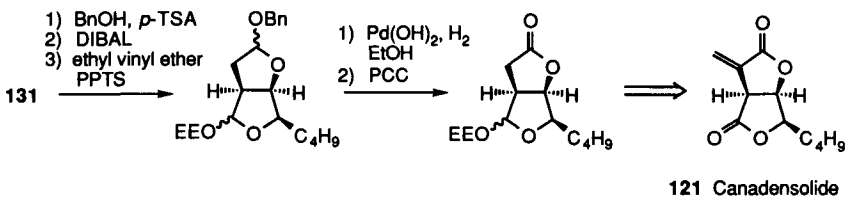
Addition of the dilithio salt of 3-furanmethanol (**122**) to *n*-valeraldehyde gave the 2-furylcarbinol, whose primary alcohol was selectively protected as its silyl ether to afford **123**. Sharpless kinetic resolution of **123** furnished the (***R***)-**123** in 41% yield with >95% enantiomeric excess, determined by the NMR spectrum of its Mosher's ester. Oxidative conversion of (***R***)-**123** to the lactol with *m*-chloroperbenzoic acid, followed by treatment with ethyl vinyl ether afforded the acetals (**124** and **125**) in a ratio of 2.4:1, owing to the anomeric effect.





Scheme 15.

Catalytic hydrogenation of the major isomer (**124**) over palladium-carbon gave the saturated ketone (**126**) with high stereoselectivity (>95%). Reduction of **126** with L-selectride in THF at -78°C furnished the desired alcohol (**127**), which was then converted to the ethoxyethyl ether (**128**). After deprotection of the silyl group on exposure to tetrabutylammonium fluoride, the resulting alcohol (**129**) was transformed to the corresponding acid (**130**) by two steps (sequential Swern oxidation and sodium chlorite oxidation). Deprotection of the ethoxyethyl ethers of **130** with 2N hydrochloric acid brought about ring transformation to give the lactol (**131**), whose further oxidation with PCC yielded the bislactone (**132**) (Scheme 15). Its spectroscopic data including the specific optical rotation were identical with those reported. (-)-Canadensolide was synthesized from the optically pure lactol (**131**) as indicated in Scheme 16 (70).



Scheme 16.

This is the first asymmetric synthesis of canadensolide and this synthetic path should be applicable to the syntheses of other types of antifungal bislactones such as avenaciolide, isoavenaciolide, and ethisolide (Figure 5).

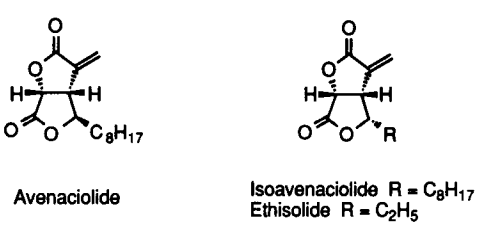


Figure 5.

4. Pumiliotoxin 251D and Swainsonine

Indolizidine alkaloids possessing the 1-azabicyclo[4.3.0]nonane skeleton have been isolated from plants and fungi as well as from animal sources and they exhibibit a wide range of structural and stereochemical features. Since this class of alkaloids has been known to exhibit interesting biological activities, intensive efforts have been devoted to their syntheses (71).

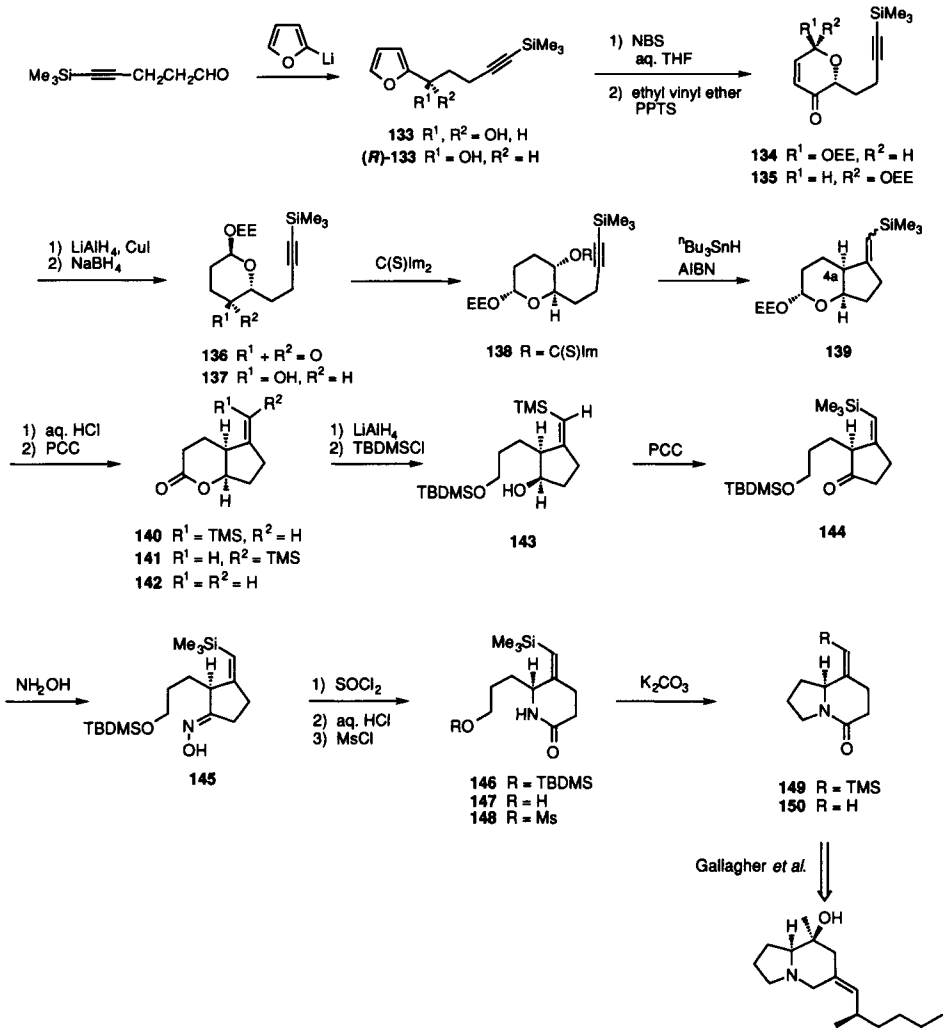
The synthesis that we have developed involves the Sharpless kinetic resolution using L-diisopropyl tartrate, titanium tetraisopropoxide, and *tert*-butyl hydroperoxide of the racemic 2-furylcarbinol derivative (**133**), readily accessible from 2-lithiofuran and 5-trimethylsilyl-4-pentyn-1-al, providing the *R*-alcohol (**133**) with high enantiomeric excess (>95%). Oxidation of (***R***)-**133** with NBS in aqueous tetrahydrofuran, followed by protection of the lactol with ethyl vinyl ether afforded the δ-lactol derivatives (**134** and **135**) in a ratio of 2.4:1. Lithium aluminum hydride reduction of the major isomer (**134**) in the presence of copper(I) iodide (72) gave the 1,4-reduction product (**136**), which was further reduced with sodium borohydride to afford the alcohol (**137**) as a single stereoisomer.

We envisioned construction of the chiral indolizidine skeleton from the corresponding chiral 2-substituted cyclopentanone *via* the piperidone derivative by using the Beckmann rearrangement with retention of the chiral center followed by an intramolecular alkylation.

The desired cyclopentanone could also be derived from the alcohol (**137**) by employing an intramolecular radical cyclization. Thus, the thiocarbonylimidazolide (**138**) prepared from **137** and thiocarbonyldiimidazole was subjected to a radical mediated 5-endo-dig cyclization to provide the olefin (**139**), in 84% yield, as a mixture of geometrical isomers. Both compounds were converted into the corresponding lactones (**140** and **141**) in two-steps. The NOE experiment on the major isomer (**140**) confirmed its stereochemistry to be *cis*-fused. Desilylation of both lactones with *p*-toluenesulfinic acid afforded the same lactone (**142**) and these results indicated that the radical cyclization of **138** proceeded in a stereoselective manner to form the *cis*-ring juncture predominantly (73) and none of the *trans* isomer could be isolated in this cyclization. Reduction of **140** with lithium aluminum hydride, followed by selective protection of the primary alcohol with *tert*-butyldimethylsilyl chloride gave the silyl ether (**143**), which on oxidation with PCC furnished the desired chiral cyclopentanone (**144**). The Beckmann rearrangement of the corresponding *anti*-oxime (**145**) using thionyl chloride in THF afforded the expected lactam (**146**) as a single compound. Removal of the silyl group of **146**, followed by treatment of the resulting alcohol (**147**) with methanesulfonyl chloride gave the mesylate (**148**), which was then subjected to an intramolecular alkylation reaction with potassium carbonate to provide the bicyclic compound (**149**).

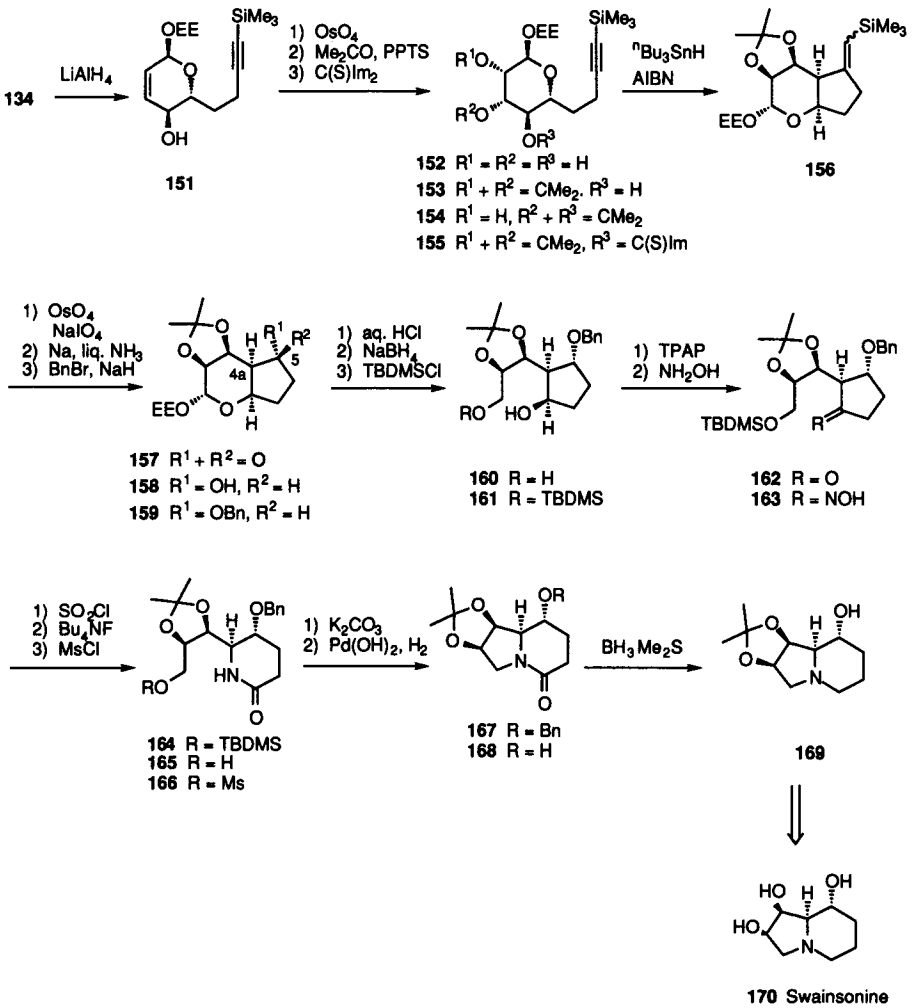
Finally desilylation of **149** with *p*-toluenesulfinic acid (74) furnished the lactam (**150**) whose spectroscopic data including the specific optical rotation were identical with those reported (75).

Since the lactam (**150**) has already transformed into (-)-pumiliotoxin 251D, this synthesis constitutes its formal synthesis (76) (Scheme 17).



Scheme 17.

This synthetic strategy was further applied to the synthesis of swainsonine, isolated from the fungae *Rhizoctonia leguninicola* (77) and *Metarhizium anisopliae* F-3522 (78) as well as from locoweed *Astragalus lentiginosus* (79) and *Swainsona canescens.* (80) This alkaloid is known to be a potential inhibitor of both lysosomal α-mannosidase and mannosidase II and to disrupt the processing of glycoproteins.



Scheme 18.

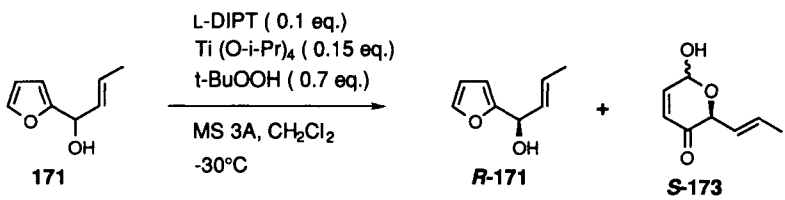
Our synthesis of swainsonine starts with the introduction of a *cis*-diol group into the ethoxyethyl ether (**151**), derived from the ketone (**134**) by lithium aluminum hydride reduction. Dihydroxylation of **151** with osmium tetraoxide proceeded diastereoselectively to give the triol (**152**), which on protection as an acetonide furnished the regioisomers (**153** and **154**) in 74% and 15% yields, respectively. Radical cyclization of the thiocarbonylimidazolide (**155**) by a process similar to that used for the synthesis of pumiliotoxin 251D furnished the bicyclic compounds (**156**) as an inseparable mixture of geometrical isomers in 63% yield. Lemieux-Johnson oxidation (81) of **156**, followed by Birch reduction of the ketone (**157**) afforded the alcohol (**158**) as the major

product. After benzylation of the hydroxyl group, the acetal (**159**) was converted into the diol (**160**) by two steps and the primary alcohol was protected as the silyl ether (**161**). Oxidation of the secondary hydroxyl group gave the ketone (**162**), which was converted into the oxime (**163**) in the usual manner. Beckmann rearrangement of the oxime (**163**) followed by intramolecular cyclization of the mesylate (**166**), derived from **164** *via* **165**, provided the indolizidine compound (**167**). Finally debenzylation of **167** under the catalytic hydrogenation condition and subsequent amide reduction of **168** with borane-dimethyl sulfide complex furnished swainsonine acetonide (**169**), which was identical with the authentic sample (82). Conversion of the acetonide to swainsonine (**170**) has been accomplished by several groups and this synthesis therefore constitutes its formal synthesis (83) (Scheme 18).

Thus we have developed an enantiocontrolled synthesis of indolizidine alkaloids, (-)-swainsonine and pumiliotoxin 251D by ring transformation of a pyranone and this strategy could be applied to the synthesis of polyhydroxylated indolizidine alkaloids, such as castanospermine, in optically active forms.

5. Asperlin

As described above, the Sharpless kinetic resolution of 2-furylcarbinol bearing an alkenyl moiety on the side chain reflected the relative reactivity of both double bonds presented in the molecule (84) (Scheme 19).

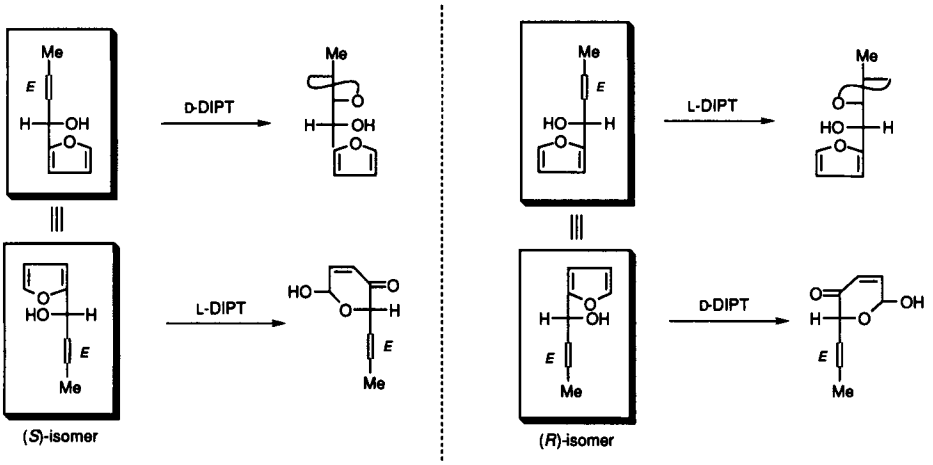


Scheme 19.

However, we found that the reactivity of the double bonds of the alcohol (**171**) varied with the chirality of DIPT employed in the reaction (Scheme 20).

The Sharpless kinetic resolution of the racemic 2-furylcarbinol (**171**) with 1.2 equivalents of TBHP in the presence of D-(-)-DIPT gave the (*S*)-epoxide (***S*-172**) and the (*R*)-pyranone (***R*-173**) in 42 and 43% yields, respectively. Conversely oxidation of the racemate (**171**) using L-(+)-DIPT afforded the optically pure (*R*)-epoxide (***R*-172**) and the (*S*)-pyranone (***S*-173**) in 41 and 41%

yields, respectively. It is noteworthy that none of the epoxide diastereomers was isolated indicating that the epoxidation proceeded in an entirely diastereofacial-selective manner. When the epoxidation

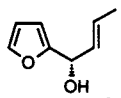
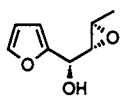
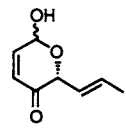
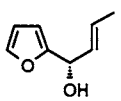
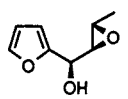
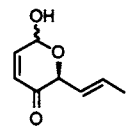
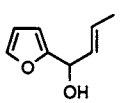
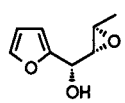
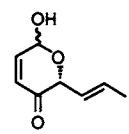
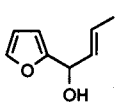
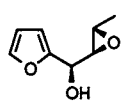
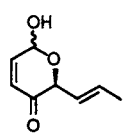


Scheme 20. The Fischer projection of the Sharpless epoxidation of (*E*)-(2-furyl)but-2-en-1-ol (**171**).

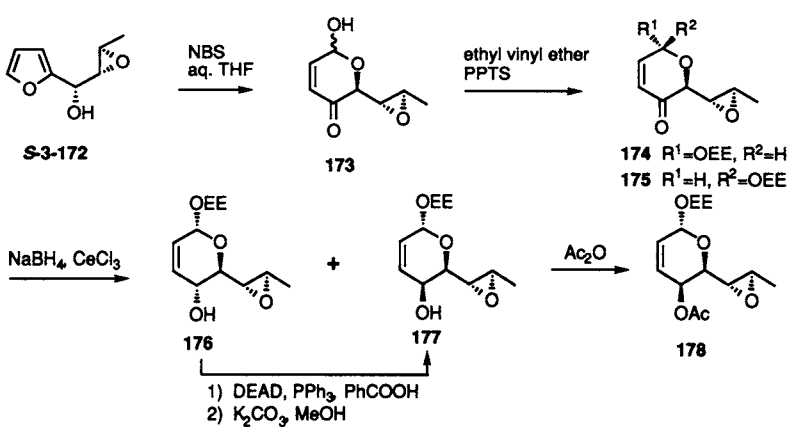
for the optically active (*S*)-alcohol was carried out with 1.2 quivalents of TBHP in the presence of L-(-)-DIPT the (*S*)-pyranone, arising from the oxidation of the more reactive furan double bond, was isolated in 85% yield, as expected. However oxidation of the (*S*)-alcohol using D-(+)-DIPT resulted in the (*S*)-epoxide in 89% yield. These results clearly indicated that the furan double bond of the (*S*)-alcohol formed a matched pair with the titanium-L-(+)-DIPT complex resulting in the formation of the (*S*)-pyranone. However use of D-(-)-DIPT resulted in the almost exclusive epoxidation of the alkenyl double bond indicating that the alkenyl double bond of the (*S*)-alcohol formed a matched pair with the titanium-D-(-)-DIPT complex (Table 6).

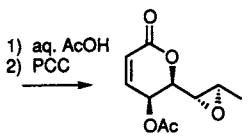
By application of this methodology to natural product synthesis and also in order to determine the stereochemistry at C-2 and C-3 positions of the (*S*)-pyranone, a synthesis of antitumor antibiotic asperlin, isolated from *Aspergillus nidulans*, (85) was investigated as follows. Oxidative ring transformation of the epoxide (**172**) and subsequent protection of the lactol (**173**) as a ethoxyethyl ether afforded the pyranones (**174** and **175**) in 60 and 27% yields, respectively. Sodium borohydride reduction of the major α-anomer (**174**) in the presence of cerium chloride gave the α-alcohol (**176**), which was converted into the β-alcohol (**177**) under the Mitsunobu reaction conditions, followed by hydrolysis of the resulting benzoate in the usual manner. After acetylation, the obtained acetate (**178**) was converted into asperlin (**179**) by hydrolysis of the acetal and subsequent oxidation with PCC (86) (Scheme 21).

Table 6.[a] The Sharpless oxidation of (*E*)-(2-furyl)but-2-en-1-ol (**171**)

Entry	Starting Material	DIPT	Products (Yield)	
1		D		
	***S*-171** (97%ee[c])		***S*-172** (89%)	***R*-173** (1.4%)
	$[\alpha]_D$ +56° ($CHCl_3$)		$[\alpha]_D$ -42.7° ($CHCl_3$)	
2[b]		L		
	***S*-171** (97%ee[c])		***R*-172** (0.4%)	***S*-173** (85%)
3		D		
	171 (racemate)		***S*-172** (42%)	***R*-173** (43%)
			$[\alpha]_D$ -43° ($CHCl_3$)	
4		L		
	171 (racemate)		***R*-172** (41%)	***S*-173** (41%)
			$[\alpha]_D$ +43.4° ($CHCl_3$)	

[a] All reactions were carried out at -20°C with 25 mol % of $Ti(O\text{-}i\text{-}Pr)_4$, 30 mol % of DIPT, and 120 mol % of TBHP in the presence of CaH_2 and 3A molecular sieves, except for the Entry 2.
[b] 150 mol % of TBHP were employed. [c] Determined based on hplc analysis using the chiral column CHIRALCEL OB (Daicel Chemical Industries LTD.).





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Scheme 21.

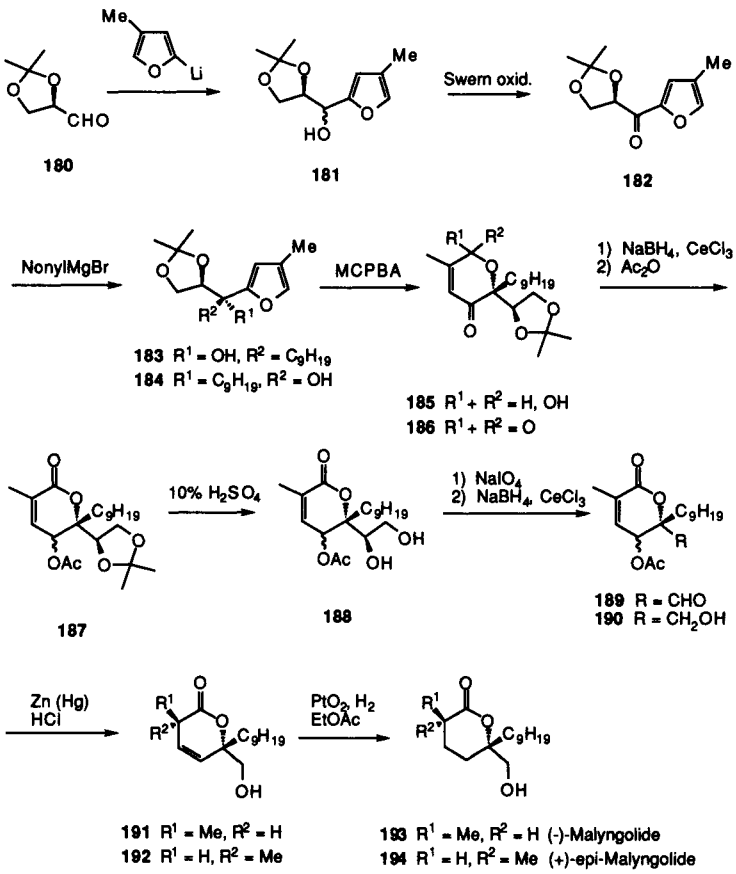
To our knowledge this is the first example of the Sharpless epoxidation of the unsymmetrical divinyl carbinol leading to the formation of the epoxide on the less reactive double bond in a regio- and diastereofacial-selective manner.

V. Utilization of (*R*)- or (*S*)-1,2-Isopropylideneglyceraldehyde as Chiral Sources in the Synthesis of Optically Active 2- Furylcarbinols

Although we have developed a general synthetic route to optically active 2-furylcarbinols by employing the Sharpless oxidation, this strategy could not be applied to tertiary 2-furylcarbinols. However (*R*)- or (*S*)-1,2-isopropylideneglyceraldehyde were easily derived from D-mannitol and L-ascorbic acid, respectively. These aldehydes are both recognized as useful chiral building blocks and are widely employed as starting materials in organic syntheses. We therefore utilized these aldehydes in the syntheses of optically active 2-furylcarbinols and their conversion into the natural products as follows.

1. (-)-Malyngolide

(-)-Malyngolide (**193**) (87) was isolated from the lipid extract of a shallow-water variety of *Lyngbya majuscula*. This antibiotic having a δ-lactone ring system with a chiral quaternary carbon center exhibits antimicrobial activity against *Streptococcus pyogenes*. The oxidative conversion of a tertiary 2-furylcarbinol into a pyranone seems to provide a concise synthetic route to this natural product. Thus, the chiral synthesis of (-)-malyngolide was successfully achieved using (*R*)-2,3-*O*-isopropylideneglyceraldehyde (**180**) (88) as the chiral source (Scheme 22). The key step in this total synthesis (89) is the introduction of the stereogenic center at the carbon bearing the lactonic oxygen and this was accomplished by employing the highly diastereoselective addition of organometallic reagents to carbonyl compounds, in which the chelation effect played an important role in controlling the stereochemistry. Addition of 2-lithio-4-methylfuran to (*R*)-2,3-*O*-isopropylideneglyceraldehyde (**180**), followed by oxidation of the resulting alcohol (**181**) afforded the ketone (**182**).



Scheme 22.

The nucleophilic addition of nonylmagnesium bromide to the ketone (**182**) at ambient temperature gave the *tert*-alcohol (**183**) as the major product together with **184**, through the expected α-chelation transition state as depicted in Figure 6.

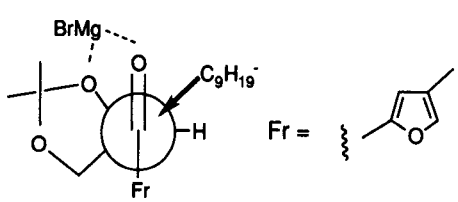


Figure 6.

Conversion of **183** on oxidative treatment with *m*-chloroperbenzoic acid yielded the pyranone (**185**), which was further oxidized with PCC to give the lactone (**186**). Sodium borohydride reduction of **186** in the presence of cerium(III) chloride and subsequent acetylation of the resulting alcohol with acetic anhydride provided the acetate (**187**). After removal of the acetonide group by acid treatment, the resulting diol (**188**) was converted into the primary alcohol (**190**), *via* the aldehyde (**189**) by treatment with sodium periodate and subsequently with sodium borohydride in the presence of cerium(III) chloride. Reductive deacetoxylation of **190** with zinc amalgam in ethereal hydrogen chloride (90) afforded the olefins (**191** and **192**) in 59.5 and 29.7% yields, respectively. Finally, hydrogenation of the major isomer (**191**) over platinum oxide provided (-)-malyngolide (**193**), which was identical with the natural product in all respects. The hydrogenation of **192** gave (+)-epimalyngolide (**194**) (91).

2. Goniothalamin, Argentilactone, Goniodiol, 9-Deoxygoniopypyrone, Goniopypyrone, (+)-Goniofufurone, (+)-8-Acetylgoniotriol, (+)-Goniotriol, and (+)-Altholactone

Argentilactone (**195**) (92), isolated from *Aristolochia argentina*, has 6-substituted 5,6-dihydro-α-pyrone structure similar to goniothalamin (**90**) (see IV-1) (Figure 7). In the syntheses of these compounds, the aldehyde (**205**) seems to be a common versatile intermediate. Although its chiral synthesis has been reported by Just in 1986 (93), the yield was not sufficiently high to allow its further conversion to the natural products. We have also concentrated our attention to the synthesis of the aldehyde (**205**) by application of the oxidative ring transformation of the 2-furylcarbinol which is easily accessible from (*R*)-2,3-*O*-isopropylideneglyceraldehyde.

Addition of 2-lithiofuran to (*R*)-2,3-*O*-isopropylideneglyceraldehyde (**180**) gave the adduct, which was oxidized with chemical manganese dioxide to afford the ketone (**196**). L-Selectride reduction of the ketone according to the Jurczak's protocol (94) furnished the 2-furylcarbinol (**197**), stereoselectively. Treatment of **197** with NBS in aqueous THF, followed by protection of the lactol as a ethoxyethyl ether gave the ethers (**198** and **199**). Reduction of the major isomer (**198**) with sodium borohydride in aqueous THF afforded the alcohols (**200** and **201**) in a ratio of 5.2:1, respectively. Mesylation of the alcohol (**200**) with methanesulfonyl chloride, and subsequent reduction of the mesylate with lithium aluminum hydride furnished the dihydropyran (**202**) in 64 % overall yield. After deprotection of the ethoxyethyl ether on acid treatment, the resulting lactol was oxidized with PDC in DMF to give the α,β-unsaturated lactone (**203**). Acidic removal of the acetonide in **203** provide the diol (**204**), which was exposed to sodium periodate to give the desired aldehyde (**205**). Since the aldehyde (**205**) was found to be too unstable to isolate, we therefore explored sequential reactions which involved *in situ* generation of the aldehyde (**205**), followed by a Wittig olefination to synthesize goniothalamin and argentilactone (95). Treatment of the aldehyde (**205**) with 10 equiv. of benzylidenetriphenylphosphorane yielded (+)-goniothalamin (**90**) and its olefinic isomer (**206**) in a ratio of 1:3.1, respectively. The (*Z*)-olefin was easily isomerized to (+)-goniothalamin by employing a photoreaction in the presence of diphenyl disulfide (96) in benzene in 64.5% yield together with 32.5% yield of the recoverd starting (*Z*)-olefin (**206**). The spectroscopic data including its melting point and specific optical rotation were identical with those reported.

(-)-Argentilactone (**195**) was also synthesized by using the Wittig reaction of the aldehyde (**205**) with hexylidenetriphenylphosphorane as above (Scheme 23). Although these syntheses proved the versatility of the aldehyde (**205**) as a common intermediate in the synthesis of these types of natural products, the reaction sequences involved in the above synthesis seemed to be rather long.

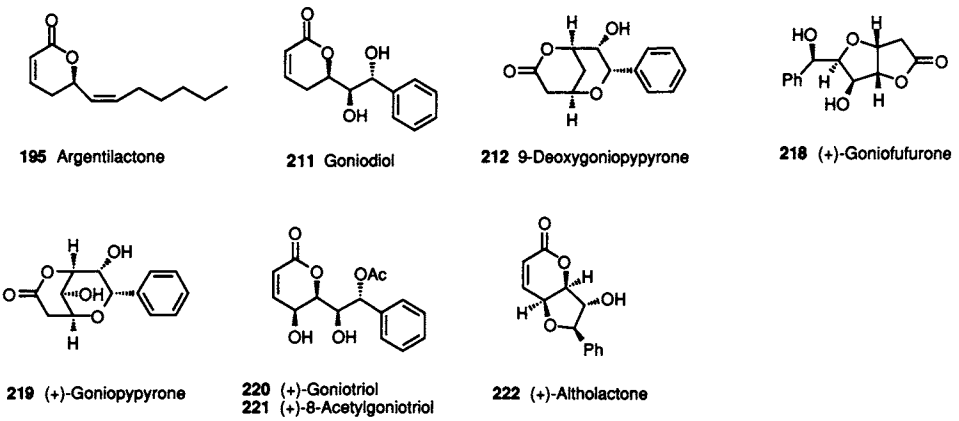
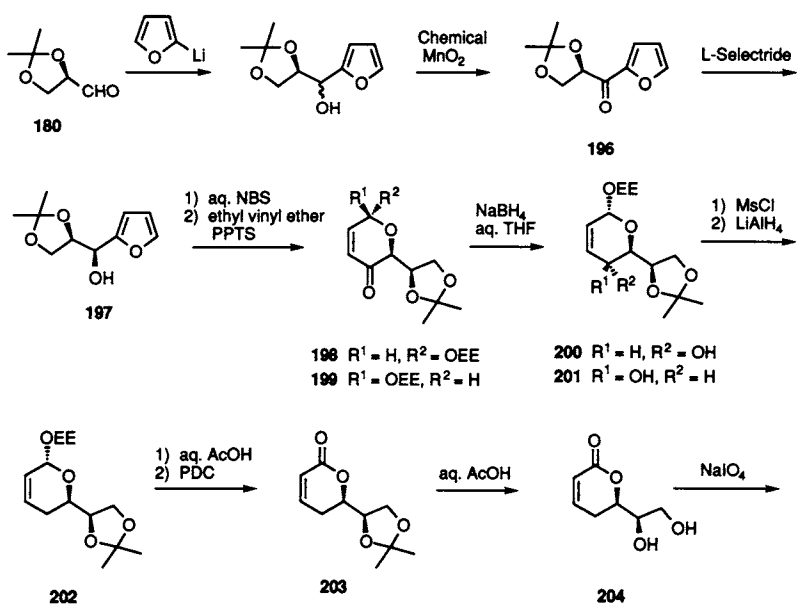
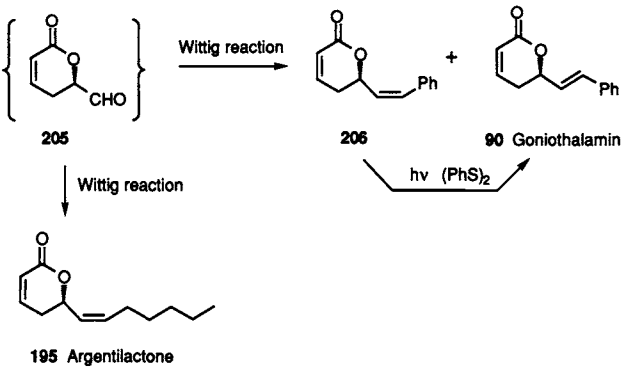


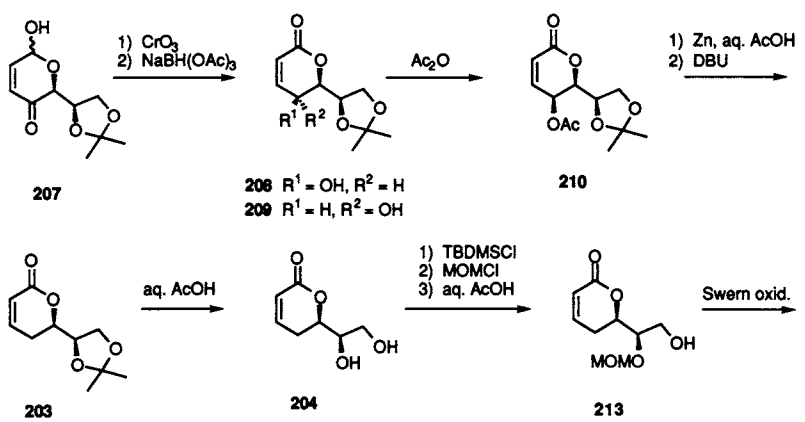
Figure 7.

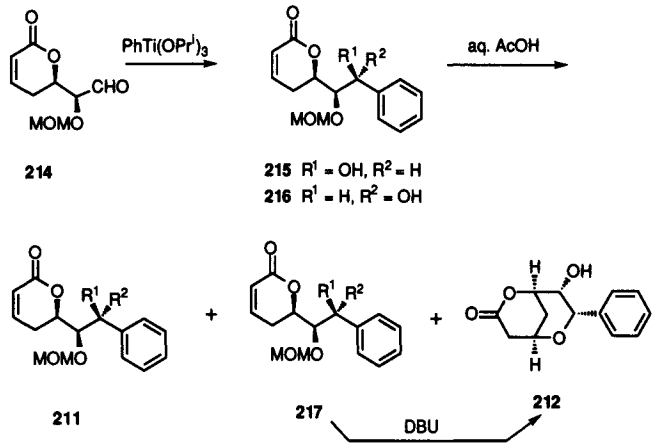




Scheme 23.

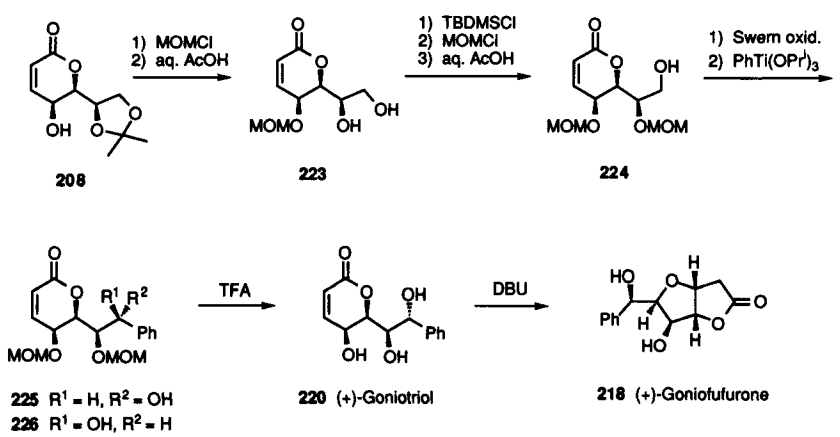
We therefore devised an alternative synthetic route to the aldehyde as follows (97). Oxidation of the lactol (**207**) obtained above with chromium (VI) oxide in acetic acid gave the unstable lactone, which, without isolation, was reduced with sodium triacetoxyborohydride in the same pot to provide the allyl alcohols (**208** and **209**) in a ratio of 7:1. Deoxygenation of the major isomer (**208**) was carried out by sequential acetylation, reductive deoxygenation of the resulting acetate (**210**) with zinc powder in acetic acid, and isomerization of the β,γ-unsaturated lactone with DBU in THF to afford the desired lactone (**203**) in good yield, which was identical with the authentic sample obtained by the above method. Using this lactone (**203**) as an intermediate, goniodiol (**211**) (98) and 9-deoxygoniopypyrone (**212**) (99) were successfully synthesized as follows. Removal of the acetonide group of **203** on acid treatment afforded the diol (**204**), which was further converted into the alcohol (**213**) by sequential selective silylation of the primary alcohol in **204**, methoxymethylation of the secondary alcohol and desilylation of the ether.

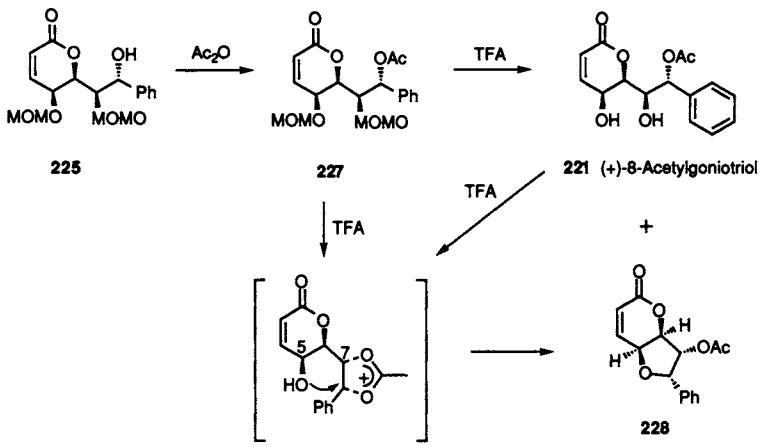




Scheme 24.

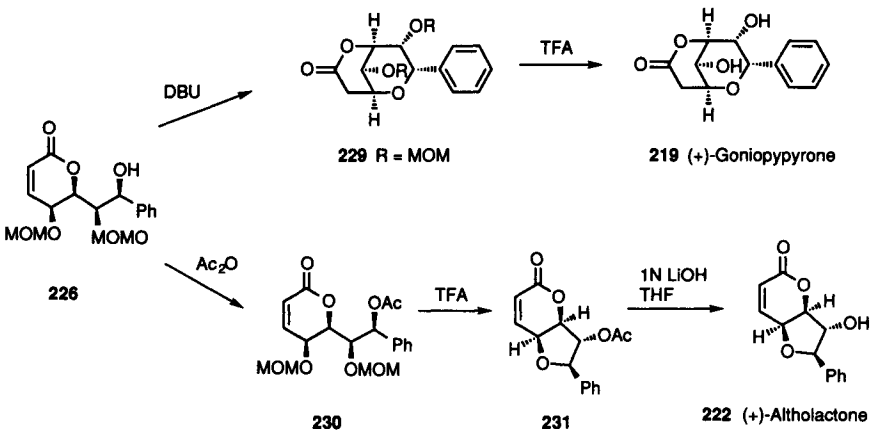
Swern oxidation of **213** followed by chemoselective phenylation of the resulting aldehyde (**214**) with triisopropoxylphenyltitanium (100) in one pot gave an inseparable mixture of diastereoisomers (**215** and **216**) in a ratio of ca. 1:1. Deprotection of the methoxymethyl group in a mixture with aqueous acetic acid provided goniodiol (**211**) and 8-epi-goniodiol (**217**) in 49.4 and 43.2% yields, respectively, together with 9-deoxygoniopypyrone (**212**) in 4.6% yield. Conversion of 8-epi-goniodiol into 9-deoxygoniopypyrone was achieved by its treatment with DBU in THF involving the Michael addition of the benzylic hydroxy group. The synthetic compounds were identical with those of natural products in all respects. Thus, the absolute configurations of goniodiol (**211**) and 9-deoxygoniopypyrone (**212**) are unambiguously determined by this synthesis (Scheme 24).





Scheme 25.

Five novel styryl lactones which are cytotoxic to human tumor cells, (+)-goniofufurone (**218**), (+)-goniopypyrone (**219**), (+)-goniotriol (**220**), (+)-8-acetylgoniotriol (**221**), and (+)-altholactone (**222**), have been isolated from the stem bark of *Goniothalamas giganteus* (Annonaceae) (101). Due to their interesting structural features and important biological activities, we have also investigated the stereoselective synthesis of those lactonic antibiotics. The strategy (102) for the synthesis of such natural products involved the use of a chiral lactonic aldehyde, readily available from the lactone (**208**) described above. Synthesis of goniofufurone was easily achieved as follows. Protection of the hydroxyl group of **208** as the methoxymethyl ether, followed by acid removal of the acetonide afforded the diol (**223**), which was further converted into the alcohol (**224**) by sequential protection and deprotection. Swern oxidation of the alcohol (**224**) generated *in situ* the unstable aldehyde, which without isolation was treated with triisopropoxyphenyltitanium (100) to provide the alcohols (**225** and **226**) in 78 and 19% yields, respectively. Removal of the two methoxymethyl groups of **225** with trifluoroacetic acid (TFA) furnished goniotriol (**220**) in 88% yield as colorless prisms, which was identical with the natural product in all respects. Interestingly, treatment of goniotriol (**220**) with a catalytic amount of DBU brought about the ring transformation to give goniofufurone (**218**) in 60% yield. 8-Acetylgoniotriol (**221**) was prepared by acetylation of **225**, followed by treatment of the acetate (**227**) with TFA in 59% yield, together with 20% of the bicyclic compound (**228**). The formation of the bicyclic compound can be rationalized by assuming that the participation of the neighboring hydroxyl group at the C-7 position resulted in the generation of the acetoxonium ion intermediate (103), which could be subsequently cyclized by nucleophilic attack of the hydroxyl group at the C-5-position with inversion of the stereochemistry at the benzylic position (Scheme 25).



Scheme 26.

Using the stereoisomeric alcohol (**226**), goniopypyrone and altholactone were synthesized. The intramolecular Michael addition of the hydroxyl group of **226**, mediated by DBU, afforded the bicyclic compound (**229**), which on treatment with TFA yielded goniopypyrone (**219**) in 88% overall yield. On the other hand, acetylation of **226** followed by TFA treatment of the acetate (**230**) gave the bicyclic compound (**231**) involving the acetoxonium ion. Finally hydrolysis of the acetate with lithium hydroxide provided altholactone (**222**) in good yield (Scheme 26).

Thus we could devise the facile synthetic route to the important key intermediate (**214**) and its utilization in the synthesis of the lactonic antibiotics, where an oxidative ring transformation of 2-furylcarbinol into a pyranone was again incorporated as a key reaction.

3. Necic Acid, Monocrotalic Acid

Pyrrolizidine alkaloids, a large class of natural products, exhibit attractive biological activity such as hepatotoxic and carcinogenic activities. Much effort has, therefore, been devoted to develop new methods and strategies for their synthesis. Some of these alkaloids occur in nature as macrocyclic compounds having 11-, 12-, 13-, and 14-membered rings through the diester linkage between the necine base and necic acid. Monocrotaline (104), isolated from *Crotalaria* species, is an 11-membered macrocyclic pyrrolizidine alkaloid having retronecine as the necine base (Figure 8). Since we have already established the chiral synthesis of retronecine (105), our focus was concentrated on the synthesis of necic acid, monocrotalic acid, in an optically active form.

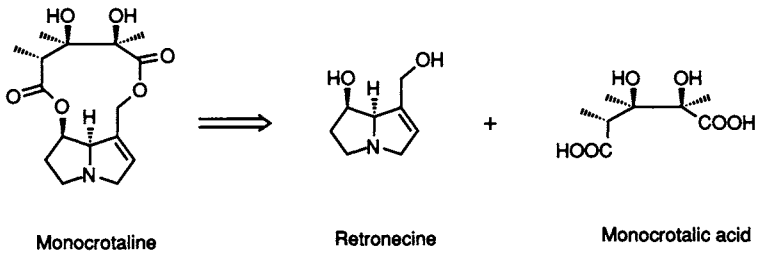


Figure 8.

Monocrotalic acid bears three contiguous chiral centers with vicinal *tert*-hydroxy groups, whose stereoselective construction seems to be a central problem of the synthesis. The strategy for the synthesis of monocrotalic acid was envisaged to employ a chiral pyranone (**235**), readily accessible from a 2-furylmethanol derivative by oxidative treatment (Figure 9).

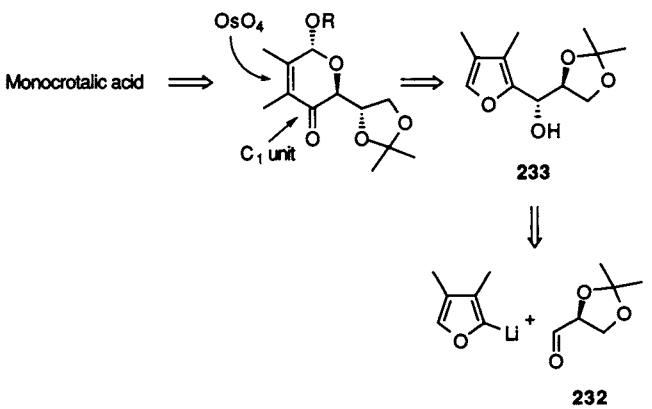
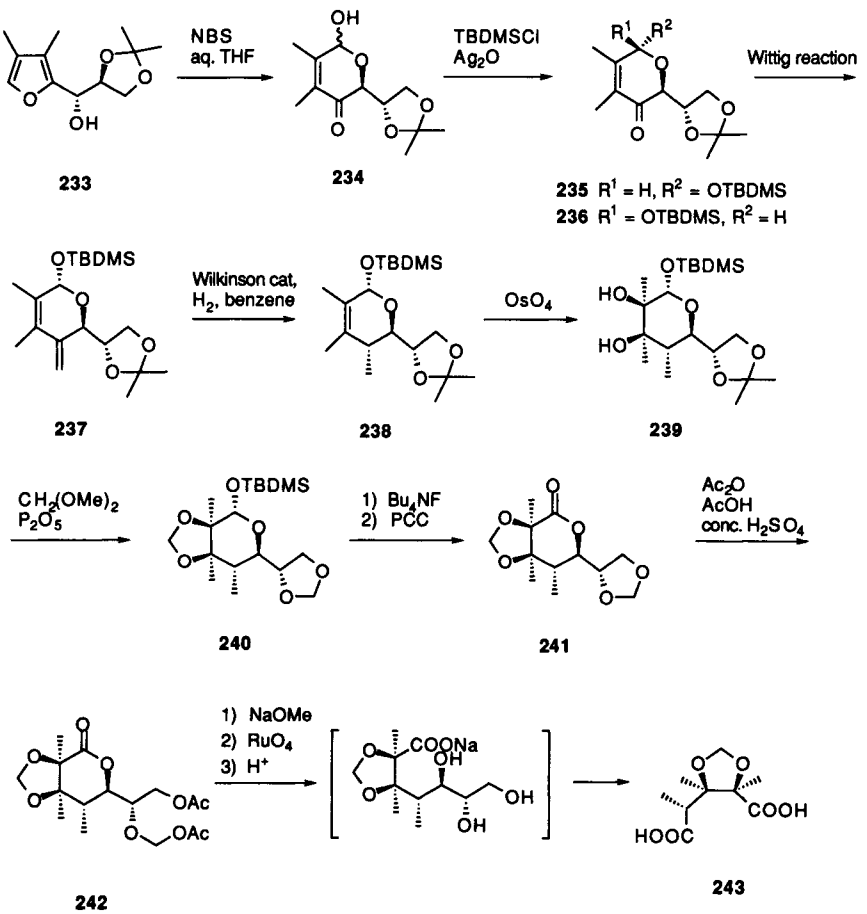


Figure 9.

Chelation-controlled addition reaction of 2-lithio-3,4-dimethylfuran to (*S*)-2,3-*O*-isopropylideneglyceraldehyde (**232**) (33, 51) in the presence of zinc bromide afforded the glycerol derivative (**233**) in 95% yield with >90% diastereomeric excess. Oxidation of **233** with NBS in aqueous THF brought about ring transformation to provide the lactol (**234**), which on silylation with TBDMSCl in the presence of silver(I) oxide gave the silyl ethers (**235** and **236**) in 68 and 25% yields, respectively. The Wittig reaction of the major α-anomer (**235**), followed by reduction of the olefin (**237**) over Wilkinson catalyst in benzene led to the formation of the α-secondary methyl with high stereoselectivity. The NMR study suggested that the stable conformation for **238** would be

depicted in Figure 10, hence its osmylation would be expected to occur from the less-hindered side of the molecule affording the diol (**239**) with the desired stereochemistry.

In fact, dihydroxylation of **238** with osmium tetraoxide furnished the diol (**239**) as the sole product. Since all the chiral centers for monocrotalic acid were thus constructed stereoselectively, preparation of the diacid function was then investigated. Protection of the diol (**239**) as a methylenedioxy group using dimethoxymethane and phosphorus pentoxide (106) was achieved using the acetal exchange reaction affording the bis(methylenedioxy) compound (**240**), which was further transformed to the lactone (**241**) by sequencial desilylation and oxidation.



Scheme 27.

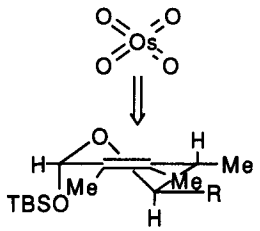


Figure 10.

Selective deprotection of the methylenedioxy group by treatment with a catalytic amount of conc. sulfuric acid in a mixture of acetic acid and acetic anhydride (107) gave the diacetate (**242**). After hydrolysis of the acetate (**242**) with sodium methoxide, the resulting compound was subjected to ruthenium tetraoxide (108) oxidation to affrod the diacid (**243**), which was identical with the authentic sample in all respects (Scheme 27).

Thus, the chiral synthesis of the necic acid, monocrotalic acid methylene acetal was successfully achieved starting from the chiral pyranone derivative, easily obtained by the oxidation of the corresponding 2-furylcarbinol (109).

VI. Conclusion

Furan is an unsaturated five membered monohetero-atom compound and the least aromatic of these class of heterocycles such as thiophene and pyrrole. Furan and its substituted derivatives are certainly highly functionalized reactive compounds and play important roles in organic synthesis as starting materials or intermediates; 1) *cisoid* 1,3-diene serves as a diene in the Diels-Alder reaction, 2) the olefin acts as a dipolarophile in the [2+3]cycloaddition reaction, and 3) the olefin reacts intra- and intermolecularly with other olefinic or carbonyl compounds to give [2+2]cycloadducts and with carbenes to give [2+1]cycloaddition products. In addition to the above characteristic properties, furan and its derivatives serve as masked 1,4-dicarbonyl compounds. Furan is classified as a π-sufficient aromatic, and it is well-recognized that oxidation of furans leads to the corresponding 1,4-dicarbonyl compounds. Although furan derivatives have great utilities in natural product synthesis, at least two major problems have to be resolved to make these compounds more useful: one is the establishment of synthetic procedures leading to poly-substituted furans with the substituents at the desird position and the other is the facile introduction of chirality with the desired stereochemistry.

Our methodology developed as above solved both the problems by converting 2-furylcarbinols into the corresponding pyranones. Using the same strategy, a number of natural products have been synthesized stereoselectively by other groups (110).

Further researches are presently under progress in our laboratory.

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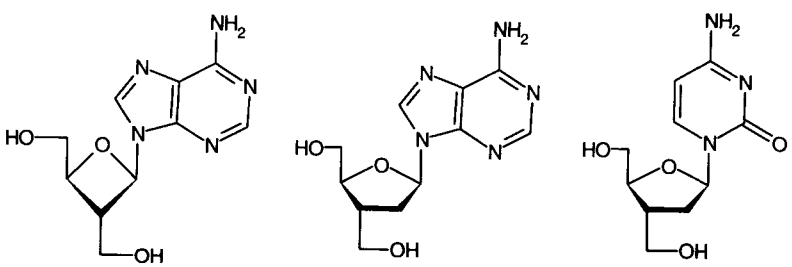
Stereoselective Synthesis of *C*-Branched Nucleoside Analogues

Pia N. Jørgensen and Jesper Wengel

1. Introduction

In the search for new anti-viral and anti-cancer agents, inhibition of genetic information by nucleoside analogues or antisense oligonucleotides displays immense pharmaceutical potential. In this context, promising anti-HIV activity of the branched nucleoside derivatives oxetanosin A [1,2], 2',3'-dideoxy-3'-*C*-hydroxymethyladenosine [3] and 2',3'-dideoxy-3'-*C*-hydroxymethylcytidine [4] (Figure) has stimulated considerable research efforts towards development of stereoselective synthesis of *C*-branched nucleosides.



Figure

In this overview, selected methods for preparation of 2'-*C*-, 3'-*C*- and 4'-*C*-branched nucleosides will be presented, followed by an account of the results in this area from our group. Only synthesis of analogues having the natural pentofuranose configuration are included. Generally, syntheses of *C*-branched nucleosides can be grouped into two categories, namely a) glycosylation of an appropriately protected nucleobase with a preconstructed *C*-branched carbohydrate, and b) direct introduction of the *C*-branching at the pentofuranose moiety of the nucleoside. We have in our group focused on the latter approach which consequently is highlighted in this report. Different chemical methodologies have been developed for the introduction of the *C*-substituent at the nucleoside level, and selected ones will be discussed in the following sections, grouped under reaction types.

2. Nucleophilic additions to ketonucleosides

Stereoselective addition of alkyl groups to carbonyl functionalities is one of the most useful techniques in synthetic organic chemistry, and application of this methodology for synthesis of *C*-branched nucleosides seems to be straightforward. Consequently, synthesis of ketonucleosides has attracted considerable attention, but in some cases (*e.g.* for 2'-deoxy-3'-keto derivatives) progress has been complicated by inherent instability of the keto derivatives, especially under basic conditions. The first successful synthesis of 2'- and 3'-ketonucleosides was reported by Cook and Moffatt [5], who oxidized 3',5'- and 2',5'-di-*O*-trityluridine by use of dimethyl sulfoxide (DMSO) and dicyclohexylcarbodiimide (DCC) in the presence of pyridinium trifluoroacetate affording the corresponding 2'- and 3'-ketonucleoside derivatives in 63% and 46% yield, respectively. The Swern modification of the Moffatt oxidation using DMSO activated by oxalyl chloride at low temperature has been used by Matsuda *et al.* [6] to give a 2'-ketonucleoside in 84% yield. Both Moffatt oxidation [7] and the Swern modification [8] have been successfully used in the oxidation of the 5'-hydroxyl functionality to generate a variety of 5'-aldehyde nucleosides. The first synthesis and isolation of a 2'-deoxy-3'-ketonucleoside was reported by Binkley *et al.* [9], who synthesised 3'-ketothymidine in 61% yield by photolysis of the 3'-pyruvoyl ester of 5'-tritylthymidine in benzene. Hansske and Robins [10] have reported the synthesis of different 2'- and 3'-(deoxy)ketonucleosides in high yields using a pre-mixed complex of CrO_3/pyridine/acetic anhydride (1:2:1) in CH_2Cl_2. An improved Cr(VI) oxidation for acid sensitive nucleosides has been reported by Froehlich *et al.* [11]. 5'-*O*-Tritylthymidine was oxidized by pyridinium dichromate (PDC) in the presence of 3Å molecular sieves in CH_2Cl_2 to give the 3'-keto derivative in 80% yield. Samano and Robins [12] describe high yielding oxidations of 3',5'- and 2',5'-bis-*O*-silyl protected nucleosides and 2'-deoxynucleosides using the Dess-Martin 12-I-5 periodinane reagent 1,1,1-tris(acetyloxy)-1,1-dihyhro-1,2-benziodoxol-3(1*H*)-one. An example of ruthenium tetroxide as oxidation reagent during synthesis of a *xylo*-configurated 2'-ketonucleoside has been published by Rosenthal *et al.* [13].

Reactions of keto nucleosides with organometallic reagents have provided the corresponding *C*-branched nucleosides. In the vast majority of these reactions, the nucleophilic addition selectively occurred from the α-face, due to steric hindrance of the base moiety. Accordingly, reactions of 3',5'-*O*-(tetraisopropyldisiloxan-1,3-diyl)-2'-keto nucleosides **1** with MeLi in THF or Me_3Al in $CHCl_3$ afforded as the only isomer 2'-*C*-methyl nucleosides **2** with β-D-*arabino* configuration in good yields (>80%) [6,14,15]. The same stereoselectivity towards the β-D-*arabino* configuration was observed using the lithium salts of (trimethylsilyl)acetylene and 1-hexyne giving 2'-*C*-(trimethylsilyl)ethynyl and 2'-*C*-(1-hexynyl) nucleosides **3** and **4** in excellent yields [16,17] (Scheme 1). On the contrary, treatment of compound **1a** with MeMgBr has been

reported to afford a mixture of **2a** (50% yield) and its 2'-epimer (43% yield) [14]. Similar results were obtained by Takenuki *et al.* [18] who suggested that the magnesium of the Grignard reagent coordinates between the 2-carbonyl oxygen and the 2'-carbonyl oxygen, facilitating the methyl carbanion attack from the sterically hindered β-face. The α-attack occurs by a non-chelation process from the more easily accessible α-face.

1

2 a, b: R = CH_3

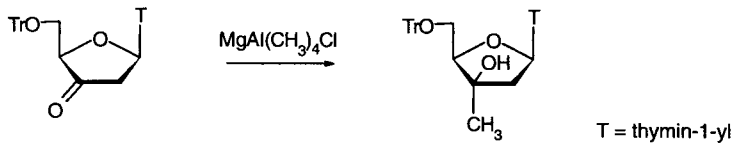
3 b, c: R = $C{\equiv}CSi(CH_3)_3$

4 b, c: R = $C{\equiv}CC_4H_9$

1-4	B
a	4-*O*-ethyluracil-1-yl
b	uracil-1-yl
c	thymine-1-yl

Scheme 1

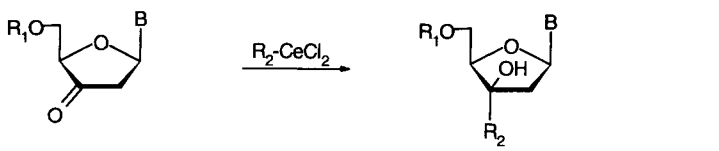
3'-Ketonucleosides are sensitive compounds prone to acid or base promoted β-elimination of the nucleobase which has posed a key problem in syntheses of 3'-*C*-branched derivatives. Addition of MeLi in THF to 2',5'-di-*O*-(*tert*-butyldimethylsilyl)-3'-ketouridine has been reported by Hayakawa *et al.* [15] to give 3'-*C*-methyl *xylo*-epimer as the only product in 79% yield. However, addition of BuLi lead to considerable β-elimination because of the higher basicity of the BuLi reagent. Based on successful addition of Me_3Al to 2',5'-bis-*O*-(*tert*-butyldimethylsilyl)-3'-ketouridine affording the 3'-*C*-methyl derivative in 64% yield [15], *Webb* [19] attempted reaction of Me_3Al with 5'-*O*-trityl-3'-ketothymidine. However, this was unsuccessful because of β-elimination of thymine or cleavage of the 5'-*O*-trityl group. Instead, he found the highly nucleophilic and less basic magnesium chlorotetramethylaluminate, prepared from equimolar amounts of MeMgCl in THF and Me_3Al in toluene, very useful. Addition of this magnesium aluminate reagent to a solution of 3'-ketonucleoside **5** in dichloromethane afforded 1-(2-deoxy-3-*C*-methyl-5-*O*-trityl-β-D-*threo*-pentofuranosyl)thymine **6** in 80% yield, as the only epimer, corresponding to nucleophilic attack from the less hindered α-face (Scheme 2).



5 **6**

Scheme 2

Organocerium reagents ($RCeCl_2$) obtained from MeMgBr, $CH_2CHMgBr$ and $Me_3SiCCLi$ and $CeCl_3$ (1:1) have been utilised as diastereoselective non-basic nucleophilic reagents by Bender and Moffett [20] in reactions with 5'-*O*-trityl-3'-ketothymidine **7a** to afford exclusively 3'-*C*-branched *threo*-configurated products **9a**, **10a** and **11a** in >90% yield. However, additions products of 5'-*O*-(*tert*-butyldiphenylsilyl)-3'-ketocytidine derivative **8c** and the more sensitive 5'-*O*-(*tert*-butyldiphenylsilyl)-3'-ketoadenosine **8b** were obtained in somewhat lower yields, but still exclusively as *threo*-epimers.



7a: R_1 = Tr

8 b, c: R_1 = TBDPS

a: B = thymin-1-yl

b: B = 6-*N*-Bz-adenin-9-yl

c: B = *6-N*-Bz-cytosin-1-yl

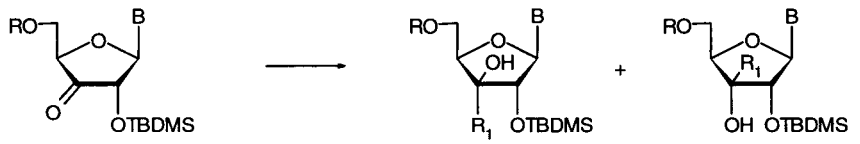
9 a, b, c: $R_2 = CH_3$

10 a, b, c: $R_2 = CH{=}CH_2$

11 a, b, c: $R_2 = C{\equiv}C{-}TMS$

Scheme 3

Jung *et al.* [21] have done some studies on the effect of the group in the 5'-position on the addition of cerium trimethylsilylacetylide to 2'-*O*-*tert*-butyldimethylsilyl-3'-ketoadenosine and -uridine (**12** and **15**). They found when having the bulky *tert*-butyldimethylsilyl protecting group at the 5'-position, the *xylo*-isomer was obtained in 81% yield, calculated from either starting nucleoside. The diastereoselectivities, as measured in the crude product, were >98:2 (**13a**:**14a**) and 95:5 (**13b**:**14b**). The preferential α-orientation of the nucleophilic attack, trans to the nucleobase, is in agreement with the results reported by Bender and Moffett [20]. Surprisingly, addition of cerium trimethylsilylacetylide to 5'-unprotected 3'-ketonucleosides **15** afforded the *ribo*-configurated derivatives **17** as the sole products which might be explained by chelation or steric effects [21] (Scheme 4).



12 a, b: R = TBDMS **13 a, b**: R = TBDMS **14 a, b**: R = TBDMS

15 a, b: R = H **16 a, b**: R = H **17 a, b**: R = H

12 - 17	B
a	adenin-9-yl
b	uracil-1-yl

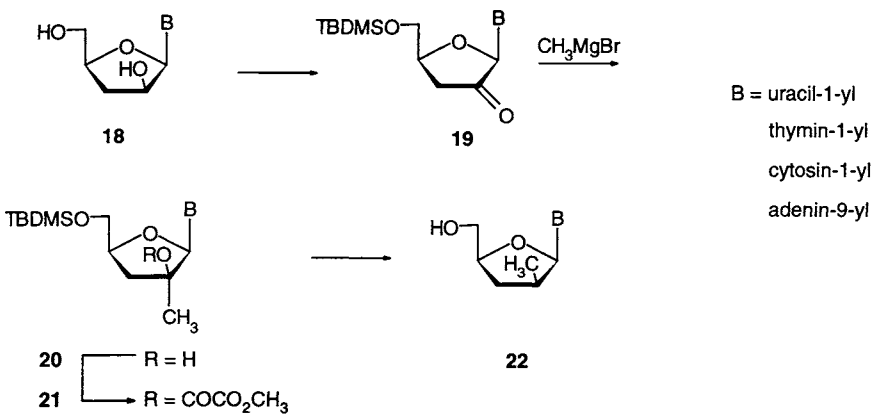
$R_1 = C{\equiv}C{-}TMS$

Scheme 4

Koole *et al.* [22] have described the synthesis of 9-(3-*C*-methyl-β-D-*xylo*-pentofuranosyl)adenine in five steps from adenosine. Reaction of the protected 3'-ketonucleoside with methylmagnesium iodide afforded the partially protected 3'-*C*-methylated *xylo*-nucleoside. As expected, the attack of the nucleophile occurred from the least hindered α-side of the 3'-ketonucloside. However, they found that the yield was highly sensitive to the nature of the solvent. Thus, using diethyl ether a relatively low yield of 35% for the addition step resulted because of partial cleavage of the 5'-*O*-monomethoxytrityl group and the formation of small amounts of the *ribo*-isomer. A change to THF as solvent completely suppressed the formation of the *ribo*-epimer and prevented the cleavage of the 5'-*O*-monomethoxytrityl group, and the desired *xylo*-isomer was obtained in 60% yield. Likewise, reaction of 2',5'-di-*O*-(*tert*-butyldimethylsilyl)-3'-keto-5-methyluridine and, to some surprise, also reaction of the unstable 5'-*O*-trityl-3'-ketothymidine with ethynylmagnesium bromide in THF proceeded with attack from the less hindered α-face of the pentofuranose ring to give 3'-*C*-ethynyl derivatives with β-D-*xylo*- and β-D-*threo*-configuration, respectively [23].

Starting from 1-(3-deoxy-β-D-*threo*-pentofuranosyl)uracil (**18**), Kakefuda *et al.* [24] have synthesised 1-(2,3-dideoxy-2-*C*-methyl-β-D-*threo*-pentofuranosyl)uracil (**22**). Silylation of the 5'-hydroxyl group followed by oxidation and treatment of the 2'-keto nucleoside with MeMgBr in THF furnished the *threo*-isomer **20** as the only product in 74% yield, corresponding to attack of the Grignard reagent from the less hindered α-face (Scheme 5). Reaction of the protected 3'-deoxy adenosine derivative 9-(5-*O*-(monomethoxytrityl)-3-deoxy-β-D-*glycero*-pentofuran-2-ulosyl)-6-*N*-(monomethoxytrityl])adenine with the Grignard reagent MeMgI in THF afforded the 2'-*C*-methyl-*threo*-isomer as the only product in 70% yield [25].

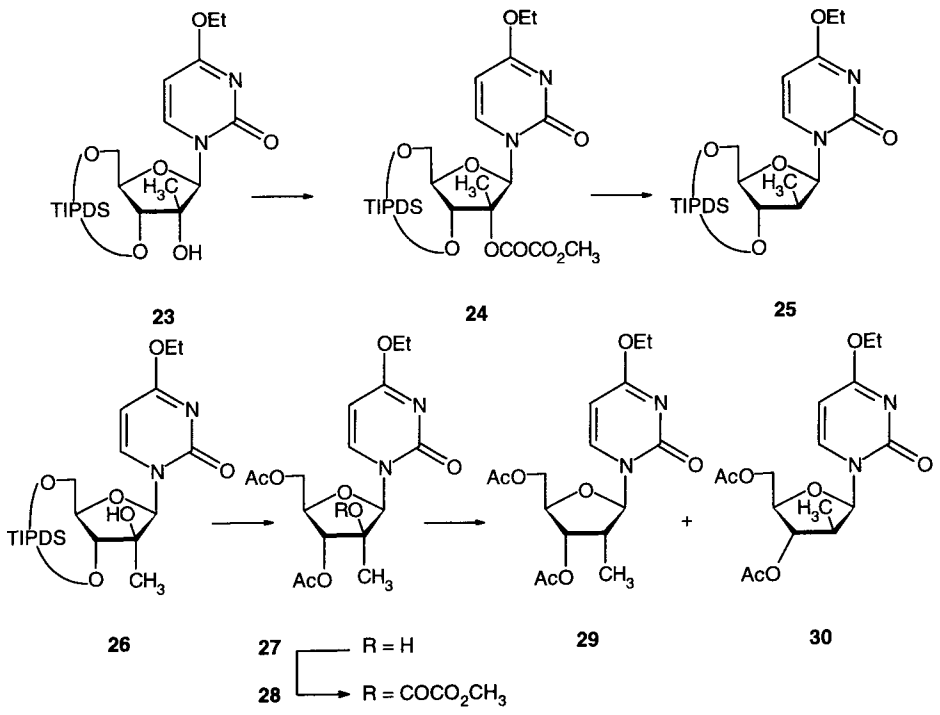
Radical deoxygenation of *tert*-alcohols, which are accessible from nucleophilic addition to 2'/3'-ketonucleosides, is a powerful method for synthesis of 2'/3'-deoxy-2'/3'-*C*-alkyl nucleosides. It has been shown that *tert*-alcohols having a substituent such as alkyl, acetylene [16,17] or cyano [26,27] group attached to the sugar moiety are effectively deoxygenated via their thiocarbonates or methoxalyl esters [28] by Bu_3SnH in the presence of AIBN in toluene. These reactions generally proceed in a stereoselective manner. Accordingly, reaction of derivative **20** with methoxalyl chloride in CH_3CN in the presence of 4-(dimethylamino)pyridine (DMAP) afforded the methoxalyl ester **21**, which was subjected to free radical deoxygenation with Bu_3SnH and AIBN in hot toluene. Only one product was obtained in 88% yield, assigned as the *threo*-configurated isomer **22**. Thus, the *tert*-radical, initially generated from **21**, was stereospecifically reduced from the α-face by Bu_3SnH to furnish **22** due to steric hindrance from the nucleobase. In a similar way, thymine, cytosine and adenine derivatives were obtained exclusively as the *threo*-epimers (Scheme 5).



Scheme 5

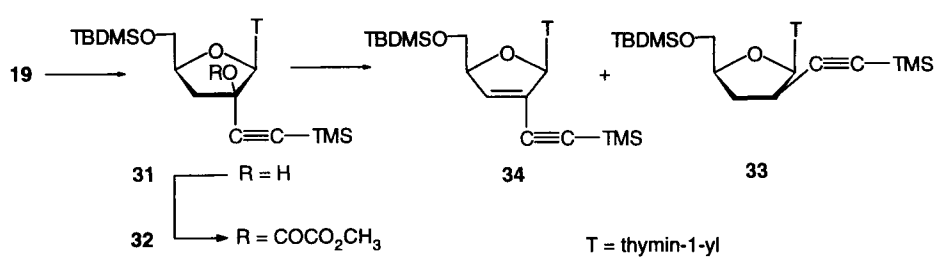
Radical deoxygenation of the methoxalyl ester **24** of 2'-*C*-methyl-3',5'-*O*-(tetraisopropyldisiloxan-1,3-diyl)-4-ethyluridine **23** afforded 2'-deoxy-2'-*C*-methyl *arabino*-configurated nucleoside **25** as the only product in 56% yield. However, acylation of 4-ethyl-1-(2-*C*-methyl-3,5-*O*-(tetraisopropyl-disiloxan-1,3-diyl)-β-D-*arabino*-pentofuranosyl)uracil **26** with methoxalyl chloride was unsuccessful even under vigorous conditions, probably due to steric hindrance from the 3,5-protecting group and the nucleobase. After conversion of **26** to the di-*O*-acetate **27** by treatment with Ac_2O and Et_3N in the presence of DMAP in CH_3CN, the methoxalyl derivative **28** was easily obtained. Subsequent free radical deoxygenation by Bu_3SnH and AIBN in hot toluene afforded a mixture of *ribo*- and *arabino*-isomers (**29** and **30**) in a ratio of 1:3, showing that the reduction occurred

predominantly from the less hindered α-face of the *tert*-alkyl radical intermediate [14,29] (Scheme 6).



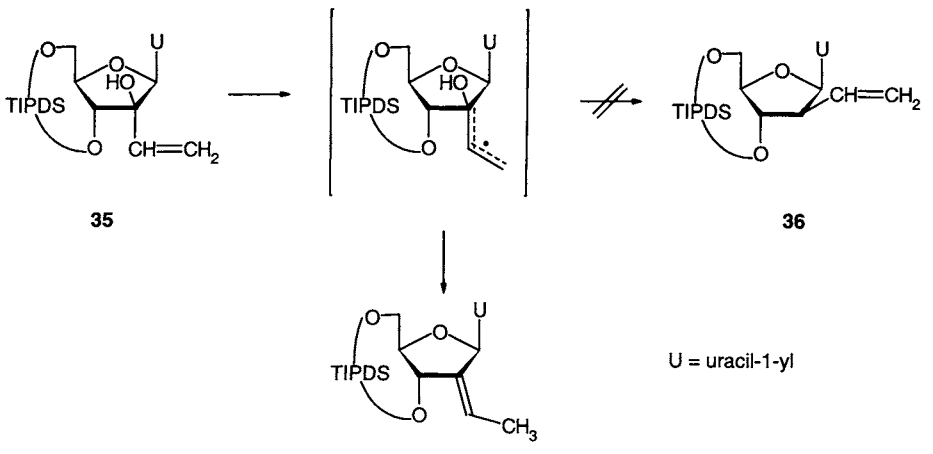
Scheme 6

Radical deoxygenation [24], *via* methoxalyl derivative **32**, of 3-deoxy-2-*C*-ethynyl-β-D-*threo* configurated thymine nucleoside **31**, obtained from reaction of the corresponding 2'-keto nucleoside **19** with lithium (TMS)acetylide in THF, afforded two nucleoside products. The less polar product, obtained in 41.5% yield, was assigned as the desired deoxygenated derivative **33** having *threo*-configuration. The second product, obtained in 15% yield, was assigned as the 2',3'-unsaturated derivative **34** which was assumed being formed during the radical reaction by tributyltin radicals or intramolecular radicals formed by abstraction of a 3'-proton.



Scheme 7

lino *et al.* [16] have showed that radical deoxygenation of the methoxalyl derivative of 1-(3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-*C*-(trimethylsilyl)ethynyl-β-D-*arabino*-pentofuranosyl)-5-methyluracil afforded the 2-deoxy *arabino*-isomer as the only product in 44% yield [16]. However, similar radical deoxygenation of the *tert*-alcohol of 1-(3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-*C*-vinyl-β-D-*arabino*-pentofuranosyl)uracil (**35**) was unsuccessful. Methoxalylation and subsequent radical deoxygenation afforded a sole nucleoside product, identified as the *Z*-form of 2'-deoxy-2'-ethylidene derivative **37** and not the expected nucleoside **36** (Scheme 8).

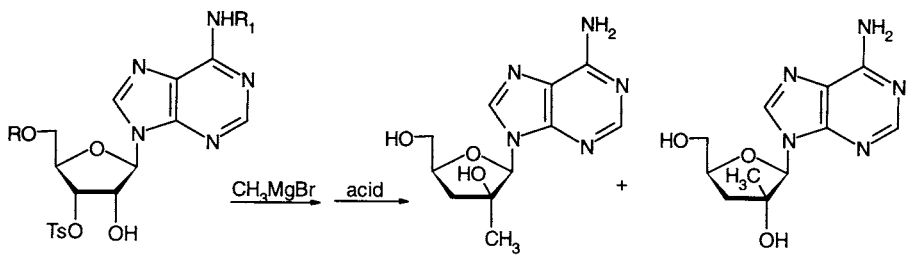


35 **36** **37**

Scheme 8

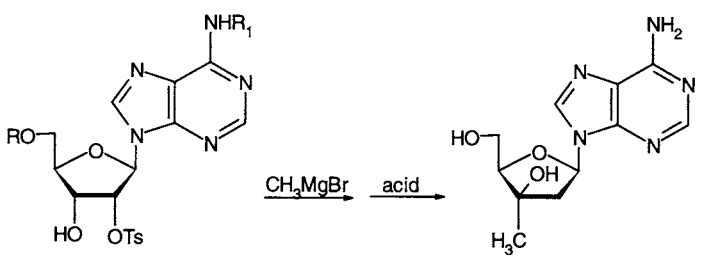
Kawana *et al.* [30] describe the deoxygenative methylation of *O*-tosylated adenosine with Grignard reagents. This reaction is an efficient one-pot procedure involving a [1,2]-hydride shift and a concerted elimination of a sulfonyloxy group [31,32]. The resulting α-deoxygenated ketonucleoside is converted into the product in the presence of an excess of MeMgX. Appropriately protected 2'-*O*- and 3'-*O*-tosyladenosines were prepared from adenosine [30]. Silylation of adenosine with *tert*-butyldimethylsilyl chloride afforded 3',5'-di-*O*- and 2',5'-di-*O*-silylated adenosine along with 2',3',5'-tri-*O*-protected adenosine in a ration of 51:41:8 after column chromatography. Protection of the amino group in the base of the two diprotected nucleosides was obtained by reaction with 4,4'-dimethoxytrityl chloride. Tosylation of the nucleosides followed by deprotection with tetrabutylammonium fluoride gave 6-*N*-(4,4'-dimethoxytrityl)-2'-*O*-tosyladenosine (**42**) and 6-*N*-(4,4'-dimethoxytrityl)-3'-*O*-tosyladenosine (**38**), respectively. Alternatively **42** and **38** were prepared from 6-*N*,5'-*O*-bis(4,4'-dimethoxytrityl)-2'-*O*-tosyladenosine (**43**) and 6-*N*,5'-*O*-bis(4,4'-dimethoxytrityl)-3'-*O*-tosyladenosine (**39**), respectively, by selective detritylation with pyridinium *p*-toluenesulfonate. The deoxygenative methylation was

carried out in dry benzene with MeMgBr in THF or MeMgI in diethyl ether. The crude products were directly deprotected with 80% acetic acid or zinc bromide before isolation by means of ion-exchange column chromatography. Reaction of 3'-*O*-tosyladenosine derivatives **38** and **39** with MeMgBr or MeMgI at elevated temperatures produced an isomeric mixture of 9-(3-deoxy-2-*C*-methyl-β-D-*threo*- and 9-(3-deoxy-2-*C*-methyl-β-D-*erythro*-pentofuranosyl)adenine (**40** and **41**) in a ratio of 3:1. Only the *threo*-isomer **44** was obtained (~50% yield) in the deoxygenative methylenation of the 2'-*O*-tosyladenosine derivatives **42/43** (Scheme 9). Koole *et al.* [33] obtained a somewhat lower yield when MeMgI in Et_2O was added to a solution of 2'-*O*-tosyl-5'-*O*-trityladenosine in THF giving 9-(2-deoxy-3-*C*-methyl-5-*O*-trityl-β-D-*threo*-pentofuranosyl)-adenine in 34% yield along with an unsaturated product in 2.7% yield. Chattopadhyaya and co-workers [34,35,36] on the other hand observed that deoxygenative methylation of 2'-*O*-tosylated 5'-*O*-trityluridine afforded the *erythro*-isomer as the only product and that reaction of the 3'-*O*-tosyl derivative with five equivalents of MeMgI afforded 1-(5-*O*-trityl-3-deoxy-β-D-*glycero*-pentofuran-2-ulosyl)uracil in 79% yield.



	R	R_1
38	H	DMT
39	DMT	DMT

40 **41**

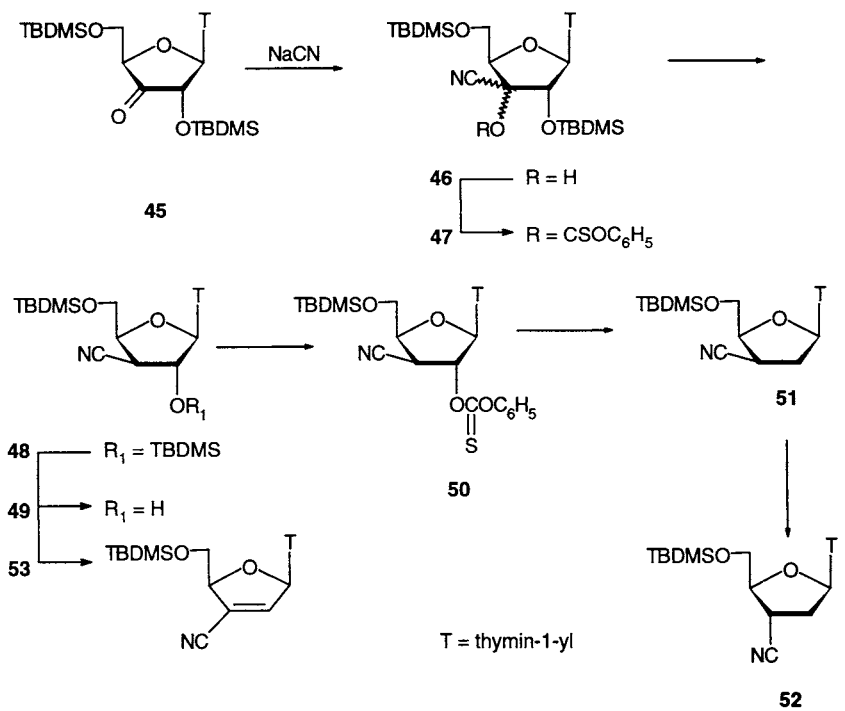


	R	R_1
42	H	DMT
43	DMT	DMT

44

Scheme 9

Synthesis of 2'-*C*-cyano- and 3'-*C*-cyano nucleosides has been attained by treatment of the corresponding keto nucleosides (**45** and its 2'-keto isomer) with sodium cyanide and sodium bicarbonate in a two-phase ethyl ether and water system to give an isomeric mixture of the two possible 2'-*C* and 3'-*C*-cyanohydrin epimers (**46** and the corresponding 2'-cyano isomer), respectively [26,27,37-39]. The 3'-*C*-cyanohydrins **46**, which decompose to the corresponding ketonucleosides on standing in solution at room temperature, were treated without further purification with phenoxythiocarbonyl chloride in the presence of DMAP to give the corresponding thiocarbonates **47**. Radical deoxygenation using Bu_3SnH in the presence of AIBN afford only one nucleoside product identified as 3'-*C*-cyano-3'-deoxy-β-D-*xylo*-pentofuranosyl derivative **48** [26,27,39]. 2'-Hydroxyl deoxygenation of **48** was achieved [26] by reaction of the nucleoside **49** with phenoxythiocarbonyl chloride in pyridine followed by treatment of this intermediate with Bu_3SnH in the presence of AIBN to give 1-(5-*O*-(*tert*-butyldimethylsilyl)-3-*C*-cyano-2,3-dideoxy-β-D-*threo*-pentofuranosyl)thymine (**51**) in 51% yield. The stronger base DMAP induced formation of the elimination product 1-(5-*O*-(*tert*-butyldimethylsilyl)-3-*C*-cyano-2,3-dideoxy-β-D-*glycero*-2-enofuranosyl)thymine (**53**) in 80% yield instead of the expected 2'-*O*-thiocarbonyl derivative **50** [27]. Epimerisation of β-D-*threo* nucleoside **51** was achieved by refluxing the nucleoside in a solution of NaOH and CH_3OH to give the β-D-*erythro* isomer **52** in 76% yield (Scheme 10).

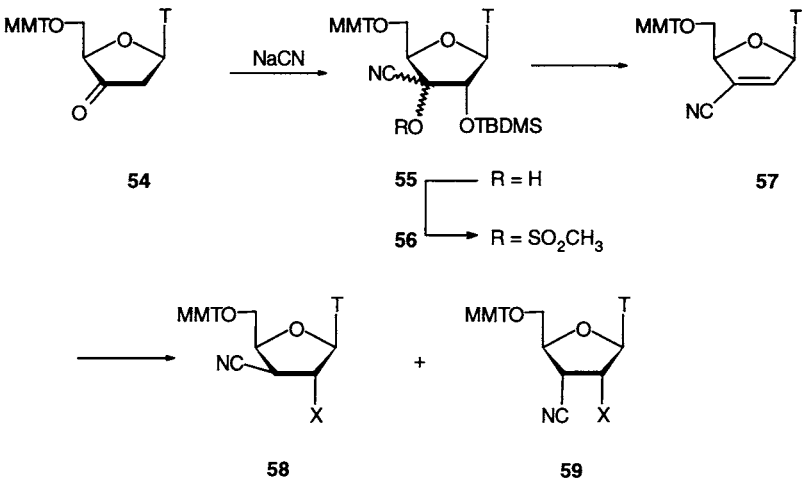


Scheme 10

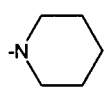
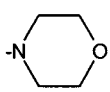
1-(3-*C*-cyano-2,3-dideoxy-β-D-glycero-2-enofuranosyl)thymine has been synthesized by Wu and Chattopadhyaya [39] from 5'-*O*-(4-methoxytrityl)-3'-ketothymidine (**54**). Treatment of the epimeric mixture of the two 3'-*C*-cyanohydrins **55** with methylsulfonyl chloride in pyridine gave an epimeric mixture **56** of 3'-*C*-cyano mesylates which was converted into 3'-*C*-cyano-3'-deoxy-2',3'-didehydrothymidine derivative **57** in 46% yield (calculated from ketone **54**) by refluxing in pyridine and triethylamine. The 3'-enenitrile **57** was shown be a good substrate for Michael addition reactions and underwent nucleophilic addition with different nitrogen- and carbon nucleophiles to give the corresponding 2',3'-dideoxy-3'-C-cyano-2'-*C*-substituted thymidine derivatives in good yields. The stereochemical course of the nucleophilic additions at the 2'-carbon were shown to be from the sterically less hindered α-face. In most cases, the *trans*-isomer **58** was the only or the predominant addition product, except for the reaction with ammonia where the *cis*-isomer **59** was the major product formed (Scheme 11).

3'-*C*-Nitro unsaturated nucleosides as substrate for Michael addition reactions has been extensively examined by Chattopadhyaya and co-workers. Because of the strong electron-withdrawing character of a nitro group, addition reactions readily occur at the β-carbon. Accordingly, 2',3'-dideoxy-2',3'-didehydro-3'-*C*-nitro nucleosides have proven synthetically useful for preparation of various 2'- and 3'-modified nucleosides. Hossain *at al* [40] describe the synthesis of 1-(2,3-dideoxy-3-*C*-nitro-β-D-*glycero*-pent-2-enofuranosyl)thymine (**64**) from 1-(5-*O*-monomethoxytrityl-2-*O*-pivaloyl-β-D-*xylo*-pentofuranosyl)thymine (**60**). Treatment of the ketone **61** with hydroxylamine hydrochloride in pyridine gave the 3'-oximino derivative **62** as an *E/Z* isomeric mixture in 80% yield. Oxidation by trifluoroperacetic acid in the presence of urea and excess Na_2HPO_4 in acetonitrile afforded 3'-*C*-nitro-olefin **63** in 76% yield (Scheme 12).

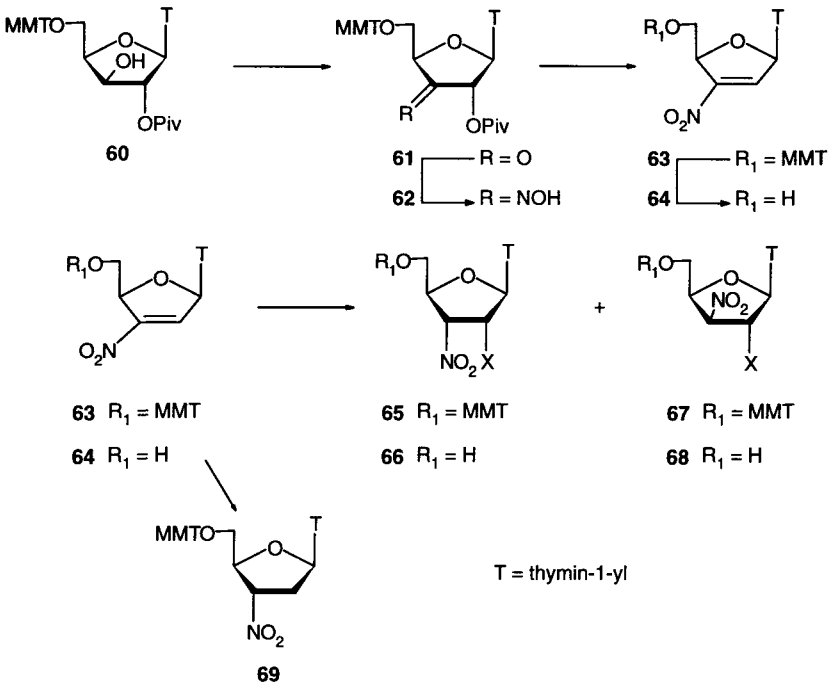
Michael addition reactions with various oxygen- and nitrogen nucleophiles afforded the *cis*-addition product **65/66** as the major product (>74% yield) with attack of the nucleophiles at the 2'-carbon exclusively from the α-face. Addition of carbon nucleophiles proceeded less stereoselectively yielding either the *cis*- (**65/66**) or the *trans*-isomer (**67/68**) as the major product, depending on the particular carbon nucleophile used. One advantage of Michael addition reactions on 3'-*C*-nitro-olefins is that the 3'-*C*-nitro group in 2'-*C*-substituted-3'-*C*-nitro-nucleosides can be easily removed to produce 3'-deoxy 2'-*C*-substituted-nucleosides. Denitration of the *trans*-isomers was easily performed with Bu_3SnH and AIBN in benzene in 50-85% yield. Denitration of the *cis*-isomers gave lower yields (~30%) under the same conditions, and in the case of 2'-alkoxy substituted nucleosides a large excess of Bu_3SnH was needed and the denitrated product was generated in very low yield (5-10%) along with several by-products identified as the corresponding oximes. Reduction of the nitro-olefin **63** with $NaBH_4$ in ethanol progressed in a stereospecific manner to give 2',3'-dideoxy-3'-*C*-nitrothymidine derivative **69** in 68% yield [40] (Scheme 12).



T = thymin-1-yl

nucleophile X	**58**	**59**
$-NH_2$	25%	42%
$-NHCH_3$	43%	19%
$-NHCH_2Ph$	73%	8%
	78%	-
	74%	-
	82%	-
$-CH(CO_2CH_3)_2$	59%	-
$-CH_2NO_2$	39%	13%

Scheme 11

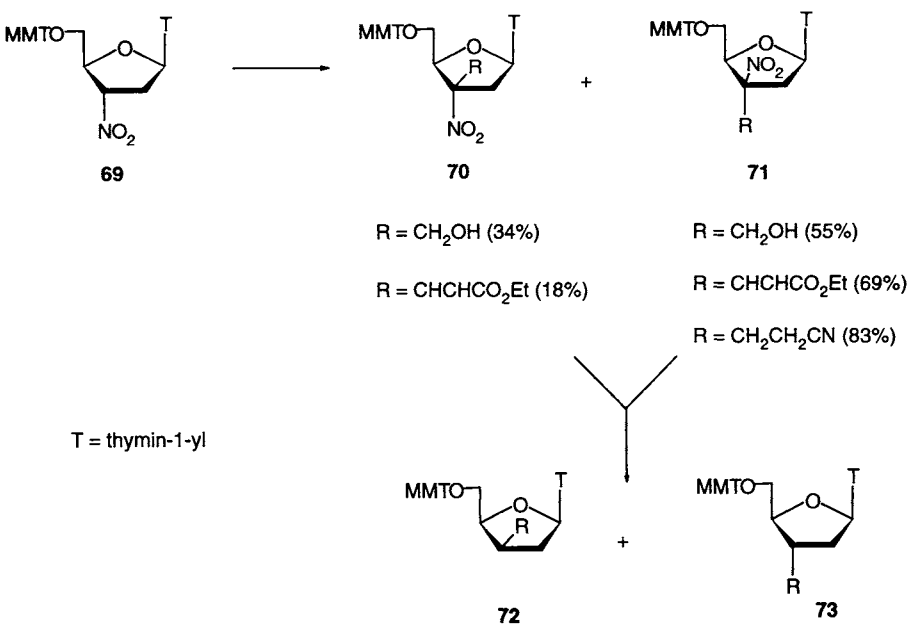


nucleophile X	R_1	*trans*	*cis*
MeNH	H	-	77%
t-BuNH	H	-	91%
$PhCH_2NH$	H	-	77%
	MMT	-	82%
OCH_3	H	-	96%
	MMT	-	83%
OCH_2CH_3	MMT	-	86%
$OCH_2CH_2OCH_3$	MMT	-	74%
OCH_2CHCH_2	MMT	-	74%
$CH(CO_2CH_3)_2$	MMT	56%	40%
$CH(CH_3CO)_2$	MMT	-	56%

Scheme 12

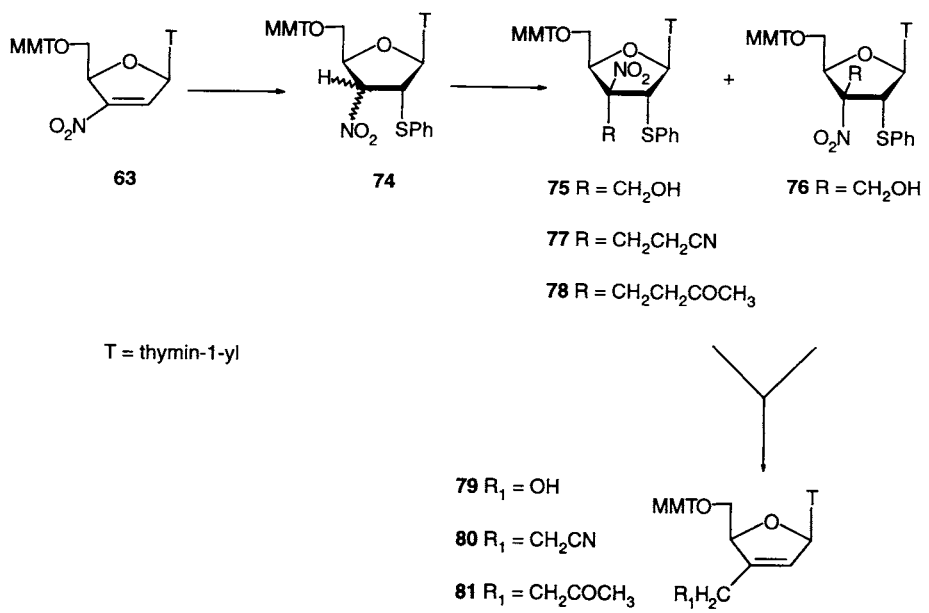
Michael addition reactions has also been accomplished with 2',3'-ene-3'-*C*-sulfones [41], 2',3'-ene-3'-*C*-nitrile and 2',3'-ene-3'-*C*-phenylselenone [42] derivatives. Generally, nucleophilic addition proceeded from the α-face yielding predominating the *trans*-2',3'-disubstituted adducts. However, Wu *et al.* [41] showed that the ratio between the *trans*- and the *cis*-isomer was temperature dependent.

Base-catalyzed addition reactions (Henry reactions) at the α-carbon of the nitro function in nucleoside **69** with various reagents containing electron-deficient functions has been described by Garg *et al.* [43]. Thus, reaction of **69** with paraformaldehyde in THF in the presence of tetrabutylammonium fluoride gave a diastereomeric mixture of 3'-(*R*)- and 3'-(*S*)-*C*-hydroxymethyl-3'-*C*-nitrothymidine (**70** and **71**) in a 1:1.6 ratio. Similarly, reaction with ethyl propiolate afforded a 1:3.8 ratio of 3'-(*R*)- and 3'-(*S*)-*C*-(ethoxycarbonylethylidene)-3'-*C*-nitrothymidine, and 3'-(*R*)-*C*-(2-cyanoethyl)-3'-*C*-nitrothymidine was isolated as the major product in 83% yield by reaction of **69** with acrylonitrile. In these base-catalysed addition reactions, the generated 3'-carbanion preferentially attacks the electron-deficient reagents from the less hindered α-face of the sugar ring. Subsequent free-radical promoted denitration of **70** and **71** by Bu_3SnH and AIBN afforded 3'-*C*-substituted-β-D-*threo*-isomers **72** as the major products, suggesting that the 3'-carbon radical generated from either of the epimers preferentially abstracts a hydrogen atom from Bu_3SnH from the less hindered α-face (Scheme 13).



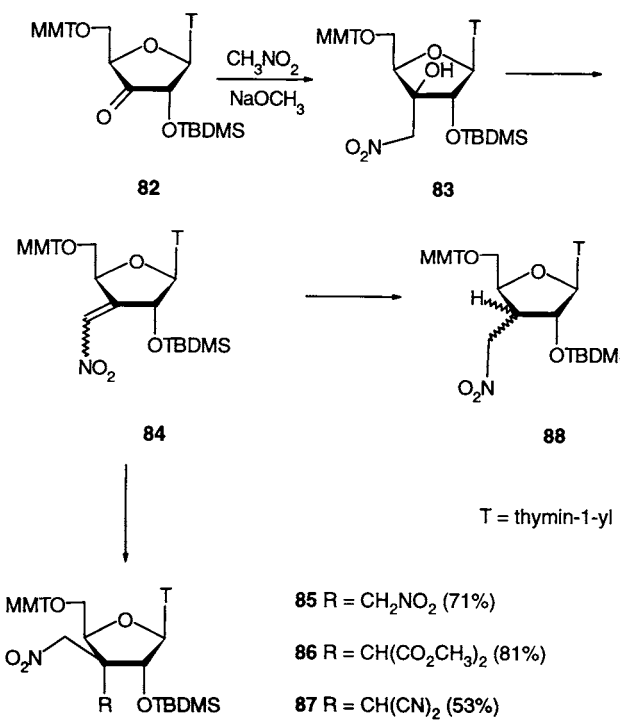
Scheme 13

Hossain *et al.* [44] describe the synthesis of 2',3'-dideoxy-2',3'-didehydro-3'-*C*-substituted thymidine derivatives using a tree-step procedure including a Michael addition of phenylthiolate to 3'-*C*-nitro-olefin **63**, followed by a base-catalysed addition reactions of an electron-deficient reagent, and finally a free-radical induced elimination the 3'-*C*-nitro and 2'-*C*-phenylthio groups. Reaction of 2',3'-dideoxy-2',3'-didehydro-3'-*C*-nitrothymidine **63** with thiophenol in the presence of 1,1,3,3-tetramethylguanidine in acetonitrile afforded an inseparable diastereomeric mixture of the *cis*- and *trans*-addition products **74** in a 1.3:1 ratio (total yield 78%). Treatment of this mixture with 35% aqueous formaldehyde in acetonitrile in the presence of 1,1,3,3-tetramethylguanidine gave 3'-(*S*)- and 3'-(*R*)-*C*-hydroxymethyl-3'-*C*-nitro-2'-(*R*)-thiophenylthymidines **75** (18% yield) and **76** (64% yield). Addition of acrylonitrile in THF in the presence of tetrabutylammonium fluoride gave 3'-(*S*)-*C*-(2-cyanoethyl)-3'-nitro-2'-(*R*)-thiophenylthymidine **77** (66% yield) and addition of methyl vinyl ketone gave 3'-(*S*)-*C*-(3-oxobutyl)-3'-*C*-nitro-2'-(*R*)-thiophenylthymidine **78** (29% yield), as the major products. Again, preferential reaction from the α-face of the sugar ring was observed. Free-radical promoted eliminations of the 3'-*C*-nitro and 2'-*C*-phenylthio groups were performed using Bu_3SnH and AIBN in dry toluene to give 3'-deoxy-2',3'-didehydro-3'-*C*-substituted thymidines **79-81** in yields of around 80% (Scheme 14). The unique electron-deficient character of nitro-olefins make them also powerful dienophiles for Diels-Alder reactions to give various cycloaddition products under various conditions [45,46].



Scheme 14

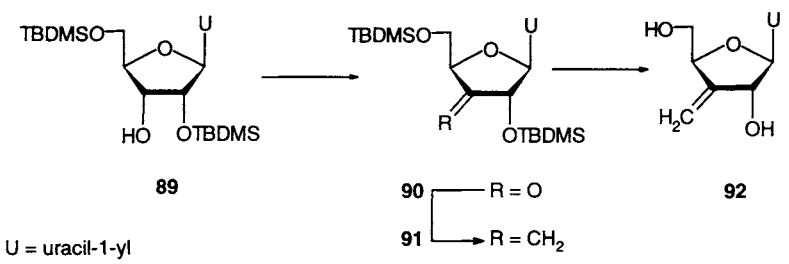
The use of a 3'-*C*-nitromethylene functionality as an intermediate in nucleophilic addition reactions to give 3'-substituted nucleosides has been described by Garg *et al.* [47]. 1-(2-*O*-(*tert*-Butyldimethylsilyl)-5-*O*-(4-methoxytrityl)-3-*C*-(*Z*/*E*)-nitromethylene-β-D-pentofuranosyl)thymine (**84**) was prepared from 3'-ketonucleoside **82** by addition of nitromethane (1 N $NaOCH_3$ in methanol), followed by treatment with acetic anhydride and DMSO at room temperature to give an isomeric mixture **84** of *Z*- and *E*-isomers in a ratio of 5:1. Michael addition reactions with various nitrogen- and carbon nucleophiles occured exclusively with attack of the nucleophiles from the α-face of the carbohydrate ring to give compounds **85-87**. Reduction of the exocyclic double bond was achieved with $NaBH_4$ in ethanol to give a mixture **88** of the *xylo*- and *ribo*-isomers (Scheme 15). The corresponding 2'-*C*-nitromethylene derivative of uridine have been prepared by Ueda *et al.* [48], and a 2-*C*-nitromethyl-β-D-*lyxo* adenine derivative has been synthesized by Rosenthal *et al.* [13].



Scheme 15

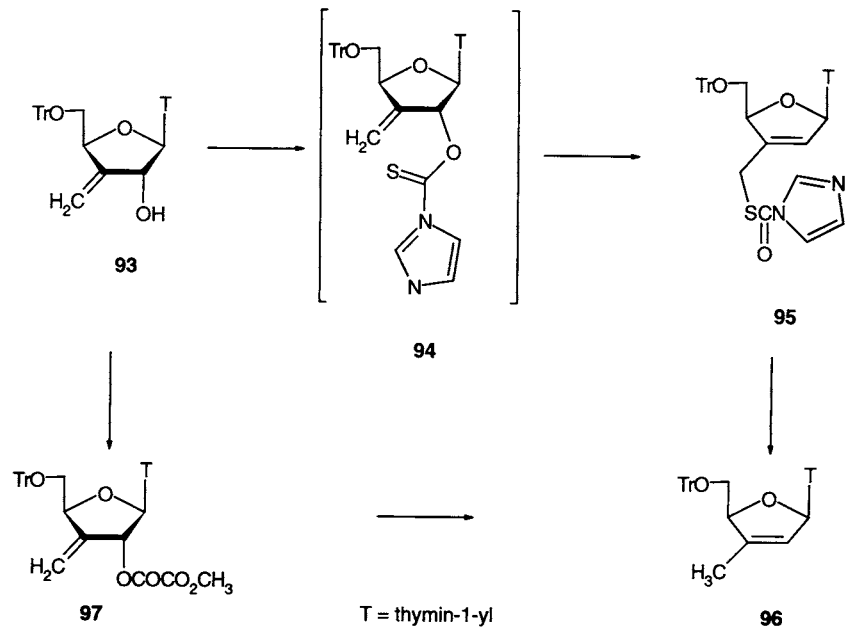
3. Miscellaneous reactions

The syntheses discussed so far have involved nucleophilic addition to ketonucleosides. Numerous other types of reactions have been applied in syntheses of *C*-branched nucleoside analogues, and some of these will be described in this section. Samano and Robins [49] have described synthesis of 3'-deoxy-3'-*C*-methyleneuridine and -cytidine starting from uridine. Protection of uridine with *tert*-butyldimethylsilyl chloride gave 2',5'-di-*O*-(*tert*-butyldimethylsilyl)uridine (**89**), 3',5'-bis-*O*-(*tert*-butyldimethylsilyl)uridine and 2',3',5'-tri-*O*-(*tert*-butyldimethylsilyl)uridine in yields of 70%, 18% and 5%, respectively. Oxidation of 2',5'-di-*O*-(*tert*-butyldimethylsilyl)uridine (**89**) with CrO_3/Ac_2O/pyridine in CH_2Cl_2 afforded 3'-ketouridine derivative **90** in 83% yield. Treatment of **90** with methyltriphenylphosphonium bromide and sodium 2-methyl-2-butoxide in diethyl ether/benzene resulted in the formation of 3'-deoxy-3'-*C*-methyleneuridine derivative **91** in 92% yield. Deprotection of **91** with teterabutylammonium fluoride in THF and purification on Dowex 50 (H^+) resin column gave a quantitative yield of 3'-deoxy-3'-*C*-methyleneuridine (**92**) (Scheme 16).



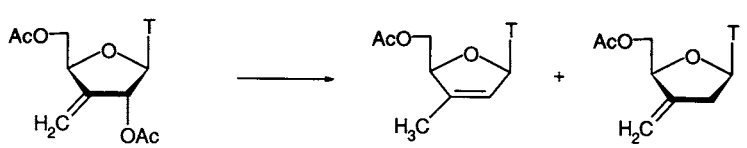
Scheme 16

Deoxygenation of the 2'-hydroxyl group in 3'-deoxy-3'-*C*-methylene pyrimidine nucleosides has proven difficult [50]. Treatment of 3'-deoxy-3'-*C*-methylene-5'-*O*-trityl-5-methyluridine with 1,1'-thiocarbonyldiimidazole in DMF afforded one nucleoside product in 84% yield. The structure of this nucleoside was assigned as 3'-*C*-((imidazol-1-yl)carbonylthiomethyl) derivative **95**, generated as a result of allylic rearrangement of the intermediate **94**, rather than the expected 2'-*O*-((imidazol-1-yl)thiocarbonyl) derivative **94**. Desulfurisation of **95** with Bu_3SnH and AIBN in hot toluene gave 3'-deoxy-2',3'-didehydro-3'-*C*-methyl-5'-*O*-tritylthymidine (**96**) in 83% yield. Conversion of nucleoside **93** into the methoxalyl derivative **97** followed by deoxygenation using Bu_3SnH and AIBN in hot toluene likewise afforded nucleoside **96**. Thus, both radical reactions are expected to proceed through the same allylic radical intermediate (Scheme 17).



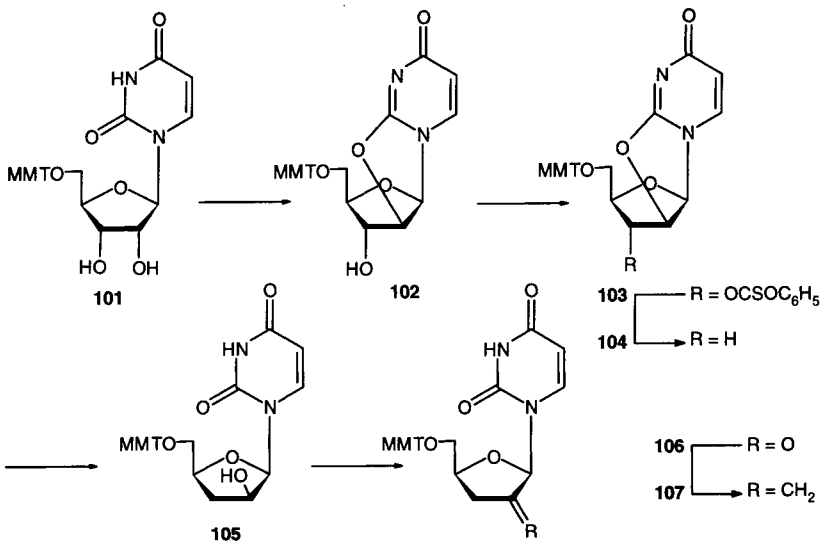
Scheme 17

Synthesis of 3'-deoxy-3'-*C*-methylenethymidine derivative **100** [50] was achieved by a method described by Tsuji *et al.* [51,52]. 2',5'-Di-*O*-acetyl-3'-deoxy-3'-*C*-methylenethymidine (**98**) was deoxygenated using triethylammonium formate as a hydride donor in the presence of Bu_3P and a catalytic amount of $Pd_2(DBA)_3$ affording in 78% yield a mixture of 5'-*O*-acetyl-2',3'-didehydro-3'-deoxy-3'-*C*-methylthymidine (**99**) and 5'-*O*-acetyl-3'-deoxy-3'-*C*-methylenethymidine (**100**) in a ratio of 5:95 (Scheme 18). Palladium mediated deoxygenation of 2'-deoxy-2'-*C*-methylene pyrimidine nucleosides were less successful. However, 2'-deoxy-2'-*C*-methylene-5-methyl-5'-*O*-trityluridine was deoxygenated as the 3'-*O*-ethoxycarbonyl derivative by triethylammonium formate in the presence of Bu_3P and $Pd_2(OAc)_2$ in THF affording in 85% yield a mixture of the *endo*- and *exo*-methylene compounds in a ratio of 28:72.



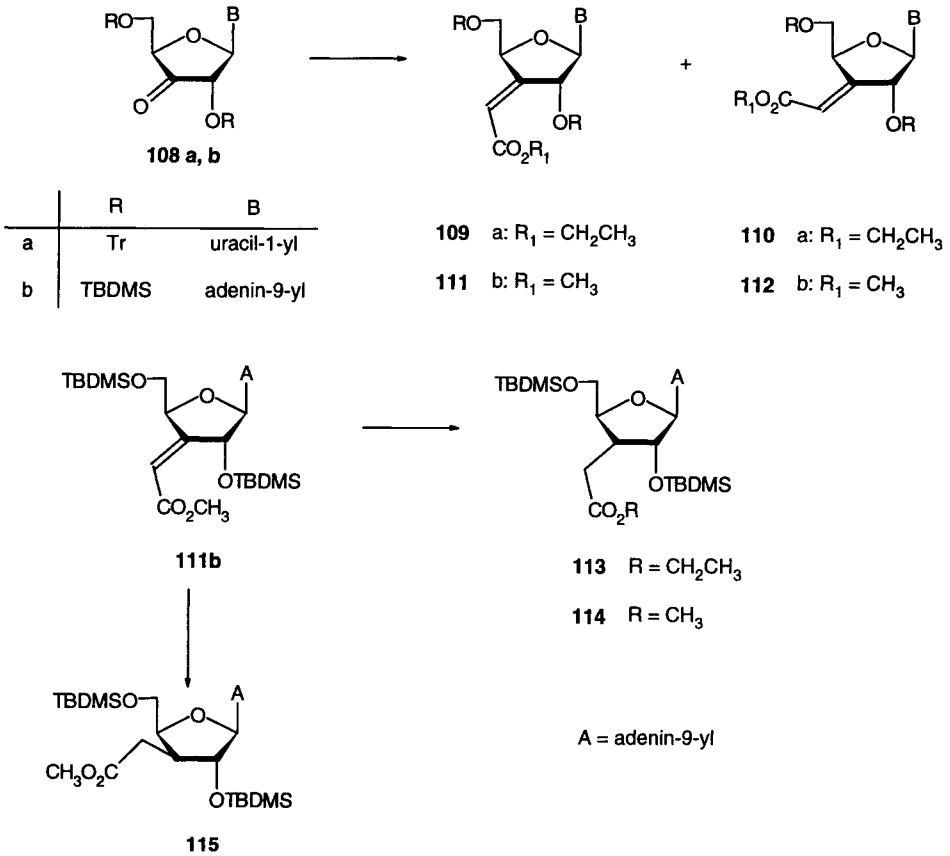
Scheme 18

An alternative method for synthesis of 2',3'-dideoxy-2'-*C*-methylene pyrimidine nucleosides has been described by Lin *et al.* [53]. Conversion of 5'-*O*-(4-methoxytrityl)uridine (**101**) to the corresponding 2,2'-anhydro analogue **102** was accomplished by reaction with diphenyl carbonate and sodium bicarbonate in DMF. Concomitant treatment with 3'-*O*-phenoxythiocarbonyl chloride and DMAP in anhydrous acetonitrile afforded 3'-*O*-phenoxythiocarbonyl derivative **103** which was reduced by Bu_3SnH and AIBN in toluene to the corresponding 3'-deoxy nucleoside **104**. Treatment of compound **104** with 1N NaOH in 50% ethanol afforded the 3'-deoxy-*threo* nucleoside **105**. Oxidation of **105** with CrO_3/Ac_2O/pyridine complex in CH_2Cl_2 followed by methylenation by reaction with methyltriphenylphosphonium bromide and BuLi in THF yielded 1-(2,3-dideoxy-2-*C*-methylene-5-*O*-(4-methoxytrityl)-β-D-*glycero*-pentofuranosyl)uracil (**107**) in an overall yield of 3.9% from uridine (Scheme 19).



Scheme 19

Synthesis of 3'-deoxy-3'-*C*-ethoxycarbonylmethylene-2',5'-di-*O*-protected nucleosides **109-112** has been achieved through reaction between 3'-keto nucleosides **108** and ethoxycarbonylmethyl-(triphenyl)phosphorane affording a single isomer in >88% yields. However assignment of the configuration gives rise to some disagreement. On the basis of NMR data, Auguste and Young [54] argue that the isomer has the *E*-configuration, whereas Lee and Wiemer [55] assign the *Z*-configuration to the single isomer achieved. By use of the sodium enolate of phosphonoacetate in a Horner-Wadswort-Emmons condensation, a 5:1 mixture of the two stereoisomers in 62% yield was obtained [55]. Hydrogenation of these olefins, either the *Z*-isomers or a mixture of the *Z*- and *E*-isomers over 10% Pd/C gave a single reduced product in 88% yield, which was assigned as the *ribo*-isomer **113/114**. However, reduction of **111b** with $NaBH_4$ in MeOH has been reported to give the *xylo*-isomer **115** (Scheme 20).

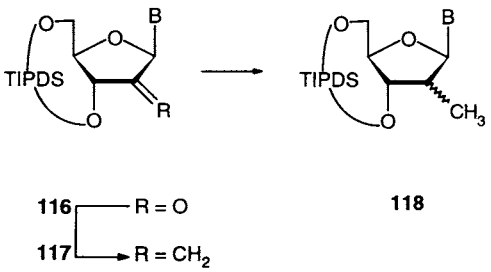


	R	B
a	Tr	uracil-1-yl
b	TBDMS	adenin-9-yl

Scheme 20

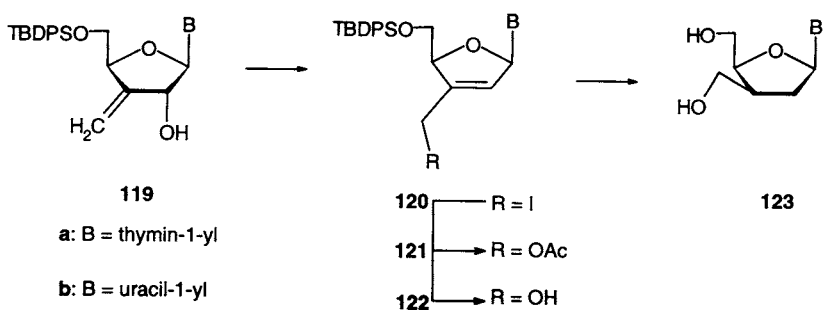
Wittig methylenation of 2'-deoxy-3'-ketonucleosides are unsuccessful because of the basicity of the ylide, leading to enone formation. However, methylenation of 2'-deoxy-3'-keto pyrimidine nucleosides have be achieved by Sharma and Bobek [56] in 60-95% yield, using the highly electrophilic reagent $Zn/CH_2Br_2/TiCl_4/THF$ in CH_2Cl_2.

The introduction of a double bond is very valuable in the sense that it can easily be modified into other functions. Cicero *et al.* [57] describe the stereoselective synthesis of 1-(2-deoxy-2-*C*-methyl-β-D-*arabino*-pentofuranosyl)uracil. Reaction of protected 2'-ketouridine **116** with methyl-triphenylphosphonium bromide gave 2'-deoxy-2'-*C*-methyleneuridine **117**, which was subjected to catalytic hydrogenation to generate an epimeric mixture **118** of the *ribo*- and *arabino*-isomers of 1-(2-deoxy-2-*C*-methyl-β-D-pentofuranosyl)uracil in quantitative yield. By selecting different catalysts for the hydrogenation step, it was possible to vary the *arabino*- to *ribo*-ratio. For example, 10% Pd on charcoal provided a 1:1 mixture of diastereomers, while the use of 5% Pd on $CaCO_3$ gave a 3:1 ratio of *arabino*- to *ribo*-isomer as estimated from integration of ^{1}H-NMR.



Scheme 21

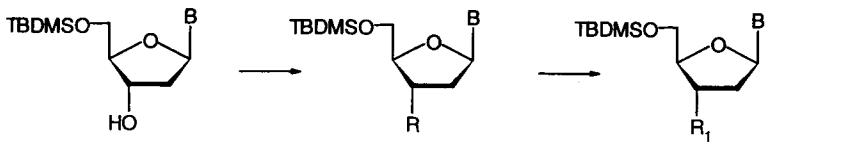
Synthesis of 2',3'-dideoxy-2'-*C*-hydroxymethyl and 3'-*C*-hydroxymethyl pyrimidine nucleosides having *threo*-configuration has been described by Ioannidis *et al.* [58,59]. The synthetic strategy was based on an allylic alcohol transposition of the corresponding 2'- and 3'-*C*-methylenenucleoside analogues. Reaction of 5'-*O*-*tert*-butyldiphenylsilyl protected 3'-*C*-methylenenucleosides **119** with 1.2 eqv. chlorodiphenylphosphine and 1.2 eqv. imidazole in CH_2Cl_2 followed by addition of 1.2 eqv. iodine in CH_2Cl_2 gave the allylic iodide **120**. Substitution of the allylic iodide with tetrabutylammonium acetate gave, after deacetylation, 5'-*O*-(*tert*-butyldiphenylsilyl)-2',3'-didehydro-2',3'-dideoxy-3'-*C*-hydroxymethyl nucleoside **122**. Catalytic hydrogenation at ambient pressure over $Rh(PPh_3)Cl$ in EtOH followed by desilylation gave the desired 1-(2,3-dideoxy-3-*C*-hydroxymethyl-β-D-*threo*-pentofuranosyl)pyrimidine nucleosides **123**. The cytosine analogues were obtained by conversion of the uracil moiety (Scheme 22).



Scheme 22

An efficient procedure using free radical chemistry for introducing an allyl group at the 3'-position of pyrimidine nucleosides has been described by Chu *et al.* [60]. 5'-*O*-(*tert*-Butyldimethylsilyl)-3'-*O*-phenoxythiocarbonylthymidine **125a** and the corresponding 2'-deoxyuridine derivative **125b** was reacted with allyltributyltin in toluene using AIBN as radical generator to give 3'-*C*-allyl-2',3'-

dideoxynucleosides **127a** and **127b** in yields of 50% and 74%, respectively. The reactions proceeded stereoselectively affording only the *erythro*-isomer corresponding to addition from the less hindered α-face of the free radical intermediate. Fiandor and Tam [61] have used photochemical radical initiation, and this methodology has been applied successfully for synthesis of 2'-*C*-allyl-2'-deoxynucleosides by Grøtli and Undheim [62]. Sanghvi *et al.* [63] used the iodo nucleoside **126a** as radical precursor. Even though the stereoselectivity was high, the expected 3'-*C*-styryl derivative **128a** was isolated in only 25% yield in the reaction of 5'-*O*-(*tert*-butyldimethylsilyl)-3'-deoxy-3'-iodothymidine (**126a**) with β-tributylstannylstyrene in the presence of AIBN. Use of 3'-*O*-phenylthiocarbonate ester **125a** as radical precursor afforded the desired nucleoside **128a** in 70% yield [63].



124

a: B = thymin-1-yl

b: B = uracil-1-yl

125 R = $OCSOC_6H_5$

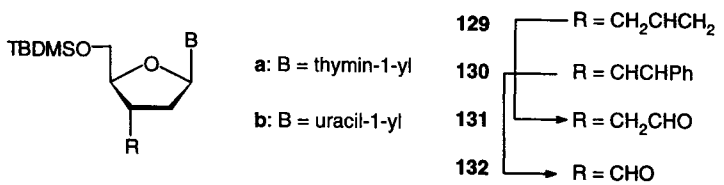
126 R = I

127 R = CH_2CHCH_2

128 R = CHCHPh

Scheme 23

The allyl group has been shown to be a useful intermediate for synthesis of backbone modified oligonucleotides since it can easily be transformed into other functionalities. Cleavage of the exocyclic double bond by successive treatment with OsO_4 and $NaIO_4$ afforded the aldehyde derivatives **131** and **132** in good yields [61,63,64,65]. Subsequent oxidation using PDC [65,66] or $NaClO_2$ in the presence of KH_2PO_4 and 2 methyl-2-butene in aqueous *t*-buthanol [67,68] give the acid derivatives. Reduction with $NaBH_4$ afforded the corresponding alcohol derivatives [63,64] which has been further converted into the corresponding amines [69].

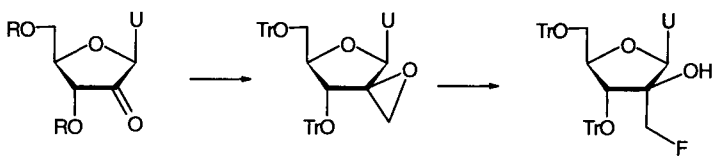


Scheme 24

Nucleophilic ring-opening of epoxides is another way of functionalisation. Reaction of di-*O*-3',5'-protected-2'-ketouridines **133** with dimethylsulfoxonium methylide afforded 2'-spiroepoxy uridine derivatives **134** [70,71] as single diastereomers. Subsequent cleavage of **134a** by KFHF gave

1-(3,5-di-*O*-trityl-2-*C*-fluoromethyl-β-D-*arabino*-pentofuranosyl)uracil (**135**) in 42% yield. The *tert*-hydroxyl group at the 2'-position was stereoselectively removed by radical deoxygenation of 3',5'-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl) protected 2'-methoxalyl derivative **137** using Bu_3SnH and AIBN in toluene to give *arabino*-nucleoside **138** in 86% yield [70]. 1-(2-*C*-Acetoxymethyl-3,5-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-β-D-*arabino*-pentofuranosyl)uracil (**139**) was obtained using a similar reaction sequence [71] (Scheme 25).

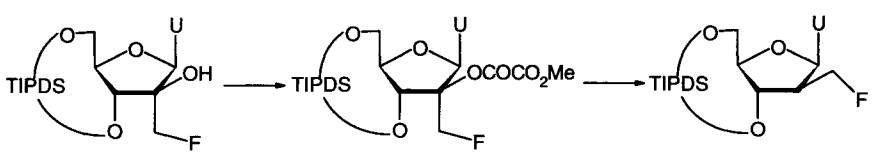
Treatment of 1-(5-*O*-trityl-2,3-anhydro-β-D-*lyxo*-pentofuranosyl)uracil (**140**), which is readily available from uridine [72], with lithium acetylide and ethylenediamine in DMSO afforded 1-(3-deoxy-3-*C*-ethynyl-5-*O*-trityl-β-D-*arabino*-pentofuranosyl)uracil (**141**) in 66% yield [72,73]. Treatment of the epoxide with 4,5-dihydro-2-lithio-5-methyl-1,3,5-dithiazine in THF/HMPA afforded the intermediate **142** in 65% yield. Replacement of the 5'-*O*-trityl group with a *tert*-butyldimethylsilyl group followed by hydrolysis with $HgO/HgCl_2$ in THF gave 1-(5-*O*-(*tert*-butyldimethylsilyl)-3-deoxy-3-*C*-formyl-β-D-*arabino*-pentofuranosyl)uracil (**144**) in 85% yield. The 3'-*C*-formyl group was further reduced with sodium borohydride to give 1-(3-deoxy-3-*C*-hydroxymethyl-β-D-*arabino*-pentofuranosyl)uracil (**146**) in 62% yield after desilylation [74] (Scheme 26).



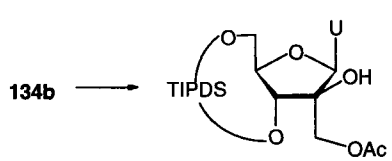
133 **134** **135**

a R = Tr

b R = TIPDS



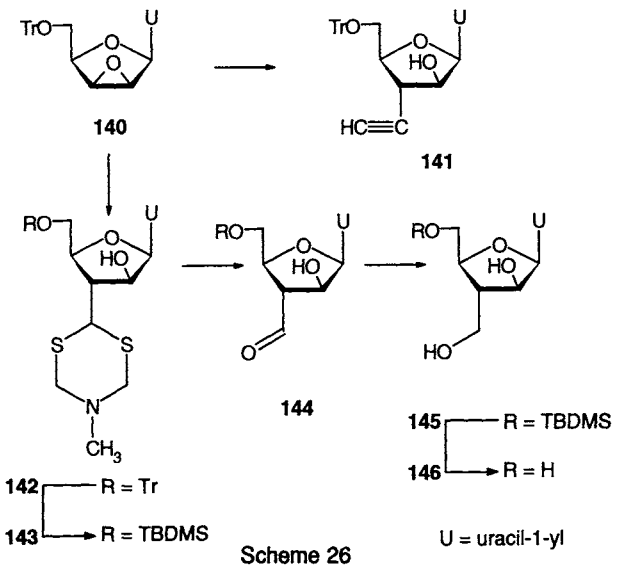
136 **137** **138**



U = uracil-1-yl

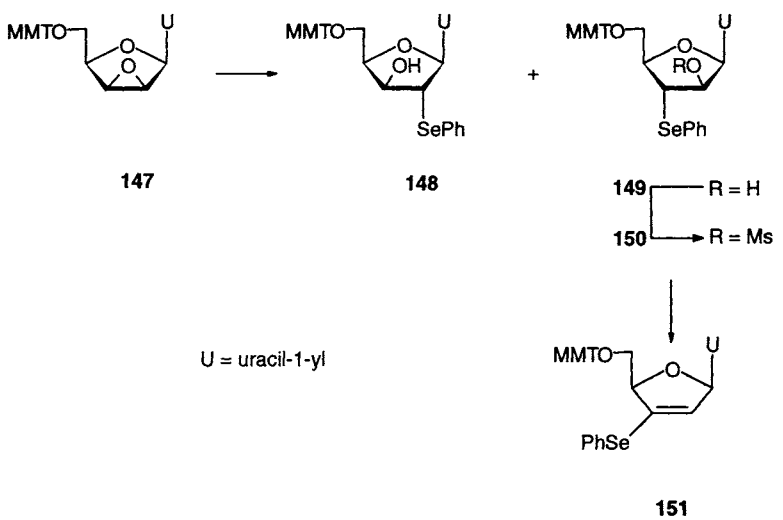
139

Scheme 25



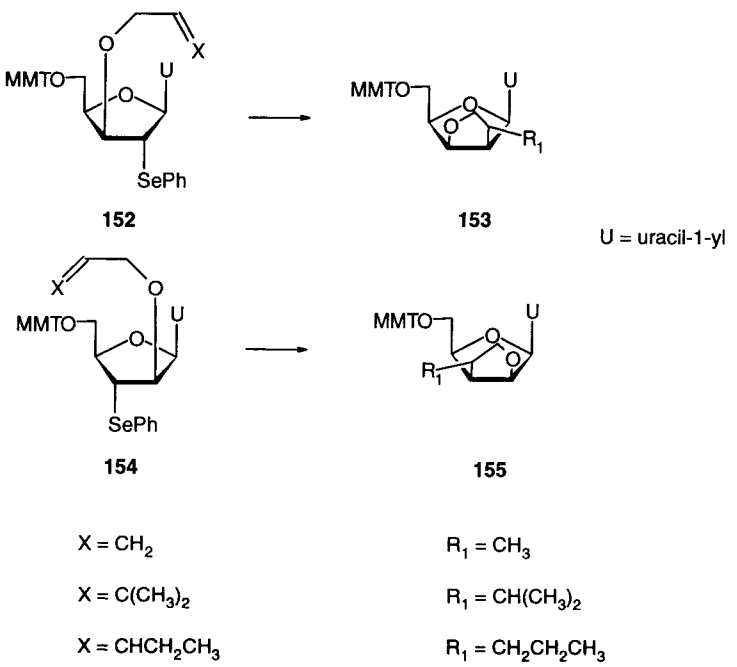
Scheme 26

Nucleophilic attack of the $PhSe^-$ ion, generated from treatment of $(PhSe)_2$ with $LiAlH_4$ in THF, on 1-(2,3-*O*-anhydro-5-*O*-(monomethoxytrityl)-β-D-*lyxo*-pentofuranosyl)uridine (**147**) gave a mixture of 2-*C*-phenylseleno and 3-*C*-phenylseleno nucleosides **148** and **149** in 26% and 55% yield, respectively [42,75,76]. Treatment of 1-(5-*O*-(monomethoxytrityl)-3-deoxy-3-*C*-phenylseleno-β-D-*arabino*-pentofuranosyl)uracil (**149**) with methylsulfonyl chloride in pyridine gave 2'-*O*-mesylate in 89% yield which upon treatment with potassium *tert*-butoxide in DMF afforded 2',3'-didehydro-3'-*C*-phenylselenouridine derivative **151** in 92% yield (Scheme 27).



Scheme 27

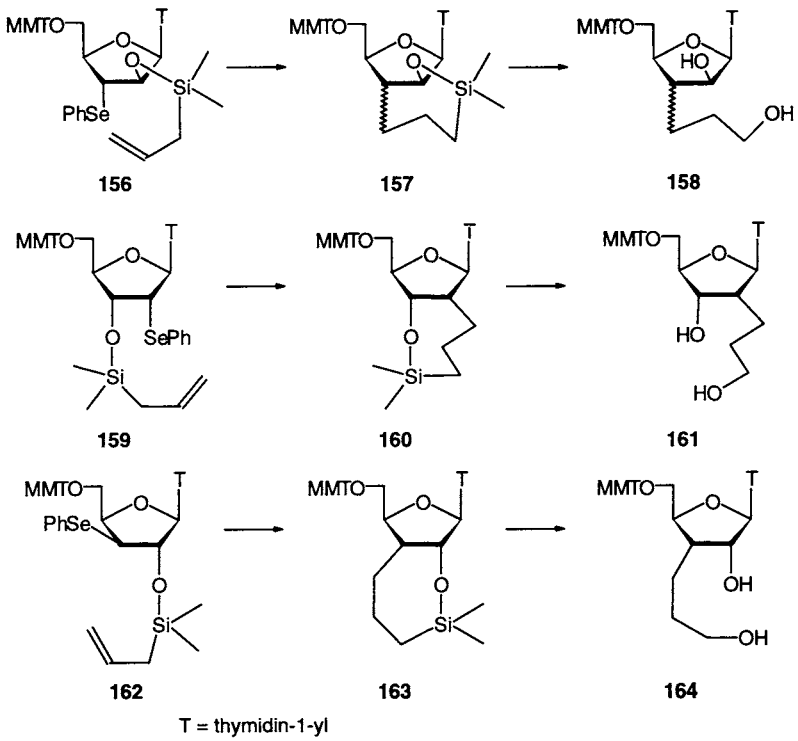
The phenylseleno group has been shown to be good free radical precursor for stereocontrolled intramolecular free-radical cyclisation. Alkylation of 1-(5-*O*-(monomethoxytrityl)-2-deoxy-2-*C*-phenylseleno-β-D-*xylo*-pentofuranosyl)uracil and 1-(5-*O*-(monomethoxytrityl)-3-deoxy-3-*C*-phenylseleno-β-D-*arabino*-pentofuranosyl)uracil with allyl bromide, 4-bromo-2-methyl-2-butene and 1-bromo-2-pentene in THF in the presence of NaH gave the corresponding 2'-*C*-phenylseleno-3'-*O*-alkyl ethers **152** and 3'-*C*-phenylseleno-2'-*O*-alkyl ethers **154** in 70-80% yields [75]. Free radical cyclisation was achieved by treatment with Bu_3SnH in the presence of AIBN in boiling benzene. Trapping of the free-radical generated at the 2'- or at the 3'-carbon by the double bond of the vicinal allylic ethers proceeded diastereoselectively to give single diastereomers of bicyclic nucleosides **153/155** in 72-95% yield (Scheme 28).



Scheme 28

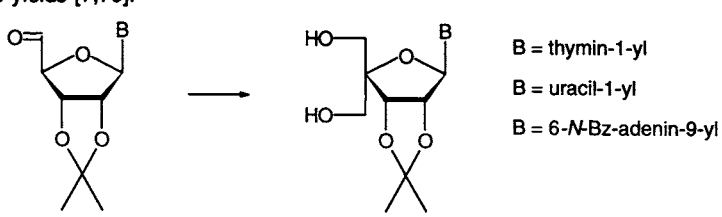
Xi *et al.* have described the intramolecular free-radical cyclisation reaction using silicon-tethered allyl [76] and acetylene [77]. It was shown that the configuration at the 2'- or the 3'-center of the silicon-tethered group dictates the stereochemistry of the radical cyclisation. Free-radical cyclisation of 2'-*O*-allyldimethylsilyl derivative **156** gave both the *cis*-fused and the *trans*-fused bicyclo derivatives **157** in a 1:1 ratio, whereas the silicon-tethered allyl groups in compounds with *ribo*- or *xylo*-configuration (**159** and **162**) exclusively afforded *cis*-fused bicyclo-derivatives **160**

and **163**. The Si-C bond of the siloxane ring was oxidatively cleavage by Tamao oxidation to give the corresponding 1,5-diols **158**, **161** and **164** in 70-89% yield (Scheme 29).



Scheme 29

Reports on 4'-*C*-branched nucleosides have been rather few. However, 4'-*C*-hydroxymethyl nucleosides (*e.g.* **166**) have been synthesised *via* crossed aldol condensation of 5'-aldehyde nucleosides **165** with formaldehyde in the presence of aqueous sodium hydroxide, followed by *in situ* Cannizzaro reduction [8,78] (Scheme 30). The rate-limiting step is the Cannizzaro reduction, and incorporation of a sodium borohydride reduction tends to accelerate the reduction and improve the yields [7,79].



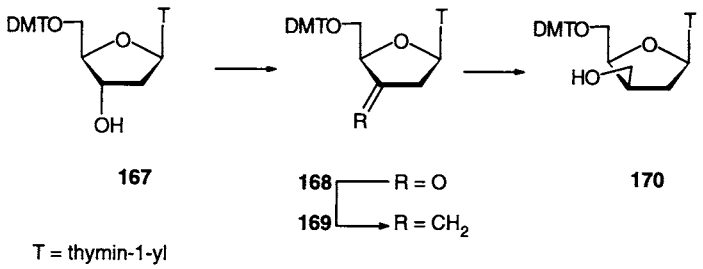
Scheme 30

4. Contributions from our group

In our group we have especially been interested in stereoselective synthesis of *C*-hydroxymethyl substituted nucleosides, following a linear strategy, and incorporation of these as monomeric substitutes into oligodeoxynucleotides (ODNs). The *C*-hydroxymethyl group is expected to be oriented either towards the major groove or the minor groove of duplexes and thus provide attachment sites for additional functionalities that might assist in facilitating transport, cell targeting, membrane permeability or enhancing the affinity towards target nucleic acids. The additional hydroxyl group also allows incorporation of the modified nucleoside into ODNs in unnatural ways. The modified oligonucleotides have been studied with respect to their hybridization properties (evaluated by the melting temperature of a duplex formed with unmodified complementary DNA or RNA strands) and their enzymatic stability towards 3'-exonucleolytic degradation. The first *C*-hydroxymethyl monomer synthesised in the group was *threo*-configurated 3'-deoxy-3'-*C*-hydroxymethyl nucleoside **170** [80] which was synthesised in three steps from 5'-*O*-(4,4'-dimethoxytrityl)thymidine (**167**). Oxidation with PDC in CH_2Cl_2 in the presence of 3Å molecular sieve powder afforded 3'-ketothymidine **168** in 81% yield. Subsequent methylenation using the electrophilic reagent $Zn/CH_2Br_2/TiCl_4/THF$ gave 3'-deoxy-3'-*C*-methylene-thymidine derivative **169** in 79% yield. Stereoselective hydroboration with borane:1,4-oxathiane followed by *in situ* oxidation with alkaline hydrogen peroxide afforded 1-(2,3-dideoxy-3-*C*-hydroxymethyl-5-*O*-(4,4'-dimethoxytrityl)-β-D-*threo*-pentofuranosyl)thymine (**170**) in 79% yield (Scheme 31). The hydroboration follows a *syn*-addition mechanism with attack of BH_3 from the less sterically hindered α-face of the pentose ring to give the desired β-D-*threo*-configurated nucleoside. Reaction of **170** with tetrazole-activated 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite afforded the phosphoramidite **171** in 87% yield which was used in automated ODN synthesis (*vide infra*).

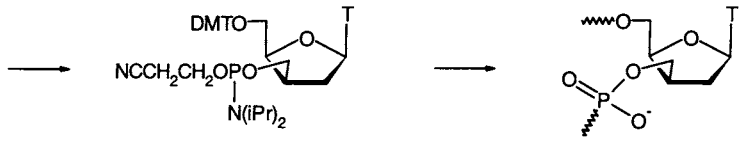
Derivatisation [81] of 1-(2,3-dideoxy-3-*C*-hydroxymethyl-5-*O*-trityl-β-D-*threo*-pentofuranosyl)-thymine (**172**) as the triflate derivative **173** was achieved in 63% yield by esterification with trifluoromethanesulfonic anhydride. The 3'-*C*-chloromethyl- and 3'-*C*-iodomethyl 2',3'-dideoxynucleosides **174** and **175** were obtained from **172** by reaction with carbon tetrachloride/triphenylphosphine and methyltriphenoxyphosphonium iodide, respectively. Arbuzow reaction of compound **175** by treatment with triethyl phosphite afforded phosphonate **176** in 63% yield. Detritylation of compounds **174** and **175** with 80% acetic acid at 100 °C afforded 1-(3-*C*-chloromethyl-2,3-dideoxy-β-D-*threo*-pentofuranosyl)thymine (**177**) in 79% yield and 1-(3-*C*-iodomethyl-2,3-dideoxy-β-D-*threo*-pentofuranosyl)thymine (**178**) in 60% yield. Additionally, the bicyclic compound **179** was isolated in 12 and 26 % yield during deprotection of **174** and **175**, respectively. Detritylation of the triflate **173** gave exclusively the bicyclic compound **179** in quantitative yield because of intramolecular nucleophilic attack of the liberated 5'-OH on the

activated 3'-*C*-hydroxymethyl functionality (Scheme 32).



167 **168** R = O **169** R = CH_2 **170**

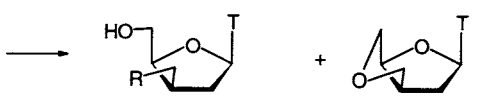
T = thymin-1-yl



171

Scheme 31

172 **173** R = TfO **174** R = Cl **175** R = I **176** R = P(O)(OEt)$_2$



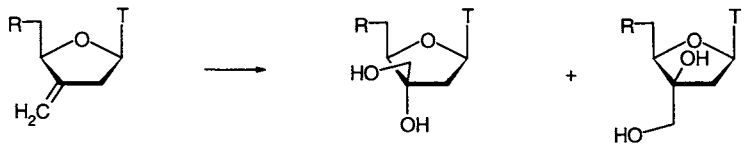
177 R = Cl **178** R = I **179** T = thymin-1-yl

Scheme 32

Diastereoselective dihydroxylation of 3'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-3'-*C*-methylenethymidine (**180a**) with a catalytic amount of osmium tetroxide in basic aqueous *t*-butanol using *N*-methylmorpholine-*N*-oxide as co-oxidant gave exclusively 5'-*O*-(4,4'-dimethoxytrityl)-3'-*C*-hydroxymethylthymidine (**181a**) in 70% yield [82-84]. The stereoselectivity is controlled by the bulky 5'-*O*-

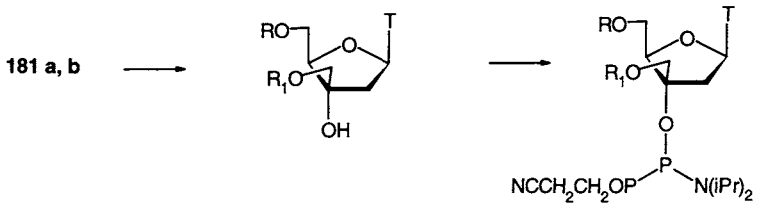
(4,4'-dimethoxytrityl) group which prevents addition of osmium tetraoxide from the β-face of the pentose ring. The same diastereoselectivity was observed with the 5'-*O*-(*t*-butyldimethyl) protecting group [85]. Dihydroxylation of 3',5'-dideoxy-3'-*C*-methylenethymidine (**180c**) under the same conditions gave a 1:1 mixture of 3'-*C*-hydroxymethyl diasteroisomers **181c** and **182c** [85]. Sharpless asymmetric dihydroxylation of 5'-deoxynucleoside **180c** proceeded stereoselectively to give a 10:3 mixture of the *erythro*-isomer **181c** and the *threo*-isomer **182c** in only 13% combined yield (Scheme 33). The additional primary hydroxyl functionality of 3'-*C*-hydroxymethyl-thymidines enables their incorporation into ODNs in different ways. Reaction of **181a** with *t*-butyldimethylsilyl chloride using imidazole as catalyst afforded 3'-*C*-(*tert*-butyldimethylsilyl)oxy-methyl-5'-*O*-(4,4'-dimethoxytrityl)thymidine (**183a**) in 81% yield which was phosphitylated to give the nucleoside phosphoramidite **184a** in 90% yield. Incorporation of **184a** afforded oligonucleotides containing the natural 5'-hydroxyl to 3'-hydroxyl phosphodiester backbone and a 3'-*C*-hydroxymethyl substituent [82,83]. Regioselective phosphitylation of **181a** afforded the primary phosphoramidite **185** in 74% yield, which was successfully applied on a DNA-synthesiser without protection of the tertiary hydroxyl group to give ODNs with an extended backbone (5'-hydroxyl to 3'-*C*-hydroxymethyl) [83]. Finally, ODNs containing a compressed phosphodiester backbone (3'-*C*-hydroxymethyl to 3'-hydroxyl) was synthesised using the phosphoramidite **184b** [85].

Oligonucleotides were synthesised by standard phosphoramidite methodology on an automated solid phase DNA-synthesiser using the modified phosphoramidites and commercial 2'-deoxynucleoside-β-cyanoethylphosphoramidites [82-85]. Incorporation of 3'-deoxy-3'-*C*-*threo*-hydroxymethylthymidine (from amidite **171**) moderately weakened the duplex stability compared to modified controls, but significantly stabilised 3'-end modified ODNs towards 3'-exonucleolytic degradation. As expected, incorporation of the modified monomer (from amidite **184b**) containing compressed 3'-*C*-hydroxymethyl to 3'-hydroxyl backbone significantly destabilised the duplex, whereas backbone extension (from amidite **185**) had a less detrimental effect. The most promising modification was 3'-*C*-hydroxymethylthymidine incorporated into oligonucleotides through 5'-hydroxyl to 3'-hydroxyl (using amidite **184a**) to give naturally linked ODN analogues. These ODNs exhibited no (middle modification) or only minor (3'-end modification) destabilisation of the duplex combined with increased stability towards 3'-exonucleolytic degradation.



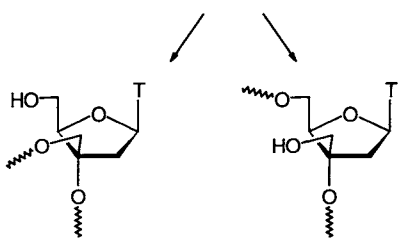
180 181 182

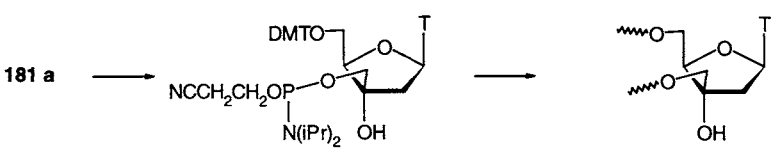
180 - 82	a	b	c
R	DMT	TBDMS	H



183 184

183 - 84	a	b
R	DMT	TBDMS
R_1	TBDMS	DMT



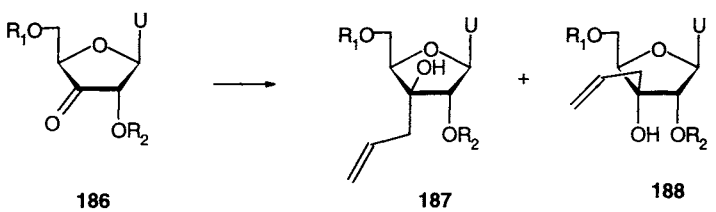


185

T = thymin-1-yl

Scheme 33

3'-*C*-Allyluridines were synthesised using cerium-assisted Grignard additions of allylmagnesium bromide to five differently protected 3'-ketouridines **186** [86]. The stereoselectivities of the reactions were controlled by the protecting group in the 5'-position. Grignard addition of the allylmagnesium bromide/$CeCl_3$ reagent to 2',5'-di-*O*-(*t*-butyldimethylsilyl)-3'-ketouridine (**186a**), as well as 2'-*O*-(*t*-butyldimethylsilyl)- and 2'-*O*-(*t*-butyldiphenylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)-3'-ketouridine, afforded exclusively the *xylo*-nucleoside derivatives **187** in moderate to high yields (52-80%). When the addition was performed on 5'-*O*-deprotected 2'-*O*-(*t*-butyldimethylsilyl)- and 2'-*O*-(*t*-butyldiphenylsilyl)-3'-ketonucleosides **186d/186e**, a mixture of *xylo*- and *ribo*-nucleosides were obtained with the *xylo*-isomers as the major products showing that the stereochemistry of the addition could not be fully controlled by the choice of 2'-*O*-protecting group. Nucleoside **187a** was stereoselectively converted into *xylo*-configurated 3'-spiro-γ-lactone nucleoside **190** by hydroboration to **189** and subsequent oxidation with PCC (Scheme 34).



186 **187** **188**

a: $R_1 = R_2$ = TBDMS 78%

b: R_1 = DMT, R_2 = TBDMS 52%

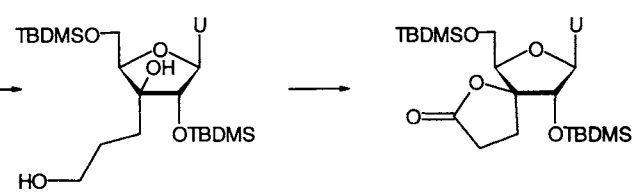
c: R_1 = DMT, R_2 = TBDPS 80%

d: R_1 = H, R_2 = TBDMS 27% 18 %

e: R_1 = H, R_2 = TBDPS 25% 15 %

U = uracil-1-yl

187 a

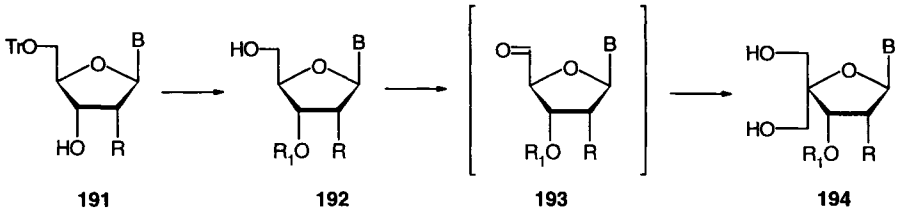


189 **190**

Scheme 34

Synthesis of 3'-*O*-ethyl-4'-*C*-hydroxymethylthymidine (**194a**) was achieved in six steps from 5'-*O*-tritylthymidine (**191a**) [87]. Reaction of **191a** with ethyl iodide and potassium hydroxide in benzene/dioxane afforded 3'-*O*-ethylthymidine (**192a**) in 56% yield after detritylation with 80% acetic acid. Pfitzner-Moffatt oxidation using DMSO and DCC in the presence of TFA afforded 5'-aldehyde **193a** which was isolated as its stable 1,3-diphenylimidazolidine derivative in 62% yield. The 5'-aldehyde was regenerated with *p*-toluenesulfonic acid and immediately subjected to aldol condensation and subsequent Cannizzaro reduction with formaldehyde in the presence of sodium hydroxide to give 3'-*O*-ethyl-4'-*C*-hydroxymethylthymidine (**194a**) in 60% yield. **194a** was converted into the phosphoramidite building block **198a** after temporary benzoylation of the more reactive 4'-*C*-hydroxymethyl group with benzoyl chloride in pyridine, 4,4'-dimethoxytritylation of the 5'-*O*-hydroxyl group, followed by debenzoylation with methanolic ammonia (Scheme 35). 3'-*O*-(*t*-Butyldimethylsilyl)-4'-*C*-hydroxymethylthymidine analogue **194b** [88] was synthesised and incorporated into ODNs in a similar way as **194a** using phosphoramidite building block **198b** [88,89]. The 3'-*O*-(*t*-butyldimethylsilyl) group could be removed after oligonucleotide synthesis to give a free 3'-hydroxyl group. Synthesis of the unstable aldehyde **193b** was optimised using Swern oxidation (oxalyl chloride/DMSO/diisopropylethyl amine) which generally proceeded in higher yield compared to Pfitzner-Moffatt oxidation. The crude aldehyde was used immediately in the crossed aldol condensation with formaldehyde which was followed by sodium borohydride reduction. For incorporation of 4'-*C*-hydroxymethylthymidine into oligonucleotides with natural 5'-hydroxyl to 3'-hydroxyl backbone [88,89], 3'-*O*-(*t*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-hydroxymethylthymidine (**197b**) was desilylated to give 5'-*O*-(4,4'-dimethoxytrityl)-4'-*C*-hydroxymethylthymidine (**199b**) which was regioselectively monosilylated with *t*-butyldimethylsilyl chloride to give 4'-*O*-(*t*-butyldimethylsilyl)oxymethyl derivative **200b**. The desired phosphoramidite **201b** was obtained by reaction with 2-cyanoethyl-*N,N'*-diisopropylphosphoramidochloridite and *N,N*-diisopropylethylamine. 4'-*C*-Hydroxymethyl-uridine derivatives **194c**, **198c** and **201c** [90] were synthesised following the same methodology as described above, taking the additional protection of the 2'-*O*-position with *t*-butyldimethylsilyl into account (Scheme 35).

Incorporation of the phosphoramidites **198a**, **198b** and **198c** one or two times into ODNs to give analogues containing unnatural 5'-hydroxyl to 4'-*C*-hydroxymethyl linked backbones, resulted in significant decreases in the hybridisation properties towards complementary DNA when compared to unmodified controls. Incorporation of the phosphoramidite building blocks **201b** and **201c** to afford ODNs containing natural backbones and an additional 4'-*C*-hydroxymethyl functionality had no significant negative effect on the stability of duplexes, especially when RNA was used as target strand [88-90]. Incorporation of 4'-*C*-hydroxymethylthymidine (from amidites **198b** or **201b**) into the 3'-end of ODNs was found to induce considerable resistance towards 3'-exonucleolytic degradation.



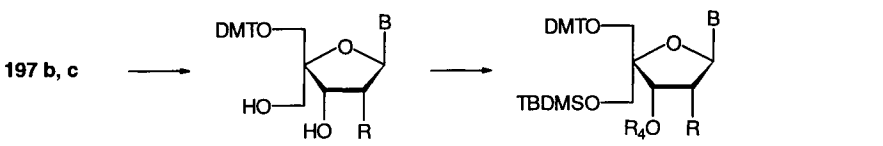
191 **192** **193** **194**

195 $R_2 = H, R_3 = Bz$

196 $R_2 = R_3 = Bz$

197 $R_3 = H$

198 $R_3 = P(OCH_2CH_2CN)N(iPr)_2$



199 b, c

200 $R_4 = H$

201 $R_4 = P(OCH_2CH_2CN)N(iPr)_2$

191, 199	a	b	c
R	H	H	OH

192- 198	a	b	c
R	H	H	TBDMS
R_1	Et	TBDMS	TBDMS

191- 201	a	b	c
B	thymin-1-yl	thymin-1-yl	uracil-1-yl

Scheme 35

5. Conclusion

From this summary it is evident that synthesis of *C*-branched pentofuranose nucleosides has attracted considerable attention in the last years. A number of short and elegant synthetic routes to a variety of derivatives have been developed. Unfortunately, the results from especially antiviral testings have generally been disappointing. However, we believe that further development and evaluation of *C*-branched nucleoside analogues as monomeric substitutes in functionalised antisense ODNs should be undertaken in light of the many synthetic routes available and the promising results described above.

6. References

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Bioactive Marine Macrolides

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1. INTRODUCTION

The macrolides are macrocyclic lactones. Since a macrocycle is usually defined as a compound having a ring constituted with twelve or more atoms, our discussion in this chapter is focused on those marine compounds containing a 12- or higher-membered lactonic ring. The first marine macrolides were the aplysiatoxins reported in 1974 by Kato and Scheuer as toxic constituents of the sea hare *Stylocheilus longicauda*. This discovery was soon followed by the report of another class of macrolides, aplasmomycins, the boron-containing microbial metabolites. These compounds have drawn much attention because of their unusual structures and significant biological properties.

More than 200 marine macrolides have been recorded in the two decades since the first report of the aplysiatoxins. In this review we have aimed to present all known marine macrolides with emphasis on their structural features, sources, and biological activities. We were able to compile 205 compounds by covering publications up to the end of July, 1996. These compounds have been classified into five main structural groups: prostaglandin lactones and related compounds, polyol macrolides, polyether macrolides, macrodiolides, and alkaloidal macrolides. This classification is only for the convenience of our discussion here but does not define compounds to specific categories. All nitrogen-containing macrolides are treated as alkaloidal macrolides. Within each group the macrolides are further classified according to their structural relationship and are presented in order of structural complexity.

Table 1 summarizes the sources and number of macrolides isolated from them. As is the case with other marine natural products, sponges are the most prolific sources of diverse macrolides. On the other hand, macroalgae, tunicates, and coelenterates, which are the major sources of other bioactive compounds, have so far yielded relatively small numbers of the macrolides. All the macrolides isolated from mollusks are presumably related to the metabolites of their prey organisms.

The majority of marine macrolides exhibit pronounced biological activity such as cytotoxic/antitumor, antiviral, antifungal, or immunomodulatory activity. Some of them are exceedingly potent

cytotoxins or *in vivo* active antitumor compounds which are now under extensive investigation as cancer chemotherapeutic agents. For example, bryostatin 1 is now in phase II clinical trials and halichondrin B is at the preclinical stage as anticancer drugs. Some of the macrolides, e.g. latrunculins and swinholides, are also used as important tools in biochemical research. However, in most cases they are extremely minor constituents, and supply of the samples is a limiting factor for their further evaluation and development. Chemical synthesis may be a solution, but most of them are highly complex molecules with unassigned stereochemistries. These situations offer great challenge to synthetic chemists. An excellent recent review by Norcross and Paterson (93) describes all synthetic works on major bioactive marine macrolides covering the literature up to the close of 1994.

Table 1. Source organisms and number of marine macrolides

Organisms	Number of macrolides	Representative compounds
Bacteria	11	Macrolactins, aplasmomycins
Cyanobacteria	11	Aplysiatoxins
Dinoflagellates	22	Amphidinolides, zooxanthellatoxins
Macroalgae	12	Polycavernosides, ecklonialactones
Sponges	78	Spongistatins, halichondrins, halichondramides, swinholides
Bryozoans	21	Bryostatins
Tunicates	14	Iejimalides, aplidites
Coelenterates	6	Lituarines
Mollusks	38	Pectenotoxins, kabiramides

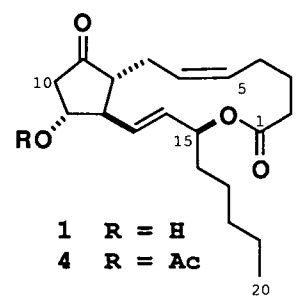
2. PROSTAGLANDIN LACTONES AND RELATED COMPOUNDS

Prostaglandin 1,15-lactones and related compounds are the simplest members of more than 200 marine macrolides known to date. A total of 13 macrocyclic lactones of this class have been reported from a nudibranch and several species of algae. They are 13- and 16-membered macrolides derived from C_{20} fatty acids and 14-membered macrolides derived from C_{18} fatty acids.

2.1 Prostaglandin 1,15-lactones

Since the first discovery of prostaglandins from a gorgonian coral by Weinheimer and Spraggins (1) in 1969, a number of prostaglandins and related compounds have been reported from marine invertebrates and algae (2). Some of them have structures identical with the mammalian prostaglandins, while many others have different structures. For example, punaglandins (2) contain a chlorine atom at C_{10} and some clavulones (3) possess a bromine or iodine atom at the same position of the prostaglandin skeleton.

Simple 13-membered lactonic derivatives of prostaglandins were first reported by Cimino *et al.* (4) in 1989. They were PGE_2-1,15-lactone (**1**), PGE_3-1,15-lactone (**2**), and PGE_3-1,15-lactone 11-acetate (**3**) isolated from the nudibranch *Tethys fimbria*.



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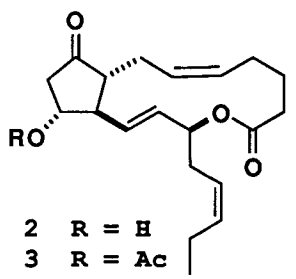
4 R = Ac



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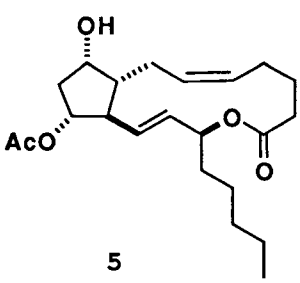
3 R = Ac



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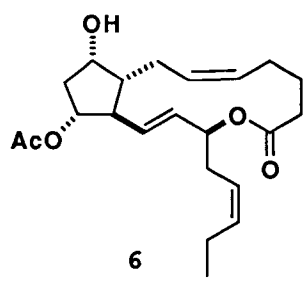

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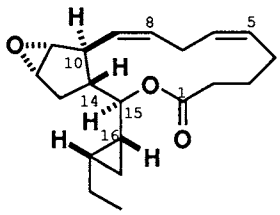
The structure and absolute configuration of PGE_2-1,15-lactone (**1**) was confirmed by comparison with a synthetic sample which had earlier been reported (5). The structures of **2** and **3** were established by spectroscopic analysis and comparison with **1**. The authors (6) subsequently isolated from the same mollusk and its egg masses three additional compounds (**4**-**6**), two of which belonged to the lactones of PGF series, $PGF_{2\alpha}$-1,15-lactone 11-acetate (**5**) and $PGF_{3\alpha}$-1,15-lactone 11-acetate (**6**). The biological role of these

lactones in the mollusk has been suggested to be the storage of prostaglandins. The Italian workers (7) have demonstrated that the lactones were biosynthesized from free prostaglandins in the mantle of *T. fimbria* and converted back to the prostaglandins upon detachment of cerata during the behavioral defense mechanism. The prostaglandins were also suggested to have a role in the reproduction of the mollusk (6).

2.2 Hybridalactone

Hybridalactone (**7**) is a 13-membered lactone based on a C_{20} fatty acid skeleton. It is therefore closely related to the structures of prostaglandin 1,15-lactones except for the positions of cyclization of both the five-membered ring and the lactone. Higgs and Mulheirn (8) first isolated the lactone from the red alga *Laurencia hybrida* and assigned the structure and partial stereochemistry on the basis of spectroscopic data and results of chemical degradation.



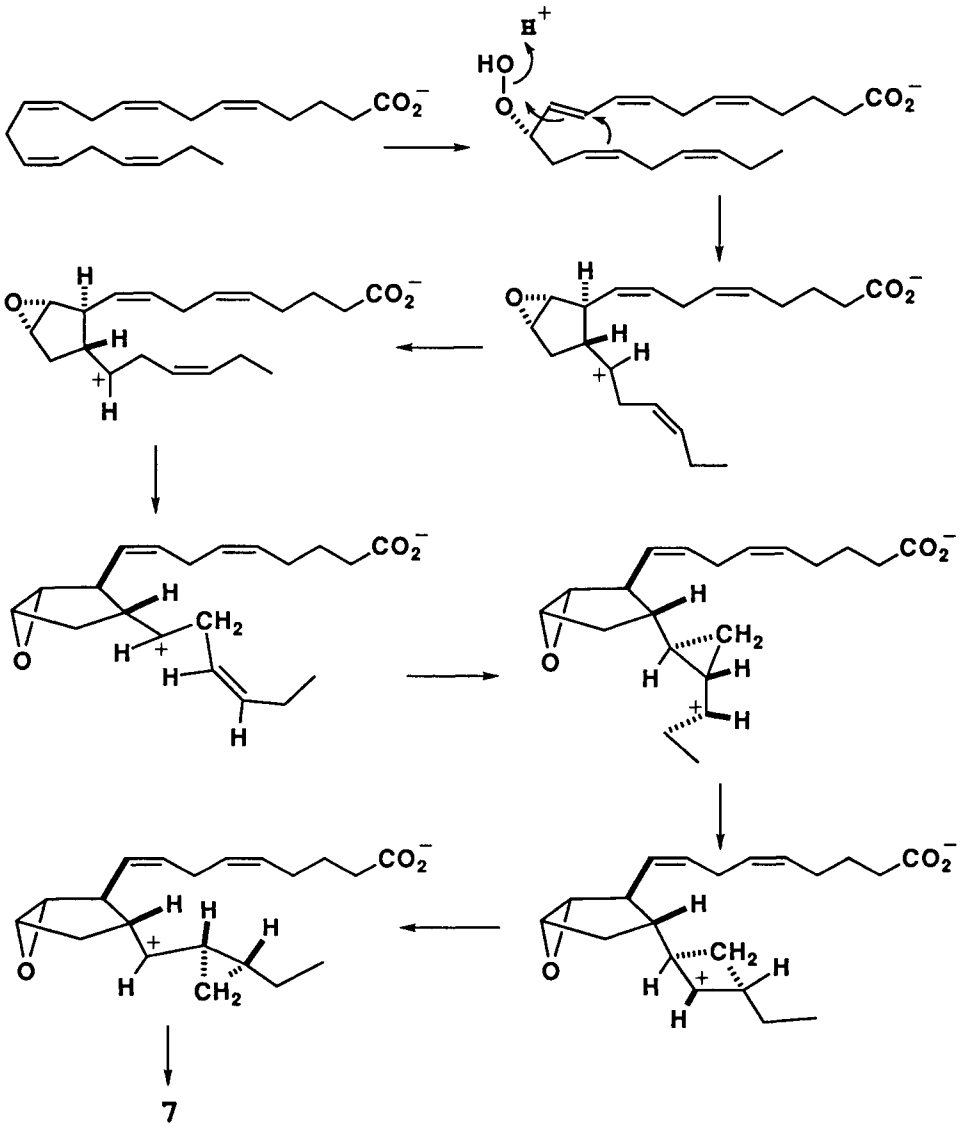
7

Corey *et al.* (9) reisolated hybridalactone from the same alga and established the full stereochemistry and absolute configuration as shown in **7** by X-ray crystallography on a bromohydrin derivative. The stereochemistry was in accord with that predicted by biosynthetic considerations (Scheme 1) and with molecular mechanics calculations. Based on their predicted stereostructure, Corey and De (10) initiated and completed the synthesis prior to their elucidation of the correct stereochemistry with a natural sample of hybridalactone. The biogenesis of **7** from eicosapentaenoic acid as proposed by Corey is shown in Scheme 1.

2.3 Ecklonialactones

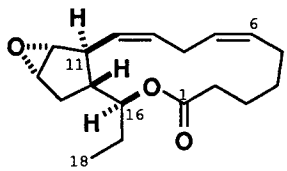
A group of macrolides having a closely related structure to hybridalactone are known as ecklonialactones (**8-13**) derived from brown algae. They are 14- and 16-membered lactones derived from C_{18}

Scheme 1. Corey's proposed biogenesis of hybridalactone (**7**).



and C_{20} fatty acids, respectively. The 14-membered macrolides ecklonialactone A (**8**) and B (**9**) were first reported by Kurata (11) from the brown alga *Ecklonia stolonifera* collected in Japan. The structure and relative stereochemistry of **8** were secured by single crystal X-ray analysis. The structure of **9** was confirmed by spectroscopic comparison and chemical correlation with **8**. Kurata's group subsequently reported the isolation from the same alga four

additional compounds, ecklonialactones C-F (**10-13**) of which C (**10**) and D (**11**) were hydration product of the 14-membered lactones **8** and **9**, respectively, while E (**12**) and F (**13**) were 16-membered lactones derived from a C_{20} fatty acid (12).



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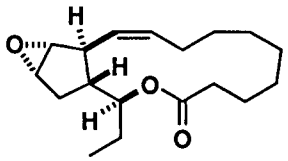

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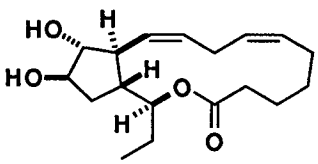

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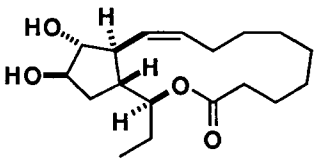

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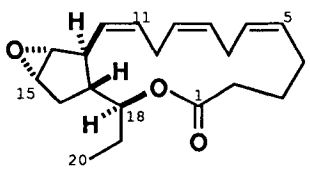

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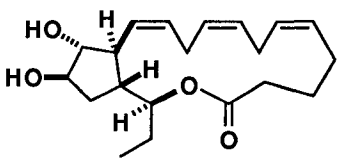

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On the other hand, Gerwick *et al.* (13) isolated ecklonialactones A (**8**), B (**9**), and E (**13**) from the brown alga *Egregia menziessi* collected at an Oregon coast. The Oregon group (14) established the absolute stereochemistry of these macrolides by CD analysis (15) of a dibenzoate derivative prepared by treating **10**, which was derived from **8**, with *p*-bromobenzoyl chloride. Gerwick (14) proposed a similar biogenetic scheme for ecklonialactones with that (Scheme 1) for hybridalactone.

According to Kurata *et al.*, ecklonialactones A (**8**) and B (**9**) showed weak feeding-deterrent activity against abalone, while C-F (**10-13**) showed no such activity. It was also reported that **8** and **9** exhibited weak cytotoxicity against B16 murine melanoma but **10** and **11** did not.

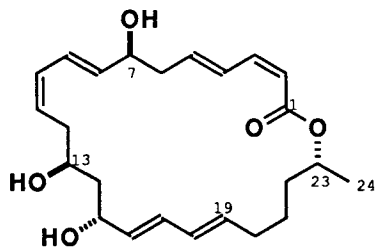
3. POLYOL MACROLIDES

Polyol macrolides are usually macrocyclic lactones having many hydroxyl groups. They may be exemplified by axenomycin and monazomycin, which are known as macrolide antibiotics (16). For the convenience of our classification, here we group together those macrolides having at least two hydroxyl groups and those having relatively simple ring structures. A few exceptions are amphidinolides O (**28**), P (**29**), and Q (**26**) which have only one hydroxyl group. These compounds will be discussed here along with other amphidinolides. Some of the compounds included in this section may best to be called as polyene macrolides in the conventional classification. The majority (28 compounds) out of a total of 33 compounds included in this section have been discovered from cultured organisms, *i.e.*, dinoflagellates, bacteria, and a cyanobacterium. Only a few have been reported from sponges and a sea hare.

3.1 Macrolactins

Macrolactins (**14-19**) are metabolites isolated by Fenical and his coworkers from the culture broth of an unclassifiable deep sea bacterium (17). They are 24-membered ring macrolides based on a C_{24} linear acetogenin. Two open-chain C_{24} acid congeners have also been isolated. Macrolactins A (**14**), E (**18**), and F (**19**) were isolated as free aglycones, while macrolactins B (**15**), C (**16**), and D (**17**) were isolated as glycosides having a unit of glucose on the lactone **14**.

Macrolactins B (**15**) and C (**16**) are positional isomers of β-glucose. Macrolactin D (**17**) contains an additional substituent of succinic acid as a half-ester at $C_{6'}$ of glucose. The structures of the macrolides were easily elucidated by NMR analysis as they contained 23 contiguous protonated carbons. The stereochemistry of these compounds was determined by a combination of ^{13}C-acetonide analysis, degradation, and chemical correlation using samples of macrolactins B (**15**) and F (**19**) (18). Determination of the relative configuration of 1,3-diols by ^{13}C NMR chemical shift difference at the methyl groups of the acetonide derivatives has been advanced by Rychnovsky (19) and Evans *et al.* (20). Commercially available 99% ^{13}C-enriched acetone was used to prepare the acetonides of the macrolactins which were available only in small quantities. The absolute configuration was determined by correlation of oxidative degradation products with synthetic samples.



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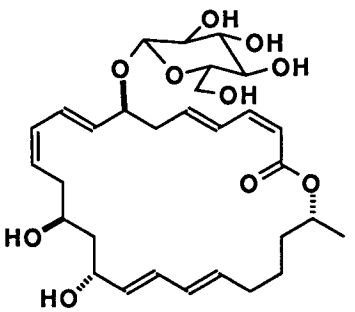

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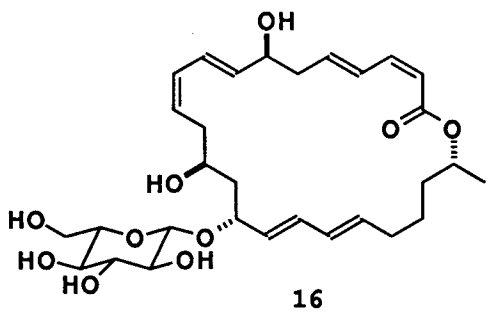

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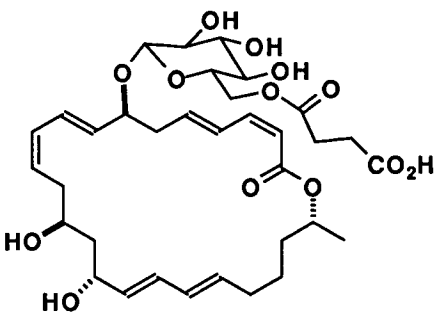

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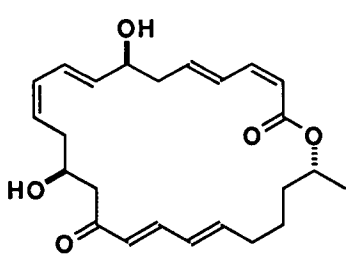

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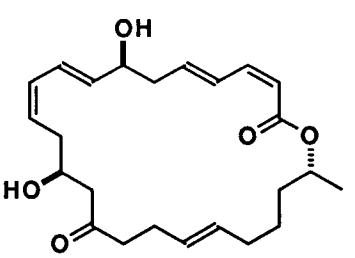


19

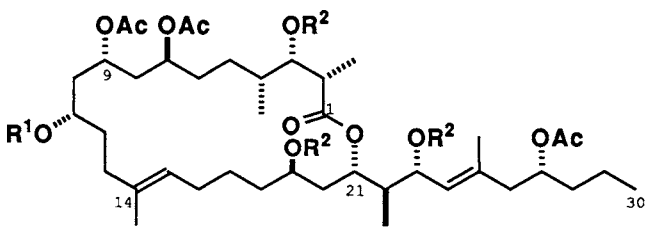
Macrolactin A (**14**) exhibits antibiotic activity against *Bacillus subtilis* and *Staphylococcus aureus* and cytotoxic activity against B16-F10 murine melanoma cell. The lactone **14** also inhibits the replication of herpes simplex virus type I and II with IC_{50} 5.0 and 8.3 μg/mL, respectively, and protects T-lymphoblast cells against HIV replication (17).

Some studies toward total synthesis of macrolactin A have been reported (21-23). No total synthesis has yet been published.

3.2 Dolabelides

Pettit and his workers (24) have extensively studied the antineoplastic constituents of the sea hare *Dolabella auricularia* collected in the Western Indian Ocean and described a number of active compounds known as dolastatins. Dolastatins are unique peptides, cyclopeptides, depsipeptides, and cyclodepsipeptides. Dolastatin 10 is currently under clinical trials as an antitumor agent.

From the same species of sea hare collected in Japan, Yamada and workers (25) recently reported the isolation and structure elucidation of two cytotoxic macrolides, dolabelides A (**20**) and B (**21**). Both compounds are 22-membered ring macrolides based on a 30-carbon chain bearing five methyl branches. The structure of **20** was determined by spectroscopic analysis, relative stereochemistry by chemical degradation and NMR studies on the resulting fragments and derivatives, and absolute configuration by applying the MTPA ester method (26) on the basic methanolysis product of **20**. The structure of dolabelide B (**21**) was secured by spectroscopic and chemical correlation with **20**. Dolabelides A and B exhibited cytotoxicity against HeLa-S3 cells with IC_{50} 6.3 and 1.3 μg/mL, respectively.



20 R^1=Ac, R^2=H

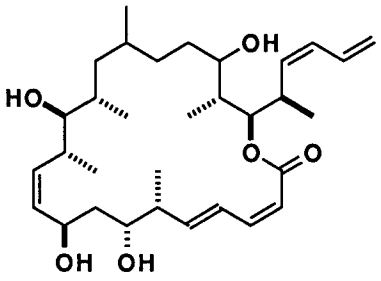
21 R^1=R^2=H

3.3 Dictyostatin 1

Another 22-membered ring macrolide of this class is dictyostatin 1 (**22**) reported recently by Pettit *et al.* (27) from a sponge of the genus *Spongia* collected in the Republic of Maldives. The same specimen also gave rise to the polyether macrolides, spongistatins, *vide infra*. These macrolides including **22** are extremely minor constituents isolated in 10^{-6} to 10^{-8}% yields. Dictyostatin 1 strongly inhibited the growth of the murine P388 lymphocytic leukemia (IC_{50} 3.8×10^{-4} μg/mL).

The structure of **22** was elucidated by NMR analysis and the

stereochemistry remains to be determined. It is composed of a 22-membered lactone on a 26-carbon chain bearing six methyl groups and four hydroxyl groups. It has five C-C double bonds, two of which are conjugated to the lactone carbonyl.



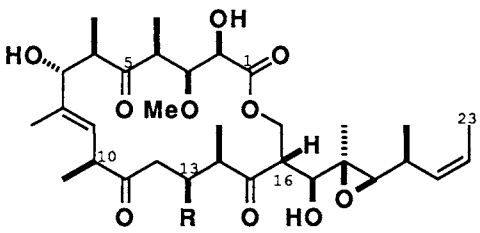
22

3.4 Tedanolides

The Caribbean sponge *Tedania ignis* is known as the fire sponge because it causes a burning sensation on contact with the skin and dermatitis for some individuals. From this sponge Schmitz *et al.* (28) discovered in 1984 a highly cytotoxic constituent, tedanolide (**23**) and determined the structure by X-ray diffraction. Tedanolide (**23**) is an 18-membered ring macrolide incorporated in a 23 carbon chain, bearing eight methyl branches, four hydroxyls, one methoxy, three ketonic carbonyls and one epoxy group. One of the methyl groups is the point of the lactonization, and in this sense it is different from those macrolides described above. The structure of **23** reveals the presence of four labile aldol units. Tedanolide is a product of mixed biogenesis involving acetate (two units C_1-C_2, C_{11}-C_{12}) and propionate (nine units). It exhibited potent cytotoxicity against KB (IC_{50} 2.5×10^{-4} μg/mL) and PS cells (IC_{50} 1.6×10^{-5} μg/mL).

More recently 13-deoxytedanolide (**24**) has been reported by Fusetani *et al.* (29) from the sponge *Mycale adhaerens* collected in Japan. The structure of **24** was elucidated by NMR connectivity studies and the stereochemistry was assumed to be the same with the parent compound **23** by comparison of NMR data. 13-Deoxytedanolide (**24**) showed the same level of *in vitro* cytotoxicity as that of **23**. Furthermore, it also showed potent *in vivo* antitumor activity against P388 leukemia in mice (T/C 189% at a dose of 0.125 mg/kg).

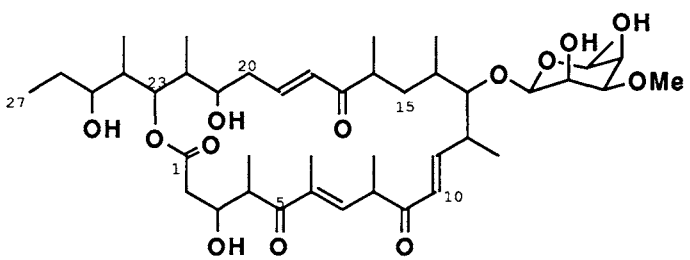
A synthetic study on tedanolide (**23**) has been recently published (30). An efficient macrolactonization of a seco-acid derivative of **23** was achieved by designing the acid through computer-aided conformational analysis.



23 R=OH
24 R=H

3.5 Maduralide

Maduralide (**25**) is a metabolite produced by a cultured marine bacterium (order Actinomycetales). It was isolated as an oil from the ethyl acetate extract of the culture by silica flash chromatography followed by silica HPLC (31). The yield of **25** was 50 mg from a 10 L culture. The structure was determined by spectroscopic analysis. It is a 24-membered ring lactone containing one sugar unit. The sugar was identified to be 6-deoxy-3-O-methyl talose pyranoside. Except for the sugar moiety no stereochemistry has been established. Like tedanolides, maduralide also arises by mixed acetate-propionate biogenesis. Maduralide showed weak antibiotic activity against *Bacillus subtilis*.



25

3.6 Amphidinolides and Related Compounds

The amphidinolides are a series of compounds reported by Kobayashi and coworkers from laboratory cultured dinoflagellates of the genus *Amphidinium*, symbiotic algae isolated from the flatworms *Amphiscolops* spp. collected in Okinawa. They have so far reported 16 macrolides, amphidinolides A-H and J-Q. All of them exhibited cytotoxic activity as shown in Table 2. Some of them are extremely

potent. More recently, Shimizu's group also reported the discovery of some closely related compounds from cultured *Amphidinium* sp., a free-swimming alga collected in the Caribbean.

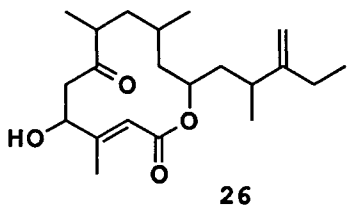
The lactonic ring size of the amphidinolides varies from 12 to 29. The majority of them possess odd-membered macrolides, differing from the macrolide antibiotics which generally have even-numbered lactone ring. Most of the amphidinolides contain, besides the lactone ring, one or two small ether rings as shown in Table 2. In the discussion below these macrolides are divided into several groups according to the ring size and other structural relationships.

Table 2. Molecular formulas, ring sizes, and cytotoxicity of the amphidinolides

Amphidinolide	Molecular formula	Ring size[a]	Cytotoxicity (IC_{50} μg/mL)	
			L1210	KB
A	$C_{31}H_{46}O_7$	20 (3)	2.4	
B	$C_{32}H_{50}O_8$	26 (3)	0.00014	
C	$C_{41}H_{60}O_9$	25 (5,5)	0.0058	
D	$C_{32}H_{50}O_8$	26 (3)	0.019	
E	$C_{30}H_{44}O_6$	19 (5)	2.0	
F	$C_{35}H_{52}O_9$	25 (5,5)	1.5	3.2
G	$C_{32}H_{50}O_8$	27 (3)	0.0054	0.0059
H	$C_{32}H_{50}O_8$	26 (3)	0.00048	0.00052
J	$C_{24}H_{38}O_4$	15	2.7	3.9
K	$C_{27}H_{40}O_5$	19 (3,5)	1.65	2.9
L	$C_{32}H_{50}O_8$	27 (3,6)	0.092	0.1
M	$C_{43}H_{66}O_9$	29 (3,5,5)	1.1	0.44
N	$C_{33}H_{54}O_{12}$	26 (6)	0.00005	0.00006
O	$C_{21}H_{28}O_6$	15 (6)	1.7	3.6
P	$C_{22}H_{30}O_5$	15 (6)	1.6	5.8
Q	$C_{21}H_{34}O_4$	12	6.4	

a Number in the parenthesis indicate the sizes of cyclic ethers.

3.6.1 Amphidinolide Q. Amphidinolide Q (**26**) is the latest and simplest member of this series reported so far (32). The 12-membered ring lactone is smallest in the size not only of the ring but also of the molecule as a whole. It is composed of a 16-carbon chain having branches of four methyls and one exomethylene. The structure was established by 2D NMR analysis. No stereochemistry has yet been reported. It showed weak cytotoxicity (Table 2).

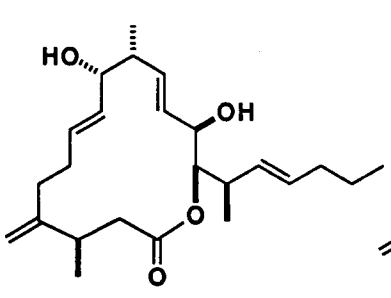


26

3.6.2 Amphidinolides J, O, and P. Amphidinolides J (**27**), O (**28**), and P (**29**) are 15-membered ring macrolides. Amphidinolide J is based on a 20-carbon chain while amphidinolides O and P bear a 17-carbon chain. The planar structure of **27** was elucidated by 2D NMR analysis and the absolute configuration by synthesis of the ozonolysis products (33). Ozonolysis of **27** gave three fragments which retain all of the chiral centers. All possible diastereomers of the three fragments were synthesized to determine the absolute configuration of **27**.

The structures of amphidinolides O (**28**) and P (**29**) are closely related, differing only at C_{11}, which is either in the form of a ketone or a methylidene. Their structures were also elucidated by extensive NMR analysis. The relative configurations shown in the structures **28** and **29** were determined by NMR (coupling constants and NOESY) and by molecular mechanics calculations (34).

A biosynthetic study of amphidinolide J (**27**) has recently been reported by Kobayashi *et al.* (35). The study was carried out by feeding ^{13}C-labeled sodium acetate to a culture of *Amphidinium* sp. Their conclusion was that **27** was generated through non-successive mixed polyketides, differing from normal polyketide biosynthesis, and that might explain the generation of the odd-numbered macrocyclic lactone ring.



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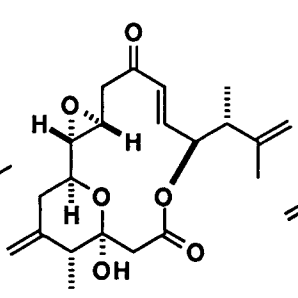

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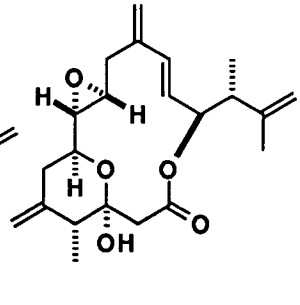
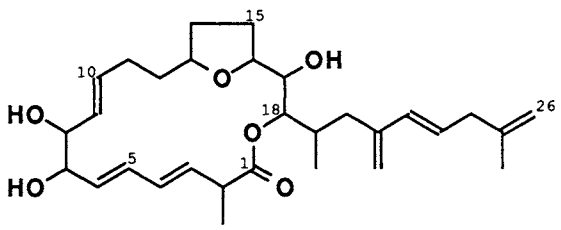


29

3.6.3 Amphidinolides E and K. Amphidinolides E (**30**) and K (**31**) are 19-membered ring macrolides. Both contain a tetrahydrofuran ring. The macrocyclic ring of E (**30**) is framed on a 26-carbon system having four C_1 branches, while amphidinolide K (**31**) is based on a C_{22} chain with five C_1 branches. Both structures were determined by NMR analysis (36, 37). No stereochemistry has yet been reported for **30**, while a partial stereochemistry has been depicted for **31** by NOESY data (37). Both compounds showed rather weak cytotoxicity as shown in Table 2.



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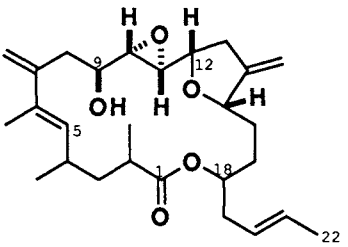
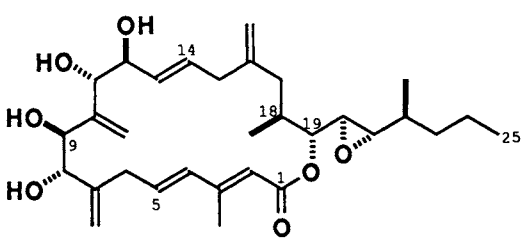


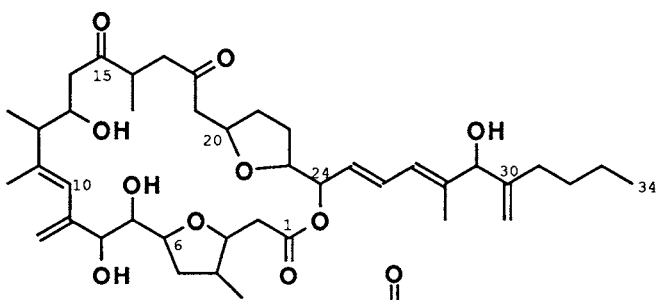
31

3.6.4 Amphidinolide A. Amphidinolide A (**32**) is the only 20-membered ring macrolide and the first one reported in the series. It was isolated as a crystalline compound. The planar structure was deduced from NMR data (38). A detailed NMR study of the compound was published later (39). As a result, the initial assignment of the ^{13}C NMR data has been corrected. The authors have proposed the relative stereochemistry of **32** on the basis of NOESY experiments. As an initial step toward stereochemical assignment and to complete the total synthesis of **32**, O'Connor and Williard (40) achieved a stereospecific synthesis of a potential C_{10}-C_{19} fragment which contained three out of nine chiral centers.



32

3.6.5 Amphidinolides C and F. These two 25-membered macrocyclic lactones have been isolated from two different species of the genus *Amphidinium*. Amphidinolide C (**33**) was reported as the first 25-membered macrolide (41). The basic structure **33** was established by extensive NMR studies. The 25-membered macrocyclic lactone ring with two tetrahydrofuran rings is constituted on a 34-carbon chain having seven C_1 branches. The structure of amphidinolide F (**34**) was also determined by NMR analysis and shown to be identical with **33** in the macrocyclic portion, differing only in the side chain which was six carbon shorter than **33** (42). The stereochemistry of the two compounds has not yet been elucidated but it was suggested to be the same since they showed very similar ^{1}H and ^{13}C NMR spectral data for the ring portions. It is interesting to note that the reported cytotoxicities for **33** and **34** are significantly different in spite of their close structural resemblance. Amphidinolide C (**33**) is extremely potent, while amphidinolide F (**34**) is 250 times less active as shown in Table 2.



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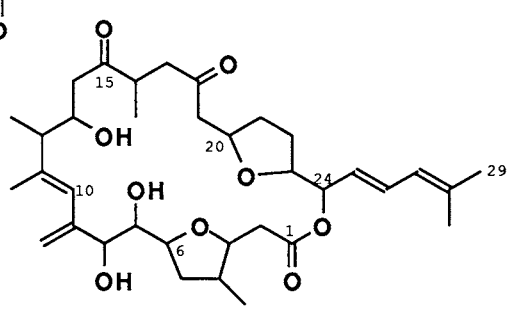


34

3.6.6 Amphidinolide B and Related Compounds. Included in this section are 26-membered ring amphidinolides B (**35**), B_2 (**36**), B_3 (**37**), D (**38**), and H (**39**) and the 27-membered ring amphidinolides G (**40**) and L (**41**). All these compounds have the same carbon skeleton based on a 26-carbon chain with six C_1 branches. Amphidinolide B

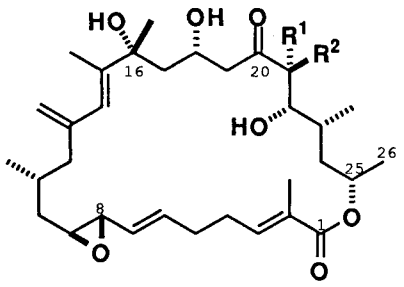
(**35**) was first reported in 1987 (43), and the initial planar structure was revised in 1989 (44). More recently, Shimizu and coworkers have reported the isolation of three isomeric compounds designated as amphidinolide B_1, B_2, and B_3 from a culture of a free-swimming dinoflagellate, *Amphidinium* sp. which was collected in U.S. Virgin Islands (45). They noted that the NMR data of B_1 was almost identical with those reported (43) for amphidinolide B and that the structure deduced from the 2D NMR spectra was in agreement with the revised planar structure of amphidinolide B (44). As they succeeded in obtaining a sample of B_1 in a crystalline form, X-ray diffraction analysis was carried out to determine the relative stereochemistry (45). The X-ray structure showed a rectangular shape by the formation of a hydrogen-bond bridge in the middle between 21-OH and the epoxide O. The identity of amphidinolide B_1 with amphidinolide B (**35**) was firmly established by direct comparison of the two samples by HPLC, NMR, and optical rotation (46). Using the X-ray data and by synthesis of a degradation product corresponding to the C_{22}-C_{26} segment of **35**, Ishibashi *et al.* (46) have determined the absolute configuration of amphidinolide B as shown in **35**.

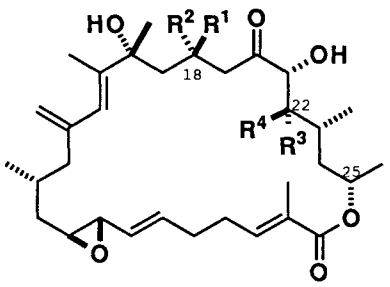
Shimizu *et al.* (45) have determined the structures of amphidinolides B_2 (**36**) and B_3 (**37**) as epimers of amphidinolide B by NMR comparison. They also suggested the identity of amphidinolide B_2 (**36**) with amphidinolide D (**38**), which had earlier been reported by Kobayashi *et al.* (44), to be the C_{21} epimer of amphidinolide B (**35**). If this is correct, amphidinolide D should have the structure of 22-*epi*-amphidinolide B (**36**) rather than the 21-epimer (**38**). This problem remains to be clarified.

Two more compounds isomeric to amphidinolide B (**35**) are amphidinolide H (**39**) and G (**40**) reported by Kobayashi *et al.* (47). The structures of these compounds were elucidated by NMR analysis to be 26- and 27-membered isomeric macrolides, respectively. The difference from the planar structure of **35** is that both **39** and **40** are oxygenated at C_{26} but have no hydroxyl group at C_{16}. No stereochemical correlation between these compounds and amphidinolide B has been reported. Another 27-membered macrolide of this class is amphidinolide L (**41**) isolated from *Amphidinium* sp., the same species from which amphidinolides H (**39**) and G (**40**) have been obtained (48). Structure **41** was determined by NMR analysis and the partial absolute configuration for the tetrahydropyran ring portion was elucidated by synthesis of the degradation product corresponding to the segment C_{21}-C_{26}. The configurations in this portion of **41** are in

agreement with those of amphidinolide B (46).

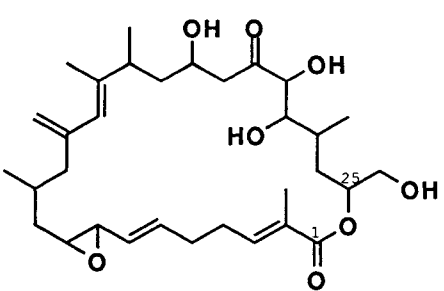
As shown in Table 2, in general these 26- and 27-membered ring amphidinolides exhibit potent cytotoxicity against L1210. However, Shimizu *et al.* (45) have reported a much lower level of cytotoxicity (IC_{50} 0.122 μg/mL) for amphidinolide B against HCT 116 human colon tumor cell line. It is not known whether this difference (1000 times) is due to the difference in the sensitivity of cell lines or due, at least partly, to the technical errors inherent in these assays.





	R^1	R^2
35	OH	H
38	H	OH

	R^1	R^2	R^3	R^4
36	OH	H	OH	H
37	H	OH	H	OH



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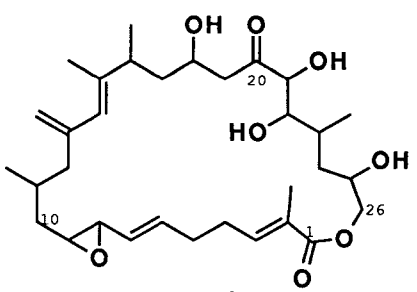

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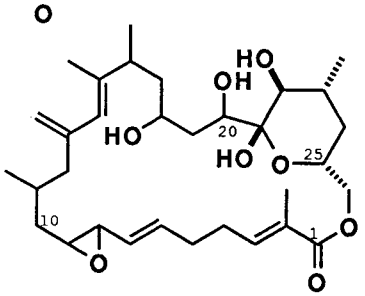
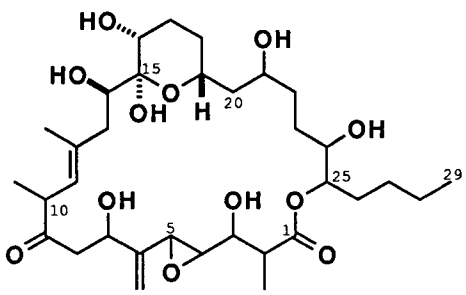
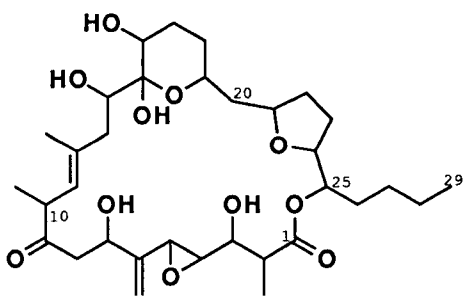


41

3.6.7 Amphidinolide N and Caribenolide 1. Amphidinolide N (**42**) and caribenolide 1 (**43**) are closely related 26-membered ring macrolides isolated from two different sources of *Amphidinium* sp., the former from a species symbiotic with a flatworm (49) and the latter from a free-swimming species (50), the same alga from which amphidinolides B_1-B_3 have been isolated by Shimizu *et al.* (45). Structures of both compounds have been deduced from extensive NMR analyses. Both compounds are based on the same carbon skeleton (C_{29} chain + $4C_1$) and contain more hydroxyl groups than other members of the amphidinolide series. The difference in the planar structures between **42** and **43** lies only in the C_{21}-C_{24} portion where **42** has two hydroxyl groups, while **43** has a tetrahydrofuran ring. Only the partial relative stereochemistry of **42** has been established but the complete stereochemistry has yet been described for either compound. Both compounds are reported to exhibit highly potent cytotoxicity. Furthermore, caribenolide 1 showed *in vivo* antitumor activity (T/C 150% at a dose of 0.03 μg/kg) against P388 murine leukemia.

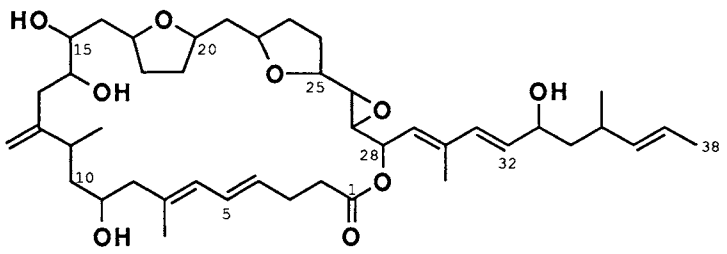


42



43

3.6.8 Amphidinolide M. Amphidinolide M (**44**) is a 29-membered ring macrolide, the largest macrocyclic lactone in the series. It was obtained as an amorphous solid. The structure elucidated by NMR analysis contains, besides a lactonic ring, two tetrahydrofuran rings, an epoxy group, four hydroxyls, two conjugated dienes, four methyls and one exomethylene on a 38-carbon chain which is also the longest in the series (51). The stereochemistry has yet to be determined. It was claimed to be the first 29-membered ring macrolide natural product. Amphidinolide M exhibited moderate cytotoxicity against L1210 and KB cell lines (Table 2).

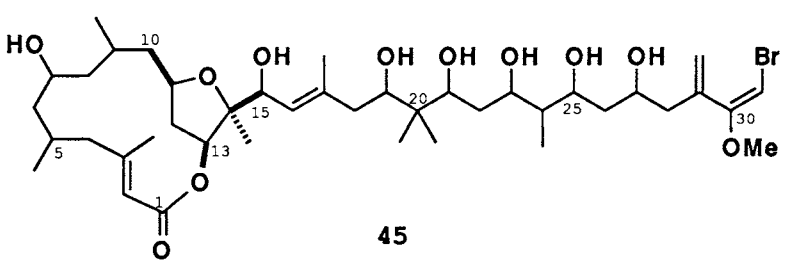


44

3.7 Oscillariolide

Since the origin of debromoaplysiatoxin (**109**), which had been discovered from a sea hare (52), was traced to the blue-green alga *Lyngbia gracilis* by Moore *et al.* in 1977 (53), increasing attention has been paid to the chemistry of blue-green algae or cyanobacteria. As a result a number of chemically and biologically interesting compounds have been reported from both marine and terrestrial cyanobacteria. The chemical investigation has been facilitated by their relatively easy cultivation in the laboratory.

Oscillariolide (**45**) is a macrolide isolated from a cultured marine cyanobacteria *Oscillatoria* sp. (54). The structure deduced from NMR analysis contains a 14-membered ring macrolide with a long side chain. The carbon skeleton consists of a 31-carbon chain with eight methyls and one exomethylene. It contains seven hydroxyl groups, one methoxy, one bromine atom, and one tetrahydrofuran ring. Except for C_5 and C_9, all other alternate carbons are oxidized, suggesting its polyketide biosynthetic origin. Geometries of the double bonds and the relative stereochemistry around tetrahydrofuran portion have been deduced from NOESY data, but the configurations at all other chiral centers remain to be elucidated. Oscillariolide showed inhibition of the cell division of fertilized starfish eggs.



45

4. POLYETHER MACROLIDE

Polyether macrolides are macrocyclic lactones containing a number of ether linkages which are usually in the form of five and/or six-membered cyclic ethers. For the discussion here we include those macrolides having two or more such ethers. Some 60 macrolides are described in this section. Some other related compounds are found in Sections 3, 5, and 6. The ring sizes of the macrolides presented here vary from 16 in polycavernosides to 42 in spongistatins. Many of the polyether macrolides together with polyethers such as okadaic acid (55) are characteristic metabolites of marine organisms, not found in terrestrial organisms. Many of them show potent cytotoxicity and some exhibit promising antitumor activity.

4.1 Laulimalides

Two 20-membered macrolides, laulimalide (**46**) and isolaulimalide (**47**), were reported by two independent groups at the same time. The group led by Moore, Scheuer, and Paul (56) isolated the compounds from an Indonesian sponge designated as *Hyattella* sp., while the group of Crews (57) isolated the macrolides from a specimen designated as *Spongia mycofijiensis*, collected in Vanuatu and named the compounds as fijianolides A (**47**) and B (**46**) after the species name. Later, isolaulimalide (**47**) was also isolated by Gunasekera and coworkers (58) from a sponge (family Thorectidae) collected in American Samoa. In all three cases latrunculin A, another macrolide (*vide infra*), was also isolated. Co-occurrence of the same combination of compounds in three different specimens raised a question on the taxonomy of the sponges. A note added in the proof of the latter paper described a re-examination of the three specimens by Bergquist (University of Auckland) and indicated the possibility of all the three sponges as being the same species of the genus *Hyattella* (58).

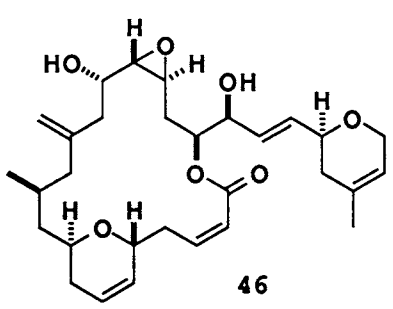
More recently we have also isolated the same combination of laulimalides and latrunculin A from a sponge designated initially as *Fasciospongia rimosa* which was collected in Okinawa. This identification was carried out by Hooper (Queensland Museum, Australia). Knowing the above-mentioned taxonomic confusion, we requested Dr. Hooper for re-examination of our sample. He noted that "its correct generic and family assignments are a real problem," and that it would probably end up with a new genus name. He tentatively assigned our sample as "*Hyattella*" *rimosa* (Lamarck)

(order Dictyoceratida: family ?Spongiidae) with the notion of "?" in front of the family name. Because of the taxonomic confusion with the sponge, we prefer to use the compound name "laulimalide" rather than "fijianolide."

Both the Hawaii and California groups have determined planar structures with partial relative stereochemistries of laulimalide (**46**) and isolaulimalide (**47**) by NMR, but their full stereostructures have remained unsolved until recently. Fortunately, we obtained laulimalide as crystals. Single crystals suitable for X-ray analysis were grown from solution of aqueous methanol. The analysis not only confirmed the previous structure of laulimalide but also gave the absolute configuration as shown in the formula **46** (59). Easy transformation of laulimalide into isolaulimalide (**47**) by acid treatment also permitted the assignment of the absolute configuration of the latter (60).

From the Okinawan sample of the sponge we isolated another related compound named neolaulimalide (**48**). 2D NMR analysis revealed that it was a 21-membered lactone, with an expansion of the ring size, but was otherwise identical with **46**. The structure and absolute configuration was confirmed by its transformation into **47** by treatment with camphorsulfonic acid in chloroform. The reaction, which was completed in about 48 hours, was much slower than the conversion of **46** to **47**, which required only two hours.

Laulimalide was highly cytotoxic (IC_{50} 15 ng/mL against KB cell line)(56), while isolaulimalide was moderately active (IC_{50} 9 μg/mL against P388)(57). Neolaulimalide also showed significant cytotoxicity (IC_{50} 0.01-0.05 μg/mL) against several tumor cell lines (P388, A549, HT29, MEL28).



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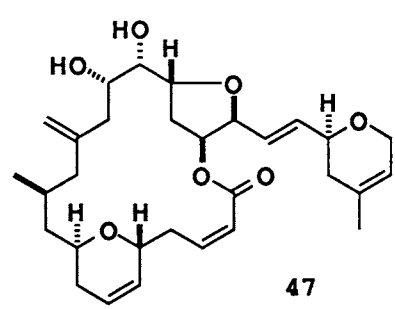

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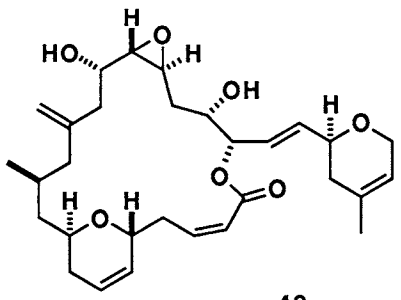
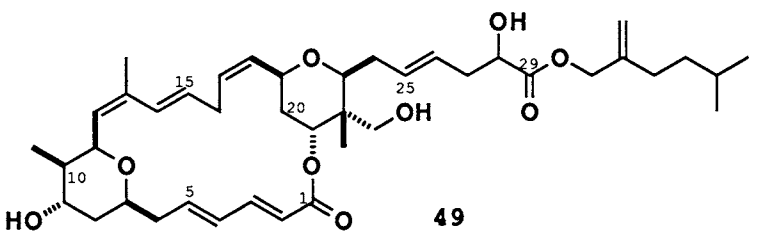


48

4.2 Lasonolide A

Lasonolide A (**49**) is a highly unsaturated 22-membered macrolide possessing two tetrahydropyran rings. It was obtained as an active constituent of a Caribbean sponge, *Forcepia* sp. whose extract inhibited the proliferation of A-549 human lung carcinoma cells as well as cell adhesion in the EL-4·EL-2 cell line (61). The structure was deduced from 2D NMR analysis. The configurations on the two tetrahydropyran rings were assigned by the NOESY experiment. However, no stereochemical correlation has been established between the two portions. Lasonolide A exhibited cytotoxicity against A-549 and P388 with IC_{50} 40 and 2 ng/mL, respectively. It also inhibited cell adhesion in the EL-4·EL-2 cell line with an IC_{50} of 19 ng/mL. Inhibition of cell adhesion is known to correlate with signal transduction activity (62).



49

4.3 Polycavernosides

Polycavernosides are toxic principles isolated from the red alga *Polycavernosa tsudai* (formerly *Gracilaria edulis*) which caused human intoxication in Guam in April, 1991. The incident affected 13 people, and three of them died. The alga has been widely consumed as a salad delicacy with no previous record of intoxication. However, similar intoxications have been recorded in Japan in the early 1980s, in which two people were killed by eating two other commonly consumed species of *Gracilaria* in two independent

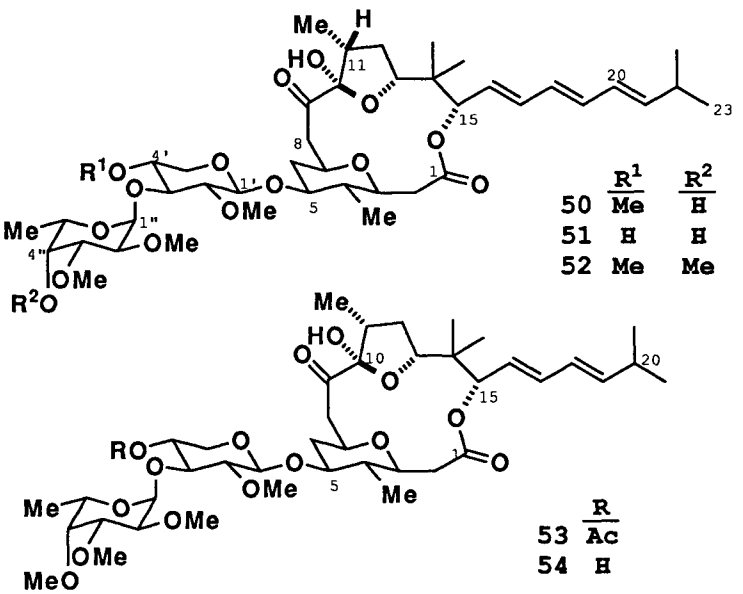
incidents. Fusetani and Hashimoto (63) have identified, from *Gracilaria verrucosa*, prostaglandins E_2 and A_2 which induced severe diarrhea in mice, the main symptom of human victims, and suggested them to be the causative agents in the Japanese incidents. Several species of *Gracilaria* are widely consumed as food in the Asia-Pacific region, and some of them are important raw materials for the commercial production of agar.

The toxicity of *P. tsudai* in Guam rapidly decreased after the incident in late April, 1991 but rose again, though at lower levels, in the same season of 1992. From the algal samples collected in June of 1991 and 1992, Yasumoto and coworkers succeeded in isolating five toxins in 100 to 400 μg quantities each. They first determined the planar structure of polycavernoside A (**50**) by extensive NMR analysis on a 400 μg sample (64). Polycavernoside A is a glycoside consisting of a 16-membered macrolide as the aglycone and two pyranose rings as sugar units. The aglycone macrolide containing two etherial rings is based on a 23-carbon chain with five methyl groups. The sugars are highly methylated fucose and xylose.

The authors subsequently reported the structures of polycavernosides A_2 (**51**), A_3 (**52**), B (**53**), and B_2 (**54**) (65). All of them contain fucopyranosyl-xylopyranosyl in the same connectivity as a common sugar moiety and the same macrocyclic unit. The difference in the structures within the polycavernoside A series lies in the differences of methylation at 4'-OH and 4"-OH groups in the sugars, while the difference between the A and B series is in the length of the conjugated double bonds in the aglycones. Polycavernoside B (**53**) differs from polycavernoside B_2 (**54**) by the presence of a 4'-OAc group instead of the 4'-OH group in the latter.

The stereochemistry of polycavernoside A (**50**) has been proposed by Murai *et al.*, by partial synthesis and NMR comparison. As an effort toward elucidation of the absolute configuration and total synthesis, Murai *et al.* have synthesized the portions of the tetrahydrofuran (66), tetrahydropyran (67), and the sugar moiety composed of *D*-xylose and *L*-fucose (68). The disaccharide was then attached onto the enantiomers of the tetrahydropyran to prepare two diastereomers whose NMR data were compared with those of **50** (69). The result is as shown in the formula **50**. The stereochemical relationship between the hydropyran ring and the hydrofuran part is based on NOE observations. The stereochemistries shown in the formulae of the other congeners are depicted on the assumption that they are the same.

Both polycavernosides A (**50**) and B (**53**) showed the same level of toxicity (LD_{99} 200-400 μg/kg) by intraperitoneal injection in mice. These compounds caused similar symptoms to those observed with human patients: diarrhea, hypersalivation, lacrymation, muscle spasms, and cyanosis.



4.4 Bryostatins

The bryostatins are the best known and most important members of the marine macrolides. A great endeavor of Pettit and coworkers in research spread over a quarter of a century has now culminated in the discovery of some twenty congeners and in the development of bryostatin 1 as an anticancer drug, which is currently in phase II clinical trials (70). Since the first report on the chemistry of bryostatin 1 (**55**) in 1982 (71), a large number of research papers have been published on the bryostatins. A computer literature survey revealed some 280 papers published in the past 14 years, that is, on average 20 papers a year. The majority of them are on the biochemical and pharmacological aspects of the bryostatins and thus are beyond the scope of the present review. Since most of the bryostatins reported to date and their development by 1991 are covered in an excellent review by Pettit (72), the reader is referred to it for details. In this section we present a brief overview with an emphasis on the advancements made since 1991.

The bryostatins are very minor constituents of the bryozoan

Bugula neritina. Thus, large scale extractions were required for their isolation. The first compound of the series, bryostatin 1 (**55**) was reported in 1982 (71). Its structure was determined by X-ray analysis. Bryostatin 1 is a 26-membered ring macrocyclic lactone in the outer skeletal perimeter, while the inner perimeter consists of a 20-membered cycle. The lactone with three tetrahydropyran rings is constructed on a 27-carbon chain with the branches of two sets of *gem*-dimethyls and two C_2 units (acetylidenes). Thus, the carbon skeleton of the bryostatins, since it contains C_2 branches, is different from those of the macrolides discussed above.

Bryostatin 2 (**56**) was described in 1983 (73). It was isolated from 500 kg of wet *B. neritina* in a yield of 314 mg, a comparable yield with that of bryostatin 1 (**55**). By that time Pettit group had already isolated bryostatins 3-17 (**57-71**) in microgram quantities and known that they were all derived from the bryopyran ring system (**75**) by spectral evidence (73). However, insufficiency of the samples precluded their immediate identification. It was not until 1996 that the structures of the last two compounds (**70, 71**) were reported together with that of bryostatin 18 (**72**) (74). The structures for bryostatins 3-15 (**57-69**) had been described by 1991 (75-84). All the bryostatins have the same skeletal and macrocyclic ring system and differ, except for a few, only in the substituents at C_7 and C_{20}. This is a striking contrast to the amphidinolides which consist of compounds having a variety of ring sizes and carbon skeletons (Section 3.6).

The structures of some bryostatins have been revised from those reported in initial publications. The isovalerate ester substituent at C_7 of bryostatins 4 (76), 5 (78), and 10 (82) has now been revised to pivalate (85). There are some confusions on the structure of bryostatin 3. The initial structure reported by Pettit *et al.* (75) in 1983 was revised by them to a structure (22-epimer of **57**) containing a γ-lactone instead of δ-lactone (86). Shortly afterward, the NCI group that worked on the large scale isolation of bryostatin 1 also published a revised structure for bryostain 3 (87). Their structure was the same as that of Pettit except for the configuration at C_{22}. The *trans* orientation for the H_{22} and H_{23} was assigned by the observed coupling constant of J = 9 Hz between the two protons. However, the structure was revised again by the NCI group (88). They also isolated a new compound from *B. neritina*, detailed NMR analysis of which revealed that the new compound should

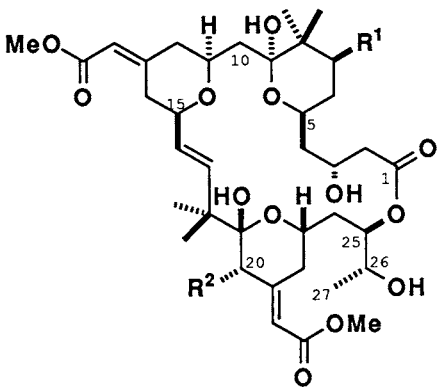
have structure **57** (20S) and that the previous sample to which structure **57** was assigned, should be the 20*R* epimer of **57**. They noted by NMR comparison that this 20*R* epimer (**73**) was actually identical with the compound reported by Pettit as bryostatin 3. Furthermore, they suggested that, in keeping with the published X-ray structures of bryostatin 1 (**55**) and bryostatin 2 (**56**) (86), their new compound should be called bryostatin 3 (**57**) and the original bryostatin 3 should be renamed as 20-*epi*-bryostatin 3 (**73**). The NCI group has reported another new bryostatin, bryostatin 3 26-ketone (**74**).

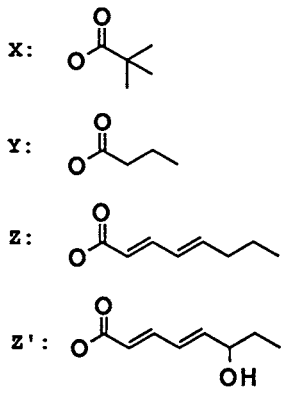
The absolute configuration of the bryostatins has been established by X-ray crystallographic analysis on bryostatin 2 7-(*p*-bromobenzoate) (86). The benzoate derivative was prepared by reacting bryostatin 2 with *tert*-butyldimethylsilylchloride to protect the C_{26} hydroxyl group followed by reaction with *p*-bromobenzoic acid and subsequent deprotection using hydrofluoric acid. The same technique was applied to convert bryostatin 2 (**56**) to the more demanding bryostatin 1 (**55**) in overall 82% yield (86).

A large-scale isolation of bryostatin 1 has been documented by the NCI group (89). In order to supply the compound for clinical evaluation, multigram quantities of bryostatin 1 was obtained from a collection of 10,000 gallons (ca. 12.6 tons) of *Bugula neritina*. A high content (10^{-3}%) of bryostatin 10 (**64**) was found in a collection of *B. neritina* from Japan (90). This was 10^4-fold higher than previous samples. Conformational analysis of bryostatin 10 (**64**) has been reported by analysis of 600 MHz ^{1}H NMR data combined with molecular mechanics calculations (91).

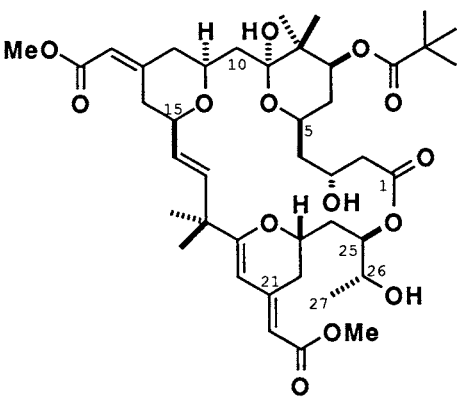
A total synthesis of bryostatin 7 (**61**) has been achieved by Masamune and coworkers (92). A recent review by Norcross and Paterson (93) describes all the syntheses of bryostatins as well as of other marine macrolides reported till 1994.

Both *in vivo* and *in vitro* antitumor activities of the bryostatins against P388 leukemia are summarized in Table 3. Biological properties of bryostatin 1 have been extensively studied. It is a powerful antitumor promoter and can act as either an inhibitor or stimulator of protein kinase C. It stimulates hematopoietic progenitor cells and has immunostimmulating properties. Most importantly, it is now in phase II clinical trials as an anticancer drug. For further details of the biological activities of bryostatin 1 the reader is referred to the reviews by Pettit (70, 72).

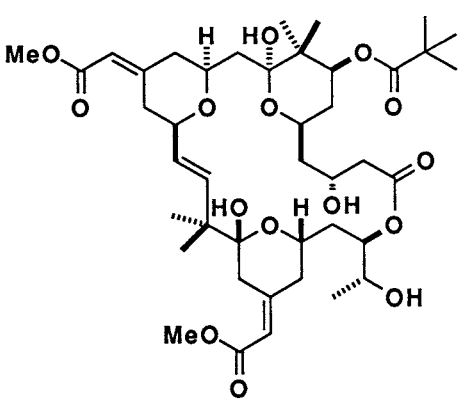




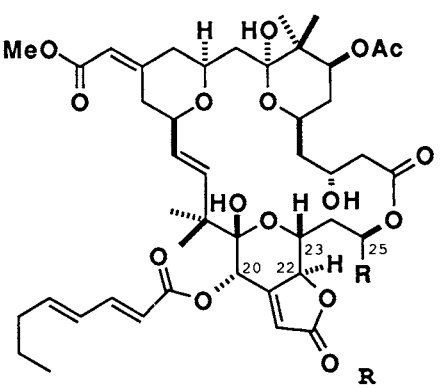
		R^1	R^2
Bryostatin 1	(55)	OAc	Z
2	(56)	OH	Z
4	(58)	X	Y
5	(59)	X	OAc
6	(60)	Y	OAc
7	(61)	OAc	OAc
8	(62)	Y	Y
9	(63)	OAc	Y
10	(64)	X	H
11	(65)	OAc	H
12	(66)	Y	Z
13	(67)	Y	H
14	(68)	X	OH
15	(69)	OAc	Z'



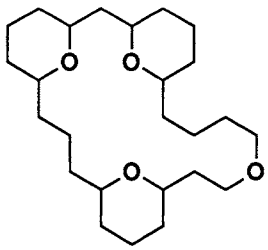
Bryostatin 16 (70)
17 (71) 21*Z*



Bryostatin 18 (72)



		R
Bryostatin 3	(57)	22*S*
20-*epi*-Bryostatin 3	(73)	22*R*
Bryostatin 3 26-ketone	(74)	22*S*



Bryopyran (**75**)

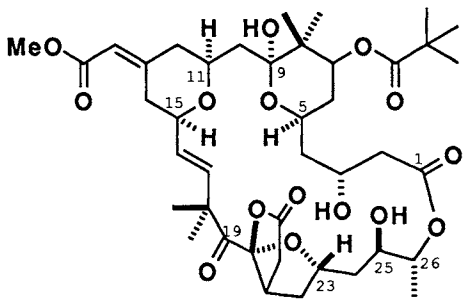
Table 3. Antitumor activity of bryostatins against P388 leukemia

Bryostatin	In vitro	In vivo
	ED_{50} (μg/mL)	%ILS (dose μg/kg)[a]
1	0.89	52-96 (10-70)
2	-	60 (30)
3[b]	-	63 (30)
4	10^{-3}-10^{-4}	62 (46)
5	$1.3x10^{-3}$-$2.6x10^{-4}$	88 (85)
6	$3.0x10^{-3}$	82 (185)
7	$2.6x10^{-5}$	77 (92)
8	$1.3x10^{-3}$	74 (110)
9	$1.2x10^{-3}$	40 (80)
10	$7.6x10^{-4}$	-
11	$1.8x10^{-5}$	64 (92.5)
12	$1.4x10^{-2}$	47-68 (30-50)
13	$5.4x10^{-3}$	-
14	0.33	-
15	1.4	-
16	$9.3x10^{-3}$	-
17	$1.9x10^{-2}$	-
18	$3.3x10^{-3}$	-

a %ILS = % increase in life span. b 20-*epi*-bryostatin 3 (**73**)

A somewhat different but closely related structure to the bryostatins is neristatin 1 (**76**) isolated also from the bryozoan *Bugula neritina* (94). The structure determined by X-ray analysis retains most of the bryostatin features, but it is the first example having a non-bryopyran ring. The macrolide has an expanded 27-membered ring as the lactonic linkage is formed with the C_{26}-OH

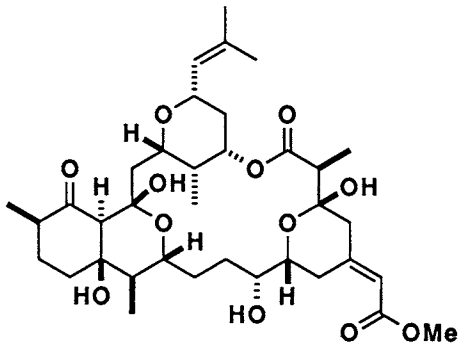
instead of the C_{25}-OH in the bryostatins. Other different features are the presence of a hydrofuran ring of the C_{20}-C_{23} segment instead of the usual hydropyran ring at the C_{19}-C_{23} segment and the presence of a γ-lactone. Neristatin 1 has the same carbon skeleton with the bryostatins. It showed binding affinity to protein kinase C but this was weaker than that of bryostatin 4. The cytotoxicity (IC_{50} 10 μg/mL) against P388 leukemia was also weak comparing to those of the bryostatins (Table 3).



76

4.5 Miyakolide

A macrolide containing some of the same structural elements of the bryostatins, miyakolide (**77**), has been discovered by us from a sponge, *Polyfibrospongia* sp. (95). The sponge collected in Miyako Island, Okinawa had earlier yielded hennoxazoles, the novel antiviral compounds having a unit of two contiguous oxazole rings (96). The structure of miyakolide (**77**) as determined by X-ray was quite different from those of the hennoxazoles (e.g. hennoxazole A, **78**), although both could be derived from polyketide biosynthesis. Like the bryostatins, it contained three tetrahydropyran rings, two hemiketal functionalities, and a methyl ethylidenoate substituent attached to one of the hydropyranes. The latter is a striking feature common to these two types of the macrolides. Miyakolide may be regarded as a 20-membered macrolide as counted along the shortest carbon chain forming the macrocycle or as a 16-membered one involving the two pyran oxygens. The inner cavity is therefore, smaller than the 20-membered ring of the bryostatins. Because of the smaller ring size and the presence of a fused carbocyclic ring, miyakolide adapts more rigid conformations than the bryostatins. Miyakolide displayed much weaker antitumor activity than the bryostatins in both *in vitro* (IC_{50} 17.5 μg/mL) and *in vivo* assays (T/C 127% at 800 μg/mL dose) against P388 leukemia.



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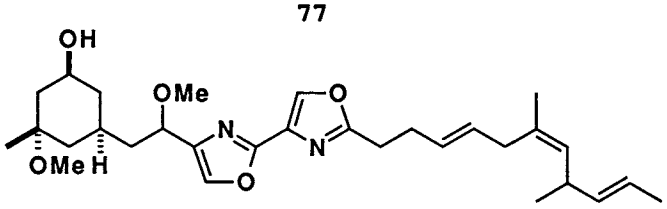


78

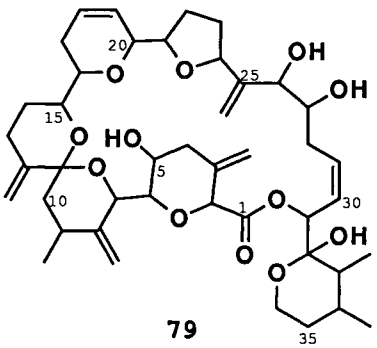
4.6 Goniodomin A

The amphidinolides discussed in Section 3.6 were the macrolides isolated from laboratory-cultured dinoflagellates. On the other hand, Murakami *et al.* (97) have reported the isolation of a polyether macrolide, goniodomin A (**79**), from a natural source of a dinoflagellate. The dinoflagellate *Goniodoma pseudogoniaulax* was collected using a plankton net at rock pool on the beach at Jogashima, southwest of Tokyo. The collected cells (320 g wet weight) were extracted, and the extract was chromatographed in the usual manner to afford 180 mg of goniodomin A. The planar structure (**79**) of goniodomin A was elucidated by NMR analysis. It is a 32-membered (outer perimeter) or 23-membered ring (inner perimeter encompassing five ether oxygens) macrolide with six ether rings. The carbon backbone is a C_{26} chain with seven C_1 branches of which four are exomethylenes.

Although the identity of **79** with goniodomin, an antifungal metabolite of unknown structure reported (98) earlier from *Goniodoma* sp., has not been established, a close structural relationship has been suggested. Goniodomin A displayed antifungal activity against *Mortierella ramannianus* and *Candida albicans* at a concentration of 0.5 μg/mL and inhibited the cell division of fertilized sea urchin eggs at 0.05 μg/mL.

Goniodomin A was also found to induce modulation of actomyosin

ATPase activity mediated through conformational change of actin (99). The authors suggest that goniodomin A is an essential tool for the investigation of the relationship between the structure and function of contractile proteins.



79

4.7 Pectenotoxins

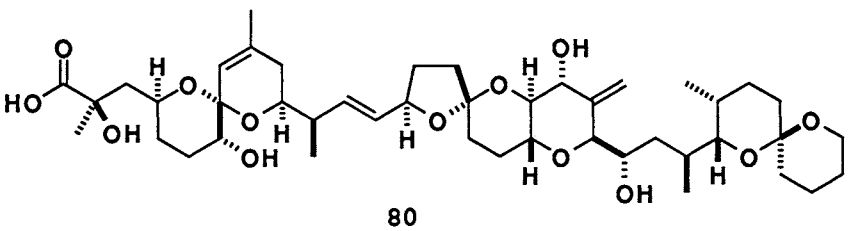
Diarrhetic shellfish poisoning (DSP) is a term coined by Yasumoto to a gastroenteritis known as a folklore in northern Japan. The disease had frequently occurred after ingestion of shellfish harvested during late spring to summer (100). Extensive investigations by Yasumoto and coworkers revealed the toxins involved in DSP and the dinoflagellates which produced the toxins (101). DSP has now been recognized as a worldwide occurrence and therefore as a worldwide problem of public health and of shellfish industries.

More than ten compounds have been isolated as causative agents of DSP from the digestive gland of the scallop *Patinopecten yessoensis*. They are all polyethers belonging to three distinctive classes, okadaic acid (**80**) and derivatives (dinophysistoxin) (100), pectenotoxins, and yessotoxins (102). Only the pectenotoxins enter discussion here as they are macrolides.

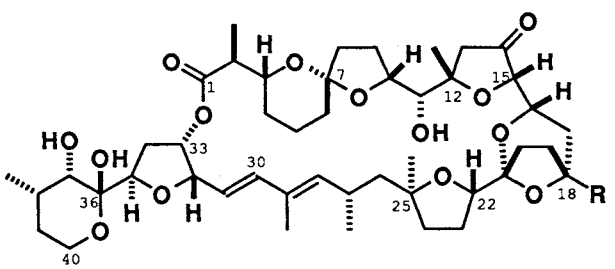
Five structures, pectenotoxins 1-4 (**81-84**) and pectenotoxin 6 (**85**) have so far been identified. The structures of pectenotoxin 1 (**81**) was determined by X-ray (100) and those of others were elucidated by spectroscopic correlation with **81** (101, 103, 104). All of them contain the same macrocyclic lactone consisting of 34 atoms in the outer perimeter of the ring and the same overall structure except for the substituent at C_{18}. Pectenotoxin 4 (**84**) differs from **81** in the C_7 configuration (104). In contrast to goniodomin A (**79**) which possesses five 6-membered ethers, five of the eight ethers in the pectenotoxins are 5-membered rings.

In a study to develop sensitive analytical methods for the determination of DSP toxins, Yasumoto *et al.* (101) detected the presence of pectenotoxin 2 (**82**) but not others in the dinoflagellate *Dinophysis fortii*. From this observation they suggested that other pectenotoxins were derived from **82** by oxidation in the hepatopancrea of the scallop.

Recently Jung *et al.* (105) isolated pectenotoxin 2 (**82**) as cytotoxic constituent from an association of two sponges *Poecillastra* sp. and *Jaspis* sp., collected in Komin Island, Korea. Pectenotoxin 2 was found to exhibit potent cytotoxicity against several human cancer cell lines including A-549 (lung), HT-29 (colon), and MCF-7 (breast) and to display differential cytotoxicity against several cell lines.



80

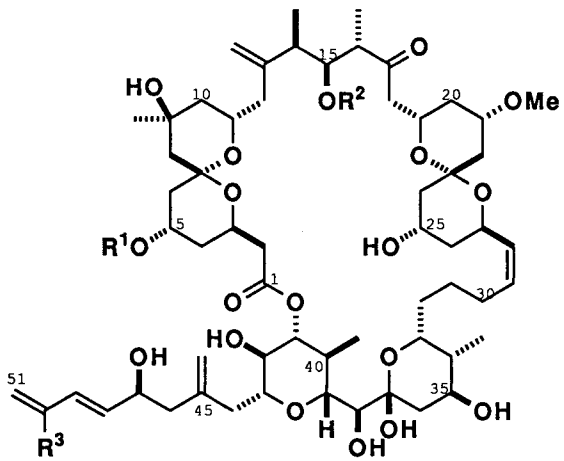


	R
81	CH_2OH
82	CH_3
83	CHO
84	CH_2OH, 7*R**
85	CO_2H

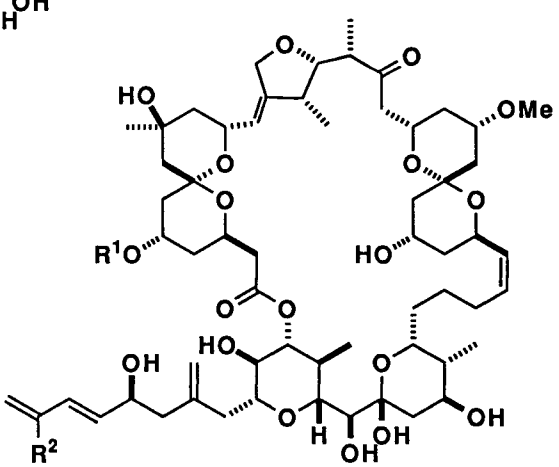
4.8 Spongistatins and Related Compounds

In 1993 three groups independently reported, from different collections of sponges, closely related polyether macrolides which exhibited potent cytotoxicity. They were, in chronological order of the papers submitted, spongistatin 1 (**86**) by Pettit (106), cinachyrolide A (**89**) by Fusetani (107), and altohyrtin A (86) by Kitagawa (108). Since the first report Pettit's group has described

the isolation and structures of eight additional compounds, spongistatins 2-9 (**87-94**) from two sponges, *Hyrtios* sp. (initialy reported as *Spongia* sp. (70, 106) collected in Maldives and *Spirastrella spinispirulifera* collected off the southeast coast of Africa (109-112). On the other hand, Kitagawa's group has reported the isolation and structures of three more altohyrtins, B (**95**), C (**87**), and 5-desacetylaltohyrtin A (**88**) from the sponge *Hyrtios altum* collected in Okinawa (113). As shown in Table 4 these compounds were very minor constituents of the sponges, and the isolations required enormous amounts of the materials except in the case of *Cinachyra* sp.



	R^1	R^2	R^3
86	Ac	Ac	Cl
87	Ac	Ac	H
88	H	Ac	Cl
89	Ac	H	Cl
91	Ac	H	H
95	Ac	Ac	Br



	R^1	R^2
90	H	Cl
92	H	H
93	Ac	H
94	Ac	Cl

Each group used NMR techniques extensively to determine the structures, including partial or complete relative stereochemistry. Kitagawa's group reported the absolute stereostructures by the

application of the MTPA (2-methoxy-2-phenyl-2-(trifluoromethyl) acetic acid) ester method and the CD exciton chirality method (114). All the compounds reported by the three groups have the same skeleton and ring system, which was named "spongipyran" by Pettit, except for spongistatins 5 and 7-9 which contain an additional hydrofuran ring.

The planar structures of the following pairs are identical: spongistatin 1/altohyrtin A (**86**), spongistatin 2/altohyrtin C (**87**), spongistatin 3/desacetylaltohyrtin A (**88**), and spongistatin 4/cinachyrolide A (**89**). Although no identities have been established between these pairs and there are some discrepancies in the relative stereochemistries reported by the three groups, it is likely that each pair is identical. In this review we have adapted the absolute stereostructures proposed by Kitagawa's group. However, there is no doubt that further work is needed to unambiguously establish the stereochemistry of this very important class of antitumor macrolides.

Table 4. Data for spongistatins, altohyrtins, and cinachyrolide A

Compound name	Compd No.	Sponge [a]	Yield [b] (mg)	$[\alpha]_D$ (MeOH)	MP °C	Cytotox.[c] IC_{50} (ng/mL)	Ref. No.
Spongistatin 1	**86**	A	13.8	+26.2°	161-162	-	106
2	**87**	A	4.3	+24.5°	140-141	-	109
3	**88**	A	2.7	+28.1°	148-149	-	109
4	**89**	B	10.7	+23.0°	153-154	0.049	110
5	**90**	B	12.9	-11.1°	186-187	0.066	110
6	**91**	B	8.4	+22.0°	139-140	3.4	111
7	**92**	B	5.3	-15.7°	166-167	2.6	111
8	**93**	B	1.8	-32.0°	158-159	0.8	112
9	**94**	B	5.4	-33.3°	164-165	0.027	112
Altohyrtin A	**86**	C	7.5	+21.7°	-	0.01	108
B	**95**	C	0.5	+45°	-	0.02	113
C	**87**	C	0.5	+31°	-	0.4	113
Desacetyl-altohyrtin A	**88**	C	4.7	+19°	-	0.3	113
Cinachyrolide A	**89**	D	1.1	+5.4°	-	<0.6	107

a A: *Hyrtios* sp., B: *Spirastrella spinispirulifera*, C: *Hyrtios altum*, D: *Cinachyra* sp.
b Yields obtained from A 400kg, B 2409 kg, C 112 kg, and D 6.6 kg.
c Cell lines were P388 for spongistatins, KB for altohyrtins, L1210 for cinachyrolide A.

These compounds show highly potent cytotoxicity as shown in Table 4. In fact Pettit has often described them as "exceptionally" or "extraordinary" potent cancer cell inhibitors. The results of the testing in the NCI 60 cell line *in vitro* screening panel are also impressive as discussed by Pettit (70), Pettit *et al.* (106, 109-112) and by Boyd and Paull (115). Furthermore, Pettit describes that an *in vivo* result of spongistatin 1 against OVCAR-3 xenograft at 25 μg/kg is even more exciting, leading to better than 70% long term survivors (70).

4.9 Halichondrins

Another series of important polyether macrolides as potential anticancer agents are the halichondrins reported first by Uemura *et al.* (116). The halichondrins were obtained as minor constituents of the sponge *Halichondria okadai*. From 600 kg of the sponge collected in Japan Uemura *et al.* isolated eight compounds, halichondrins B (**96**) and C (**97**), norhalichondrins A-C (**98-100**), and homohalichondrins A-C (**101-103**) in yields ranging from 2.1 to 35.0 mg. The structure and absolute configuration of the most abundant norhalichondrin A (**98**) was determined by X-ray on its *p*-bromophenacyl ester (116). The structures of other congeners were elucidated by NMR comparison with **98**. In *in vivo* antitumor assay halichondrin B (**96**) was most active, exhibiting T/C 244% against B16 melanoma at 5.0 μg/kg. Details of the chemistry and antitumor activity of these unique compounds are described by Uemura and Hirata in Volume 5 of this series (117). The reader is also referred to another review by Uemura (118).

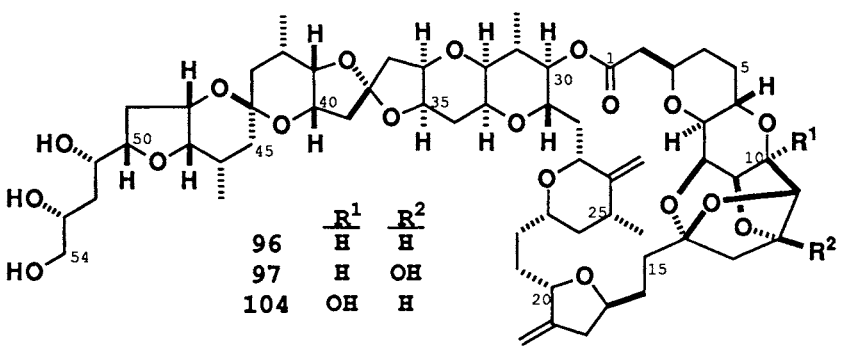
In spite of their promising activity, further development of the halichondrins as anticancer agents has appeared to be impractical because of their limited availability from natural sources and their structural complexity. Nevertheless, Pettit's group, who also discovered the compounds from a Pacific sponge in the early 1980s, have been continuing the research, and their efforts have now brought halichondrin B to the stage of preclinical development with an excellent result in the NCI (70).

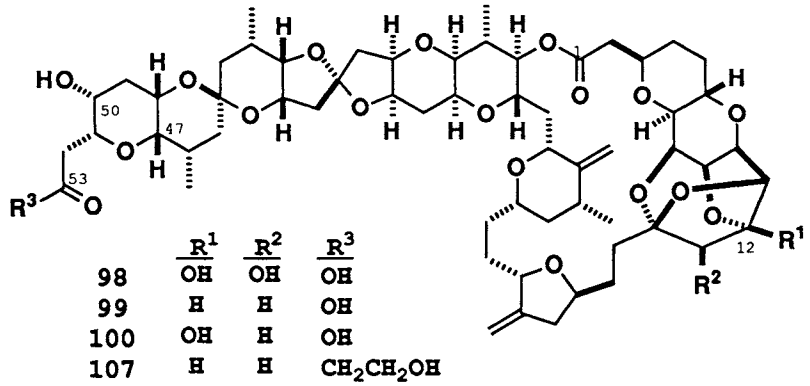
Some of the halichondrins and new related compounds have been reported from other sponges. Pettit *et al.* (119) reported the isolation of halichondrin B (**96**) and homohalichondrin B (**102**) from a new Western Pacific sponge in the genus *Axinella*. The Arizona group subsequently described the isolation of new congeners: halistatin 1 (**104**) and halistatin 2 (**105**) from Eastern Indian Ocean sponges of

the family Axinellidae, and halistatin 3 (**106**) from the micronesian sponge *Phakellia* sp. (120-122). In all three samples halichondrin B and homohalichondrin B were also present. The halistatins contain a hydroxyl group at C_{10}. On the other hand, Munro's group has also isolated a new congener, isohomohalichondrin B (**107**) together with halichondrin B (**96**) and homohalichondrin B (**102**) from a New Zealand deep-water sponge, *Lissodendoryx* sp. (123). They obtained these compounds in a yield of 2-4.5 mg each from 5 kg of the sponge, that amounted to more than ten times the yields from other sponges. Like halichondrin B and homohalichondrin B, the new congeners exhibited exceedingly potent cytotoxicity in P388 assays and a similar trend of activity in the NCI 60 cell line panel. The halichondrins caused the accumulation of cells arrested in mitosis, inhibited tubulin polymerization, and inhibited the binding of radiolabelled vinblastine and GTP to tubulin (120-123).

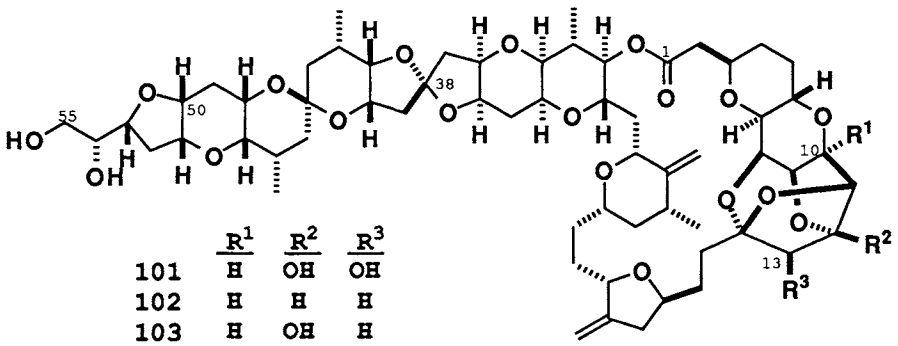
Munro's group (124) has recently described acid-catalyzed reactions of homohalichondrin B (**102**). Homohalichondrin B was treated wtih 1.5-fold molar excess of trifluoroacetic acid in CH_2Cl_2 solution at 20° C for 30 min, and the resulting four products were separated by reverse phase HPLC and characterized by NMR. They were recognized as being products derived by epimerization at the C_{38} spiroketal and/or cleavage of the two ester linkages to form a C_{11}-C_{14} furan ring at the tricyclic moiety. The C_{44} spiroketal was not affected under the conditions.

The total synthesis of halichondrin B (**96**) and norhalichondrin B (**99**) has been achieved by Kishi and coworkers (125-127). At least three other groups, Salomon, Burke, and Horita and Yonemitsu, have also been active in efforts directed toward the total synthesis of the halichondrins. Some 25 papers have been published on the syntheses since 1987, and most of them are covered in a recent review by Norcross and Paterson (93).

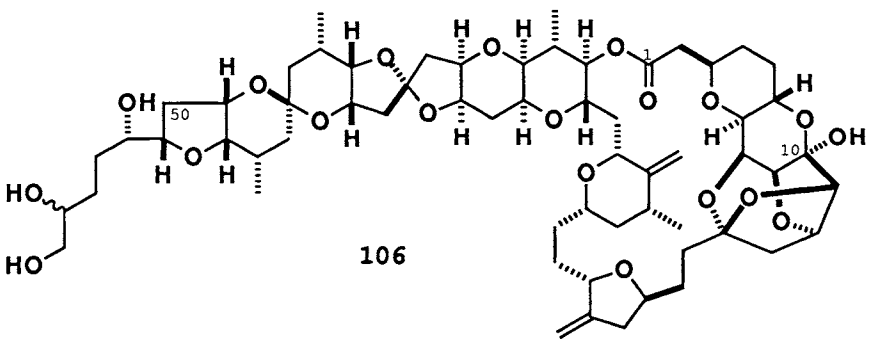




	R^1	R^2	R^3
98	OH	OH	OH
99	H	H	OH
100	OH	H	OH
107	H	H	CH_2CH_2OH



	R^1	R^2	R^3
101	H	OH	OH
102	H	H	H
103	H	OH	H
105	OH	H	H



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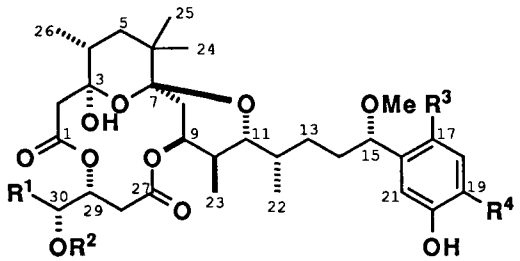
5. MACRODIOLIDES

In this section we discuss compounds having two lactonic linkages in a macrocyclic ring. Such compounds are often called dilactones. In order to distinguish them from those compounds having two independent lactone rings, we use the term macrodiolides. Many examples are known as antibiotics from terrestrial microbes (16). The currently known marine macrodiolides may be classified into three groups i.e. those represented by aplysiatoxin, aplasmomycin, and swinholide classes. Since the

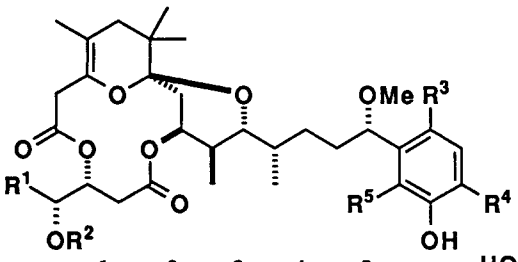
compounds of the former two classes have been known for twenty years and hence described elsewhere, they will be treated only briefly here.

5.1 Aplysiatoxins and Related Compounds

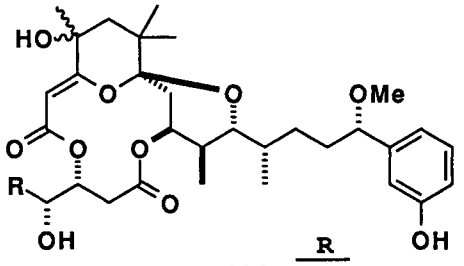
Aplysiatoxins were first discovered by Kato and Scheuer (52) as toxic constituents of the sea hare *Stylocheilus longicauda*. They isolated aplysiatoxin (**108**), debromoaplysiatoxin (**109**), their monoacetates (**110, 111**) and the anhydroaplysiatoxins (**116-121**) from the digestive gland of the sea hare. Moore *et al.* subsequently found debromoaplysiatoxin in the blue-green alga *Lyngbia gracilis* (53) and oscillatoxin A (**112**) and related compounds (**113-115, 122-124**) in a mixture of the blue-green algae *Schizothrix calcicola* and *Oscillatoria nigroviridis* (128, 129), demonstrating the algal origin of the aplysiatoxins. The absolute stereochemistries of these compounds have been elucidated by Moore *et al* (130).



	R^1	R^2	R^3	R^4
108	Me	H	Br	H
109	Me	H	H	H
110	Me	Ac	Br	H
111	Me	Ac	H	H
112	H	H	H	H
113	H	H	Br	H
114	H	H	Br	Br
115	Me	H	Br	Br



	R^1	R^2	R^3	R^4	R^5
116	Me	H	Br	H	H
117	Me	H	H	H	H
118	Me	Ac	Br	H	H
119	Me	Ac	H	H	H
120	Me	H	Br	Br	H
121	Me	H	Br	Br	Br
122	H	H	H	H	H



	R
123	Me
124	H

The blue-green alga *Lyngbia majuscula* is the causative agent of a severe contact dermatitis known as swimmer's itch in Hawaii. Aplysiatoxin (**108**) and debromoaplysiatoxin (**109**) have been identified as the major constituents responsible for the dermatitis (131). These compounds (**108, 109**) together with **112, 115** and **117** have also been known as tumor promoters (132).

Total syntheses of **108** and **109** have been reported by Kishi *et al* (133). Several other synthetic studies and formal synthesis have been published (134-138). Previous reviews on the aplysiatoxins have described isolation and structure determination studies (139, 140) and tumor promoting activity (141).

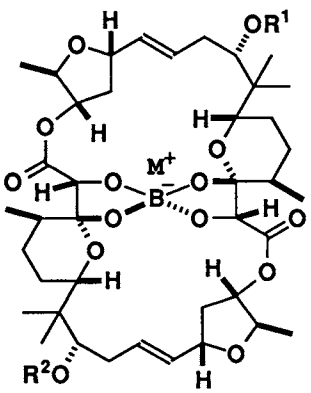
5.2 Aplasmomycins and Borophycin

Aplasmophycins are rare boron-containing natural products isolated from *Streptomyces griseus*, found in a shallow water sediment. Three congeners, aplasmomycin (**125**), aplasmomycin B (**126**) and C (**127**) have been reported (142, 143). The structure (**125**) of aplasmomycin, as elucidated by X-ray analysis on its Ag salt (144) displayed a symmetric macrodiolide complexed with boric acid (Boeseken complex). It was closely related to the only previous example, the ionophoric antibiotic boromycin (**128**), isolated from a soil microbe in the mid-1960s (145). Aplasmomycin B (**126**) is the monoacetate and aplasmomycin C (**127**) is the diacetate of **125**. The name aplasmomycin was derived from its antiplasmodial activity. *Plasmodium* is the genus name of malaria parasites. Recently a group led by Clardy and Fenical (146) reported the isolation and X-ray structural study of aplasmomycin C and deboroaplasmomycin C (**129**). They found by X-ray and NMR analysis that a pair of chromatographically separable compounds were actually conformational isomers of aplasmomycin C. The X-ray study indicates the presence of a number of geometric conformers in the solid state of aplasmomycin C.

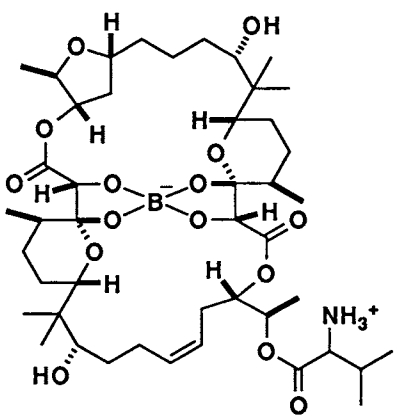
More recently Moore's group discovered another closely related compound named borophycin (**130**) as a potent cytotoxin from a marine strain of the blue-green alga *Nostoc linckia* (147). The gross structure was determined by spectroscopic methods and the relative configuration by X-ray studies in collaboration with Clardy. The authors also described the biosynthesis of borophycin which is slightly different from those of boromycin and aplasmomycin (148). All of them are acetate-derived polyketide that utilize a C_3 precursor for the starter unit and methionine for the methyl

branches on the polyketide chain. In the biosynthesis of aplasmomycin phosphoglycerate or phosphoenolpyruvate has been suggested to be the C_3 starter unit, while the unit in the biosynthesis of borophycin (**130**) was found to be derived from acetate and methionine. For details of the biosynthesis the reader is referred to the papers by Moore *et al.* (147) for borophycin and by Floss *et al.* (148) for aplasmomycin.

Two total syntheses for aplasmomycin have been reported by Corey *et al.* (149) and by White *et al.* (150). Two other groups have also described formal total syntheses which were included in the review by Norcross and Paterson (93). Chemical and biological properties of the aplasmomycins have been described in reviews by Omura (16) and Okami (151).



	R^1	R^2
125	**H**	**H**
126	**H**	**Ac**
127	**Ac**	**Ac**



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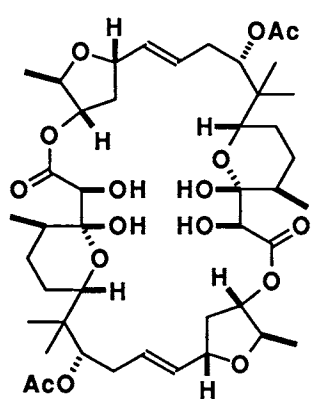

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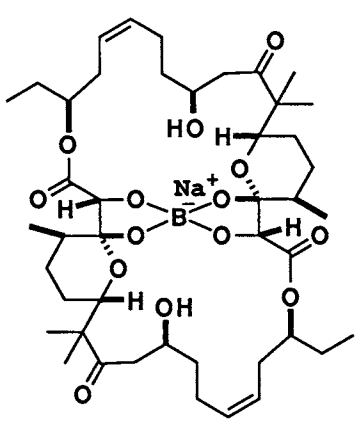

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5.3 Swinholides and Misakinolides

The swinholides and the misakinolides (=bistheonellides) are closely related macrolides obtained from different species of the sponge genus *Theonella*. Representative compounds of each group, swinholide A (**132**) and misakinolide A (**141**), have dimeric lactone structures with C_2 symmetry and are essentially identical except that **141** has two double bond units (CH=CH) less than **132**. These compounds exhibit potent cytotoxicity as shown in Table 5.

Table 5. Cytotoxicity of swinholides and related compounds

Compound name	Compound No.	IC_{50} (μg/mL) L1210	IC_{50} (μg/mL) KB	Reference
Swinholide A	**132**	0.03	0.04	153,169
B	**133**	0.30	0.04	158,169
C	**134**	0.14	0.05	158,169
D	**136**	0.19	0.01	159
E	**137**	0.35	0.16	159
F	**138**	0.03	0.01	159
G	**139**	0.06	0.07	159
Isoswinholide A	**135**	1.35	1.1	158,169
Misakinolide A	**141**	0.035	0.01[a]	154,169
Bistheonellide B	**142**	3.4	>10	174
C	**143**	5.6	>10	174
Isobistheonellide A	**144**	0.76	1.3	174

a This is a result obtained against P388.

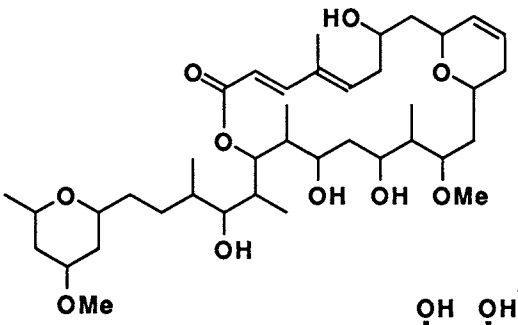
5.3.1 Swinholides. Swinholide A (**132**) was first reported by Carmely and Kashman from the Red Sea sponge *Theonella swinhoei* in 1985 (152). Based on the formula $C_{39}H_{66}O_{10}$ deduced from elemental analysis and 2D NMR connectivity study with its tetraformate derivative, they proposed a 22-membered monomeric macrolide structure (**131**). On the other hand, Kitagawa's group later isolated the same compound from the same species of the sponge collected in Okinawa (153). By forming di-, tri-, and tetra-*p*-bromobenzoate derivatives, a method which was used successfully to determine the dimeric nature of misakinolide A (154), the Osaka group demonstrated that swinholide A was indeed a dimer having a 44-membered dilactone structure (**132**) and not the monomeric structure **131**. They subsequently determined the stereostructure of swinholide A by X-ray

analysis on a dimeric derivative. Application of the MTPA ester method and the exciton chirality method on other derivatives established the absolute configuration as shown in **132** (155, 156). The X-ray structure revealed the conformational characteristics of swinholide A, in which oxygen atoms were directed toward the interior of the ring. The authors suggested its possible relevance to cytotoxic activity (157).

Kitagawa's group has also reported the isolation of minor constituents of *T. swinhoei*, swinholides B (**133**) and C (**134**) and isoswinholide A (**135**) and the elucidation of their structures by spectroscopic correlation with **132** (158). Additional congeners swinholides D-G (**136-139**) have been reported by Kobayashi's group from a sponge, *Theonella* sp. which was also collected in Okinawa (159). The latter group also isolated a seco acid corresponding to the monomer of **132**. The same acid (preswinholide A) was again described as a constituent of a Papua New Guinean sample of *T. swinhoei* (160).

Swinholide A has been suggested to be produced by a symbiotic blue-green alga (cyanobacteria) in *T. swinhoei* because of its close structural similarity, including the absolute configuration, to the blue-green metabolites, scytophycins (e.g., scytophycin C, **140**) (161) and of the fact that the sponge contained a filamentous organism which appeared to be a cyanobacterium (155). Recently, Faulkner *et al.* (162) examined this problem with *T. swinhoei* and showed that swinholide A was produced by the mixed population of unicellular heterotrophic bacteria in the sponge. By transmission electron microscopy, they found the presence of four distinct cell populations in *T. swinhoei*: eukaryotic sponge cells, unicellular heterotrophic bacteria, unicellular cyanobacteria, and filamentous heterotrophic bacteria. They separated these cells and analyzed the metabolites of each cell type by HPLC and NMR. The results clearly indicated that swinholide A (**132**) was contained only in the unicellular heterotrophic bacteria and that an antifungal cyclic peptide occurred in the filamentous heterotrophic bacteria. No major metabolites could be located in the cyanobacteria or sponge cells. This is a significant finding that opens a path of research for the mass production of potential pharmaceutical agents.

The total synthesis and synthetic studies of the swinholides have been reviewed (93). The first total synthesis was reported by Paterson *et al.* in 1994 (163-167) and the second by Nicolaou *et al.* (168) in 1996.



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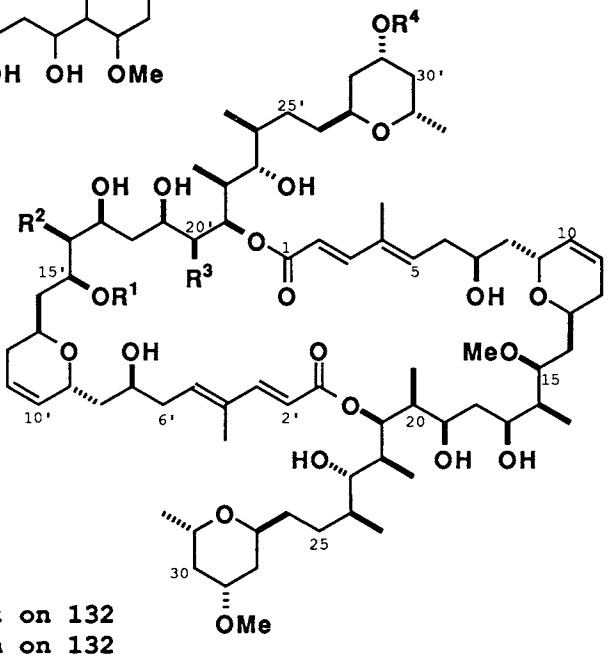
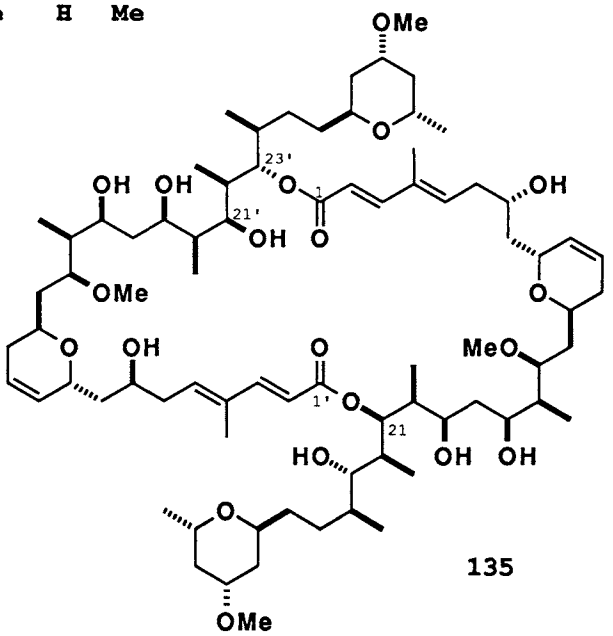


	R^1	R^2	R^3	R^4
132	Me	Me	Me	Me
133	Me	H	Me	Me
134	Me	Me	Me	H
136	H	Me	Me	Me
137	6'-OH substituent on 132			
138	2'*Z* configuration on 132			
139	Me	Me	H	Me



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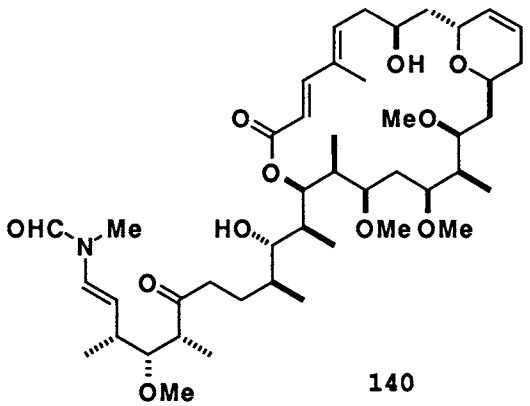


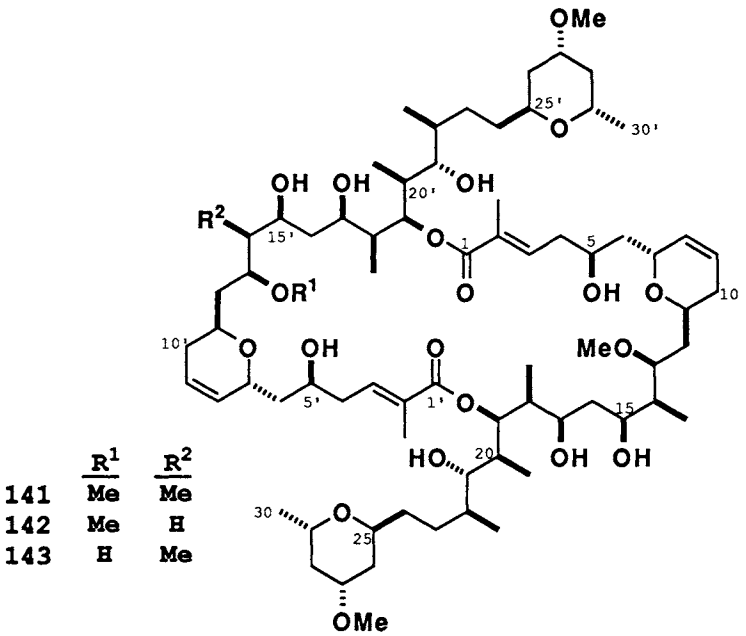
140

The structure-cytotoxicity correlation has been reported by Kitagawa *et al.* (169). Acid treatment of **132** resulted in shifts in the positions of the lactonic linkages giving rise to five diolides of different ring sizes: 19,19'-diolide, 19,21'-diolide, 17,23'-diolide, 21,23'-diolide (**135**), 23,23'-diolide. These together with monomeric lactones and derivatives prepared from **132** were assayed against L1210 and KB cell lines. The results revealed that none of them were as potent as swinholide A. Some of the derivatives and swinholide A were also submitted to *in vivo* antitumor assay against P388 mouse leukemia, but none was found to be interesting. A study of the mechanism of cytotoxicity of swinholide A was published recently (170). It was attributed to its ability to disrupt actin cytoskeleton, to sequester actin dimers, and to sever actin filaments.

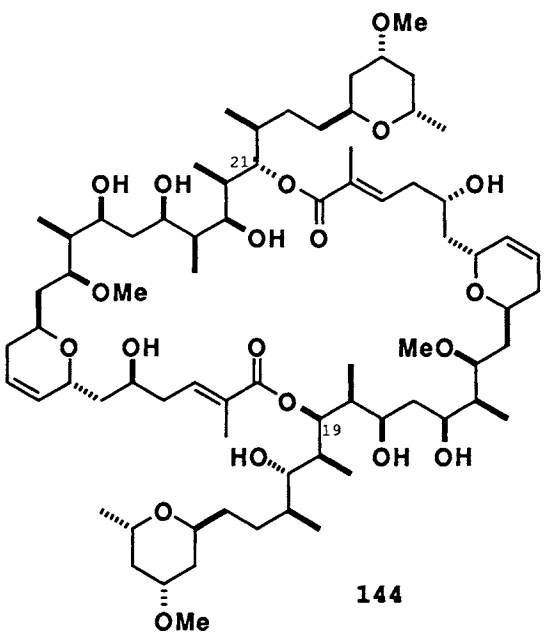
5.3.2 Misakinolides. Misakinolide A (**141**) is a 40-membered dimeric macrodiolide with C_2 symmetry as noted above. Since it has been described in Volume 5 of this series, the reader is referred to it for further details (171). This compound is sometimes called bistheonellide A. However, since it was first reported as misakinolide A (172), we recommend the initial name to be used. It would be appropriate to use the name bistheonellides for other congeners which were discovered later and published under this name.

The stereostructure and the absolute configuration of misakinolide A (**141**) were determined by chemical correlation with swinholide A (**132**) as shown in Scheme 2 (173). Treatment of **141** with methanolic sodium methoxide gave the methyl ester **145**. Ozonolysis of the ester **145** followed by treatment with dimethyl

sulfide gave a trialdehyde which in turn was treated with semicarbazide and sodium acetate in aqueous methanol to a trisemicarbazone (**146**). This product was identical with the one derived from **132** in all respects including IR, UV, ^{1}H and ^{13}C NMR spectra and optical rotation.



	R^1	R^2
141	**Me**	**Me**
142	**Me**	**H**
143	**H**	**Me**



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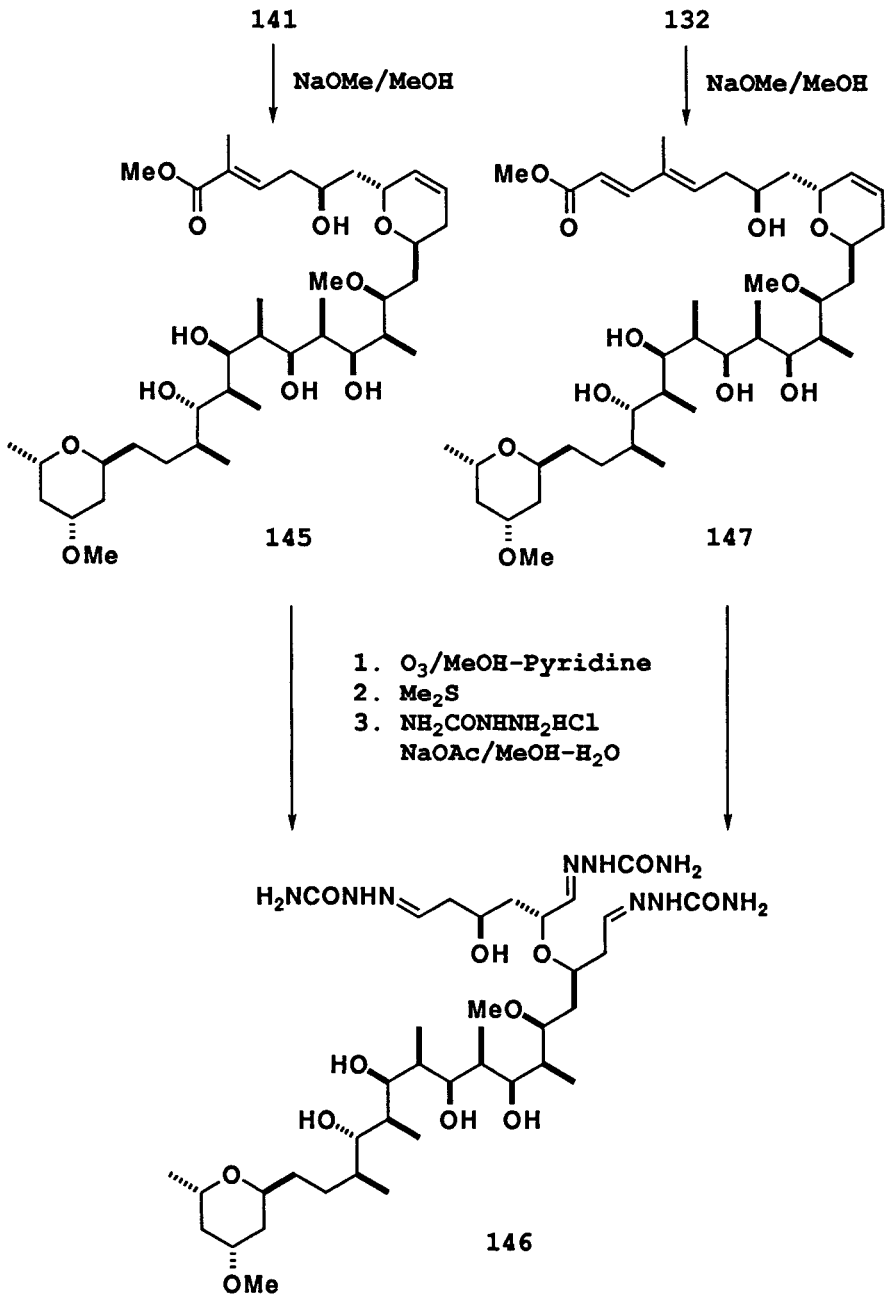
Scheme 2. Structural correlation of misakinolide A (**141**) with swinholide A (**132**)



Three congeners of misakinolide A have so far been isolated from *Theonella* spp. Bistheonellide B (14-demethylmisakinolide A) (**142**) has been reported from a *Theonella* sp. collected in Hachijo-jima, an island located in the south of Tokyo (154). On the other hand, Kobayashi *et al.* (174) have isolated from a *Theonella* sp. from Okinawa bistheonellide C (**143**) and isobistheonellide A (**144**) together with **141**, **142**, the monomeric acid of **141**, and a dimeric acid of **141** in which one lactonic linkage was hydrolyzed. The structures of these compounds were secured by spectral comparison with **141**.

As described earlier (171), misakinolide A showed potent cytotoxicity (IC_{50} 0.01 μg/mL) and moderate *in vivo* antitumor activity (T/C 145% at a dose of 0.5 μg/kg) against P388 leukemia. The cytotoxicity is comparable with that of swinholide A, but the *in vivo* efficacy of misakinolide A is better than that of swinholide A which shows T/C 115% at 0.03 mg/kg (169). Swinholide A is reported to be toxic at 0.05 mg/kg. Interestingly, recently Spector *et al.* have found that misakinolide A also acts on actin cytoskeleton but by a completely different mechanism from that of swinholide A (175).

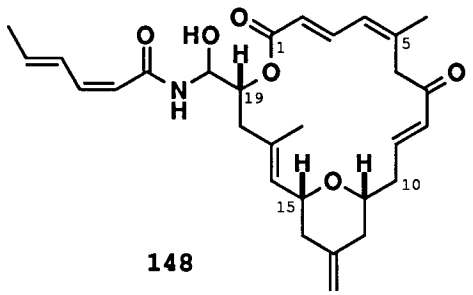
6. ALKALOIDAL MACROLIDES

Regardless of the nature of the nitrogen atom and its position in a molecule, all nitrogenous macrolides are classified here as alkaloidal macrolides. In these compounds a nitrogen atom may be found in a macrocyclic ring system or on a side chain. It occurs in a variety of functional groups, and the two most abundant forms are as an acyl amide, particularly formamide, and as an oxazole ring. These compounds have been reported mainly from sponges and mollusks and some from tunicates, dinoflagellates, and a sea pen.

6.1 Macrolides Having an Acyl Amide Other than Formamide

6.1.1 Zampanolide. In Section 4.1 we described the isolation of laulimalide (**46**) and neolaulimalide (**48**) from an Okinawan sponge. The same sponge collected at a different locality (Zampa-misaki) gave rise to a novel 20-membered macrolide designated as zampanolide (**148**) as a minor constituent (176). The structure was elucidated by 2D NMR analysis. The relative configurations at the chiral centers C11, C15, and C19 have been proposed by NOE observation. The configuration of C20 remains to be solved. The compound is unique as it contains a rare carbinol amine functionality. Although both laulimalide and zampanolide are 20-

membered macrolides which share some common structural features, the overall structures are different. Zampanolide exhibits potent cytotoxicity (IC_{50} 1-5 ng/mL) against P-388, A549, H729, and MEL28 cell lines.



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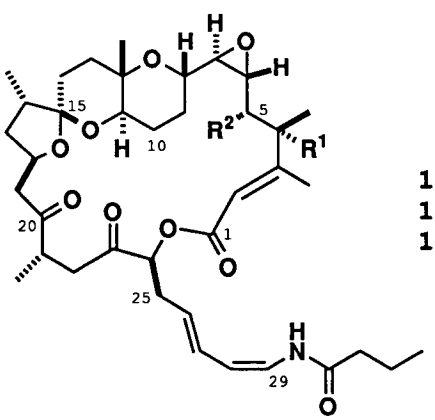
6.1.2 Lituarines. In 1992 French workers reported what appeared to be the first macrolides from an octocoral (177). The macrolides, lituarines A-C (**149-151**) were isolated from the sea pen *Lituaria australasiae* collected in New Caledonia. The structures were elucidated by NMR analysis mainly with lituarine C (**151**) and the relative stereochemistry by NOESY and ROESY experiments. The lituarines are 25-membered ring macrolides constructed on a backbone consisting of a C_{29} chain and five methyl branches. All three contain four ether rings and a butanoylamide group at the end of the chain. The lituarines showed potent cytotoxicity (IC_{50} in the range of 1-6 ng/mL) againt KB cells.

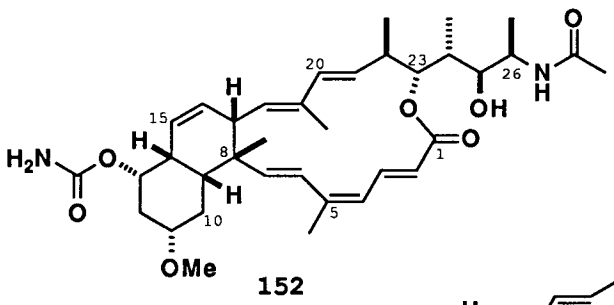


	R^1	R^2
149	**H**	**H**
150	**OAc**	**OH**
151	**OH**	**OH**

6.1.3 Superstolides. From the deep water sponge *Neosiphonia superstes* collected at a depth of 500 m off New Caledonia, Minale *et al.* isolated two highly cytotoxic macrolides, superstolides A (**152**)

and B (**153**). The major constituent superstolide A was reported first and much of the structural work was carried out with this compound (178). The gross structure was determined by extensive 2D NMR study with the lactone **152** and with its methyl ester derivatives (**154**, **155**) prepared by the treatment of **152** with sodium methoxide in methanol. The relative stereochemistry for the decaline portion was secured by NOESY data, and that for the C22-C26 portion by ^{1}H NMR coupling constant argument and by the application of Rychnosky's method (179) on the acetonide **156** derived from **154**. The absolute configuation was proposed as 8*R*, 9*R*, 11*S*, 13*S*, 14*S*, 17*R*, 22*R*, 23*R*, 24*R*, 25*S*, 26*R* on the basis of results obtained by the application of Horeau's method on **156** for the decalin portion and of modified Mosher's method using MTPA esters of **152** and **155** for the C22-C26 portion.

Superstolide B (**153**) was obtained as a minor constituent of the sponge (180). The structure as elucidated by NMR correlation with **152** reveals that it is a dehydrated derivative of superstolide A. Superstolides are 16-membered ring polyene macrolides fused with a decaline ring and containing two nitrogen atoms as an acetamide and a carbamate group. The latter functional group is rare in marine natural products. Previous examples are saxitoxin and its related marine toxins (181) and kabiramides (*vide infra*). Both superstolides A (**152**) and B (**153**) displayed potent cytotoxicity (IC_{50} μg/mL) against P388 (**152**: 0.003, **153**: 0.003), KB (**152**: 0.02, **153**: 0.005), non-small cell lung carcinoma NSCLC-N6-L16 (**152**: 0.04, **153**: 0.039), and human colon carcinoma HT29 (**152**: 0.04) cell lines.



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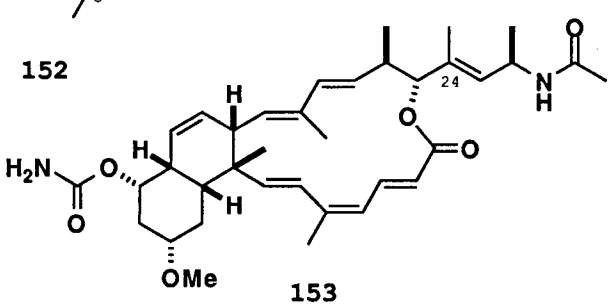

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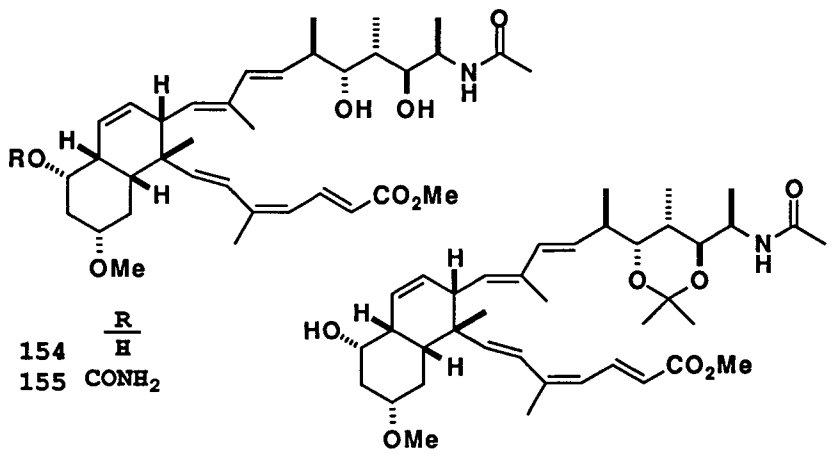
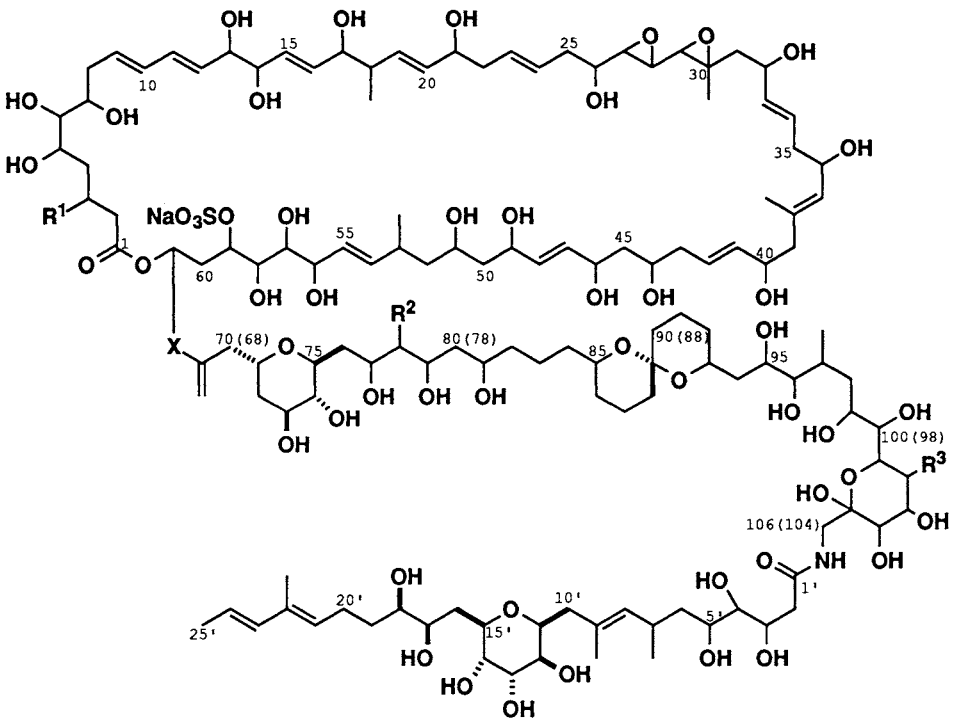
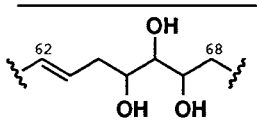
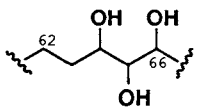


6.1.4 Zooxanthellatoxins. From the cultures of symbiotic dinoflagellates, *Amphidinium* spp. isolated from flatworms, Kobayashi's group has discovered a number of cytotoxic macrolides as described in Section 3.6. On the other hand, Nakamura and coworkers have found two potent vasoconstrictive compounds from a culture of another symbiotic dinoflagellate, *Symbiodinium* sp. isolated also from a flatworm. It turned out that the compounds designated as zooxanthellatoxins A (**157**) and B (**158**) were large polyol macrolides (A: $C_{140}H_{232}NO_{57}SNa$, B: $C_{138}H_{230}NO_{56}SNa$) comparable to palytoxin (117) in the molecular size. After the publication (182) of partial structures in 1993, Nakamura *et al.* reported the full gross structure of zooxanthellatoxin A (**157**) in 1995 (183). The structure with partial relative stereochemistry was elucidated by extensive spectroscopic analysis and chemical degradation. The gross structure of zooxanthellatoxin B (**158**) was subsequently reported (184). It was a congener having a ring of the same size and a side chain shorter than that of zooxanthellatoxin A by two carbons. Both macrolides contain the same C_{25}-acyl amide function on the side chain. The lactone ring consisting of 62 atoms is the largest, surpassing that (60-membered ring) of quinolidomicin A_1, an actinomycete metabolite (185). The stereochemistry of these interesting molecules remains to be elucidated. An approach to this end is the enantioselective synthesis of the degradation products. Recently Nakamura *et al.* determined the absolute configurations for the C11'-C18' portion of the common acyl side chain to be 11'*S*, 12'*R*, 13'*S*, 14'*R*, 15'*R*, 17'*R*, and 18'*R* by synthesis of a degradation product (186).



	R^1	R^2	R^3	X
157	H	OH	OH	
158	OH	H	H	

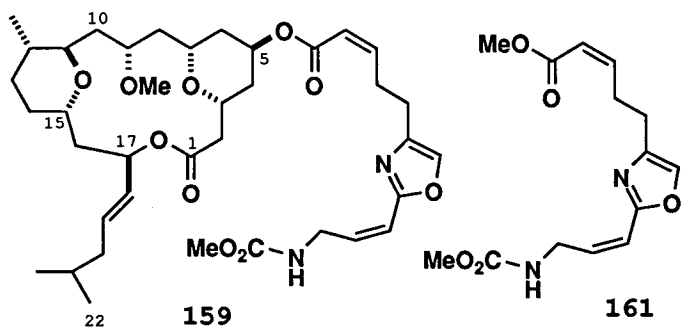
6.2 Macrolides Having Oxazole or Thiazole Rings

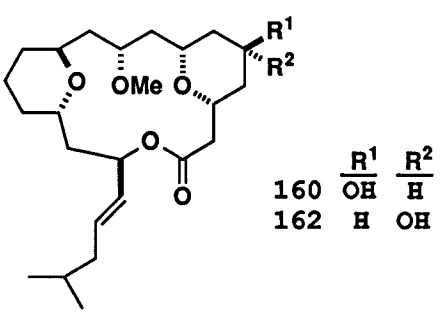
In this subsection we consider those macrolides containing one or two non-consecutive oxazole and/or thiazole rings. They are leucascandrolide A, pateamine, patellazoles, phorboxazoles, and theonezolides. The latrunculins which contain a thiazolidinone ring will also be described here. Those macrolides having three contiguous oxazoles as in the ulapualides will be discussed in the next subsection.

6.2.1 Leucascandrolide A. Leucascandrolide A (**159**) was recently reported by Pietra *et al.* (187) from the calcareous sponge

Leucascandra caveolata collected in New Caledonia. The gross structure and relative configuration were deduced from spectroscopic data. In order to determine the absolute configuration by the application of the MTPA ester method, the macrolide **159** was treated with Na_2CO_3 in methanol to give the macrolide alcohol (**160**) and the methyl ester (**161**) of the acyl side chain. Since the MTPA esters from **160** gave scattered $\Delta\delta$ = (δ (*S*)- δ (*R*)) data which precluded confident determination, the C5 epimer **161** was prepared by oxidation (PCC) of the hydroxyl to a ketone followed by reduction ($NaBH_4$). The reduction gave **162** in 87% and **160** in 5% yield. The MTPA esters prepared from **162** gave a clear result, establising the 5*S* configuration to **162** and thus 5*R*-configuration to leucascandrolide A (**159**).

Leucascandrolide A is a potent cytotoxin exhibiting IC_{50} 0.05 and 0.25 μg/mL against KB and P388 cell lines, respectively. It also showed potent antifungal activity against *Candida albicans*. Both the derivatives **160** and **161** also showed cytotoxic and antifungal activity.

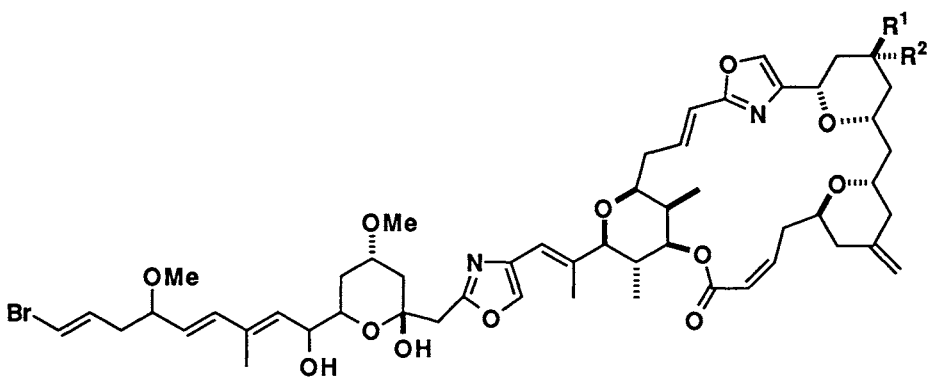




6.2.2 Phorboxazoles. Two isomeric macrolides, phorboxazoles A (**163**) and B (**164**), have been described as cytostatic and antifungal

constituents of an Indian Ocean sponge, *Phorbas* sp. (188). The structures with relative stereochemistry for the ring portions were secured by extensive 2D NMR study on the major compound, phoboxazole A. Phoboxazole B was found as the C13 epimer of **163**. These compounds contain two oxazoles, one in the macrocycle and the other in the side chain; four hydropyrans; and a bromine atom.

Both compounds exhibited potent antifungal activity against *Candida albicans* and *Saccharomyces carlsbergensis*. Neither compound was antibacterial against *Escherichia coli*, *Pseudomonas aeruginosa*, or *Staphylococcus aureus*. In the NCI 60 cell lines panel both compounds showed exceptional inhibition of cell growth. According to the authors the phorboxazoles are among the most potent cytostatic agents yet discovered.

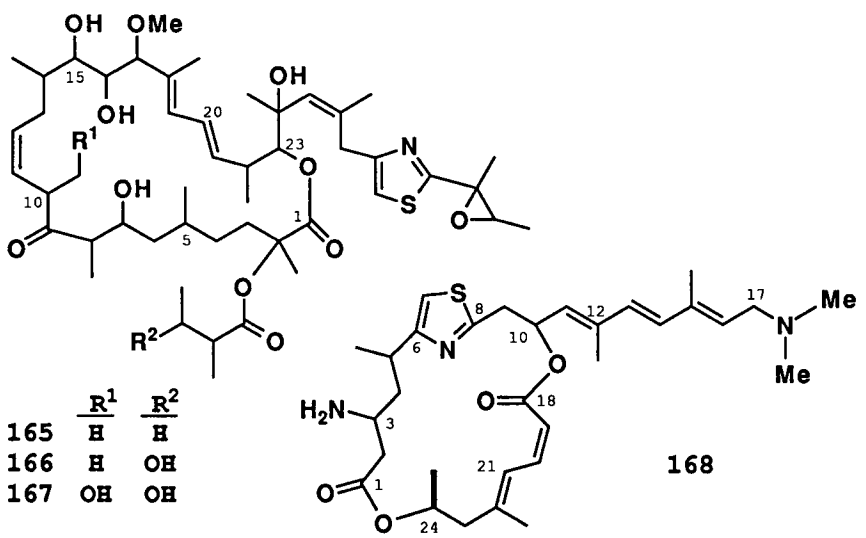


	R^1	R^2
163	OH	H
164	H	OH

6.2.3 Patellazoles. Thiazole is not uncommon as a structural element in marine natural products. Some noted examples of the thiazole-containing compounds are antitumor peptides such as dolastatins -3 and -10 from the sea hare *Dolabella auricularia* and ulithiacyclamide from the tunicate *Lissoclinum patella* (189). The latter animal has yielded a dozen cyclic peptides which contain at least one thiazole ring (189). It was also found to be a source of macrolides. In 1988 two groups simultaneously reported the discoveries of patellazoles, 24-membered ring macrolides containing a thiazole. Ireland *et al.* described patellazoles A-C (**165-167**) from the tunicate collected in Fiji (190) and Moore *et al.* reported patellazole B (**166**) from a sample of Guam (191). The former group established the gross structures by spectroscopic analysis and

partial chemical degradation of patellazole C, while the latter group elucidated the structure of patellazole B by solely spectroscopic methods. No stereochemistry has yet been reported. Patellazoles are potent cytotoxins (mean IC_{50} 10^{-3}-10^{-6} μg/mL in the NCI human cancer cell lines) and exhibit antifungal activity against *Candida albicans*.

6.2.4 Pateamine A. From a different collection of the same sponge, *Mycale* sp., which earlier yielded novel antiviral compounds, the mycalamides (192, 193), Munro *et al.* (194) isolated a potent cytotoxin, pateamine A (P388: IC_{50} 0.15 ng/mL). The gross structure elucidated by spectroscopic analysis revealed that pateamine A (**168**) was a thiazole-containing macrodiolide. It bears two additional nitrogen atoms as a primary and a tertiary amine. The thiazole ring is a part of the 19-membered macrolide ring. Except for one chiral center, full stereochemistry has not yet been disclosed at this writing. The absolute configuration at C24 as (*S*) was assigned by an asymmetric synthesis of methyl (2*Z*, 4*E*)-7-hydroxyoctadienoate and and its correlation to a degradation product (195). Besides its potent cytotoxicity pateamine A has been noted to have immunomodulating properties (195).



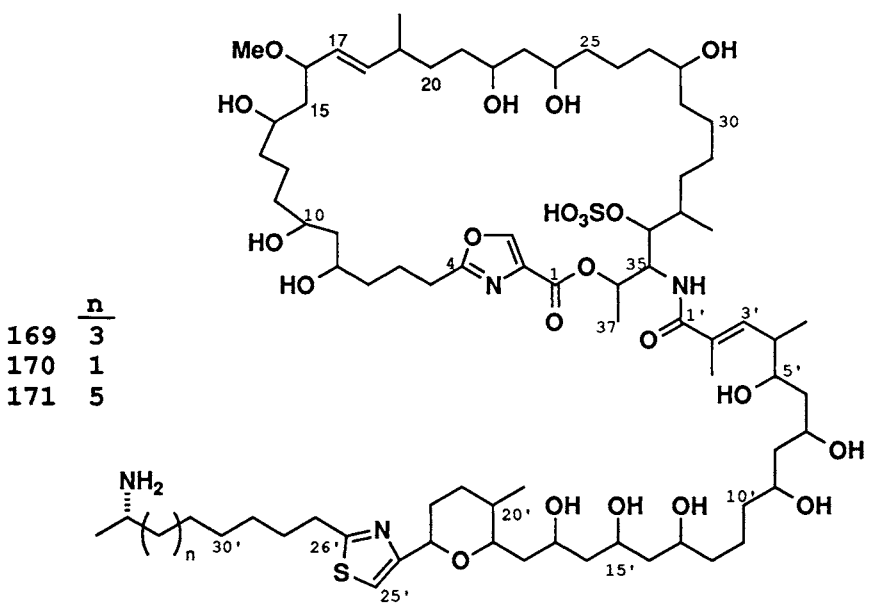
	R^1	R^2
165	H	H
166	H	OH
167	OH	OH

168

6.2.5 Theonezolides. Theonezolides are macrolides which contain oxazole and thiazole rings. Theonezolide A (**169**) was

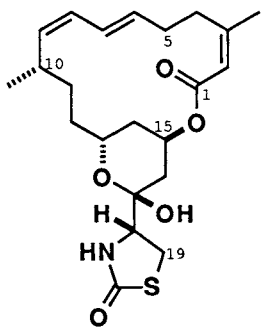
reported first as a metabolites from a sponge, *Theonella* sp. collected in Okinawa (196). The structure was elucidated by a combination of spectroscopic methods and chemical degradation. Ozonolysis of **169** gave four fragments corresponding to C4-C17, C18-C37, C3'-C24', and C26'-C36'. Identification of these fragments complemented the NMR connectivity study and helped to assign the gross structure of theonezolide A as a 36-membered ring macrolide. The oxazole-containing macrolide ring is connected through an amide bond to a long side chain bearing a thiazole and a hydropyran ring and an amino group. It also contains a sulfate group.

Two homologues, theonezolides B (**170**) and C (**171**), have subsequently been reported from the same sponge (197). The three compounds differ only in the length of the methylene portion of the side chain, but otherwise they are identical as proved by comparing the ozonolysis products of each. Thus all three of the four ozonolysis products, corresponding to the terminal portion of the side chain, were prepared by asymmetric synthesis for their identification. The synthesis also allowed the assignment of the absolute configuration at the terminal chiral center. The configurations of 22 other centers remain to be determined. Theonezolides A, B, and C were cytotoxic against L1210 (IC_{50} 0.75, 5.6, and 0.3 μg/mL, respectively) and KB cells (IC_{50} 0.75, 11, and 0.37 μg/mL, respectively).

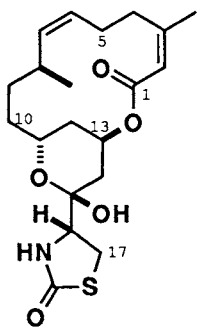


	n
169	3
170	1
171	5

6.2.6 Latrunculins. The latrunculins are 16- and 14-membered ring macrolides containing a 2-thiazolidinone as a common appendage. Six macrolides (**172-177**) of this class have been isolated from sponges and nudibrachs. Latrunculins A (**172**) and B (**173**) were first reported by Kashman *et al.* (198) in 1980 as ichthyotoxic constituents of the Red Sea sponge *Latrunculia magnifica*. The structure and relative stereochemistry of latrunculin A were determined by X-ray on the methyl ketal derivative prepared by converting the hemiketal hydroxyl group into a methoxy group (198, 199). Kashman's group also determined the absolute configuration of latrunculin A by comparison of its degradation product, ethyl 2-oxo-4-thiazolidinecarboxylate, with that of a synthetic sample prepared from L-cysteine (200).



172



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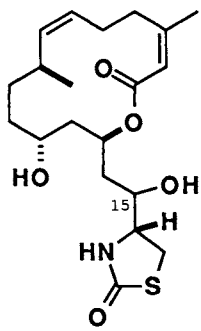

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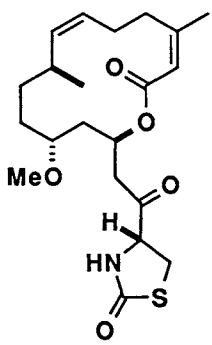
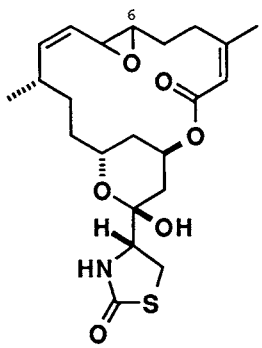


175



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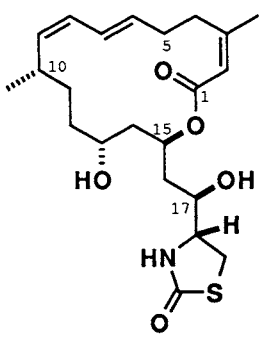


177

We recently confirmed the structure and absolute configuration of latrunculin A (**172**) by performing direct X-ray analysis with a

crystal of latrunculin A obtained from an Okinawan sponge (59).

The structure of latrunculin B (**173**) was secured by extensive NMR analysis and comparison with **172** (199, 200). Kashman's group also reported latrunculins C (**174**) and D (**175**) and 6,7-epoxylatrunculin A (**176**) from the same sponge (200, 201). Latrunculins C and D are derivatives of latrunculin B and could thus be assumed to have the same stereochemistry. In fact $NaBH_4$ reduction of **173** afforded two C15 epimeric alcohols, one of which was identical with **174**. However, the configuration at C15 still remains to be established.

From the Okinawan sponge which yielded latrunculin A and laulimalides (*vide supra*), we recently isolated a new latrunculin, latrunculin S (**177**), as a minor cytotoxic constituent (60). Its structure was correlated with latrunculin A by $NaBH_4$ reduction of **172** in the same manner as that employed by Kashman *et al.* The absolute configuration of C15 was established by the application of MTPA ester method. Latrunculins have often been reported from different sponges (56-60) and their predator nudibranchs of the genus *Chromodoris* (56, 202-204). Syntheses of the latrunculins are covered in a recent review by Norcross and Paterson (93). Two total syntheses of latrunculin A have been published, one by Smith *et al.* (205) and another by White and Kawasaki (206). Smith also achieved a total synthesis of latrunculin B and a formal synthesis of latrunculin C (205, 207). The synthesis of the common structural moiety, the tetrahydropyranylthiazolidin-2-one system, has been reported by Kashman *et al.* (200, 208). They have also described rearrangement and transformation reactions of latrunculin B (209).

Besides being toxic to fish, latrunculins A (**172**) and B (**173**) have been shown to have various biological properties. Okuda and Scheuer demonstrated that latrunculin A was highly antifungal against *Candida albicans*, but not antibacterial against *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, or *Pseudomonas aeruginosa* (202). Similar trend of the activity was also observed with latrunculin B (210). Different levels of cytotoxicity have been recorded with **172** by Scheuer *et al.* (IC_{50} 0.15 μg/mL againt KB) (56), Crews *et al.* (IC_{50} 0.072 and 0.23 μg/mL againt HEP-2 and MA-104, respectively) (203), Gunasekera *et al.* (IC_{50} 4.1 μg/mL againt P388) (58), and by Higa *et al.* (IC_{50} 0.1-0.25 μg/mL againt P388, A549, HT29, MEL28) (211). Latrunculin S (**177**) exhibited IC_{50} 0.5-1.2 μg/mL againt the latter cell lines (60, 211).

Most importantly, latrunculins are used as valuable tools in

researches in the field of cell biology. Latrunculins A and B have been shown to induce striking changes in the morphology of nonmuscle cells that are reversible upon removal of the agents. These changes occur by disruption of the microfilament organization without affecting the microtubular system (212). The latrunculins were also shown to be potent inhibitors of the microfilament-mediated processes involved in fertilization and in cell division (213). Inhibition of actin polymerization by latrunculin A was demonstrated to be effected by the formation of a 1:1 molar complex between latrunculin A and G-actin (214).

6.3 Macrolides Containing a Formamide

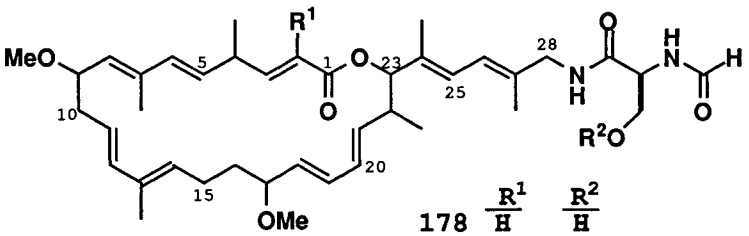
There are a number of marine macrolides which contain a formamide at the terminus of a long side chain. These compounds can be further classified into four distinctive groups: the iejimalides (four compounds), the sphinxolides (six compounds), the aplyronines (three compounds), and the ulapualides and related compounds (22 compounds). The latter is by far the largest group consisting of rather unique macrolides as they contain three contiguous oxazole rings as a common structural unit. Except for the group of iejimalides, the compounds of three other groups have closely related structural features in spite of their differences in the macrocyclic portion. Furthermore, other than the aplyronines, stereochemistries of these macrolides largely remain to be elucidated. Most of these compounds are potent cytotoxins.

6.3.1 Iejimalides. The iejimalides were reported by Kobayashi *et al.* as cytotoxic constituents of the tunicate *Eudistoma* cf. *rigida* collected at Iejima, Okinawa. The structures of iejimalides A (**178**) and B (**179**) were described in 1988 (215) followed by those of iejimalides C (**180**) and D (**181**) in 1991 (216). The structure elucidation was carried out mainly with iejimalide B by spectroscopic methods. The iejimalides are highly unsaturated 24-membered ring macrolides having an N-formylserine residue. Iejimalide B (**179**) differs from iejimalide A (**178**) by the presence of an additional methyl group at C2, and iejimalides C and D are their sulfate esters.

The serine residue was identified as the L-isomer by amino acid analysis of the hydrolysate of **179**. The *anti* relationship of H22 and H23 was the only other stereochemical implication made by the authors. Thus, the configurations at five chiral centers in the

ring portion essentially remain to be resolved. As a step toward a total synthesis and to assign the stereochemistry of the iejimalides, Helquist *et al.* recently achieved an enantioselective synthesis of the C17-C28 portion of the macrolides (217).

Cytotoxicities of iejimalides A and B have been reported against L1210 (IC_{50} 0.062 and 0.032 μg/mL) and L5178Y cells (IC_{50} 0.022 and 0.001 μg/mL) and iejimalides C and D against L1210 (IC_{50} 10 and 0.58 μg/mL) and KB cells (IC_{50} 4.7 and 0.2 μg/mL). These results reveal that iejimalides A and B have much stronger activity than their sulfate ester derivatives.

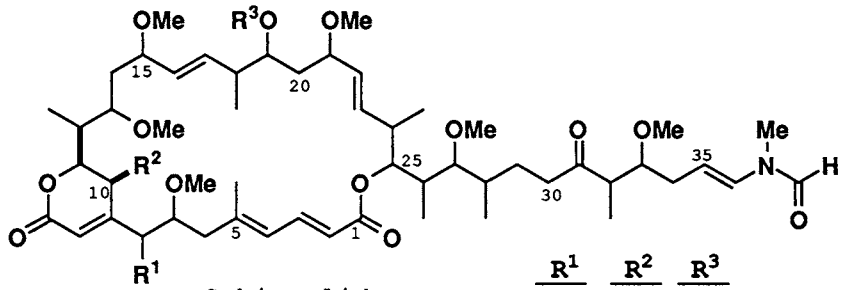


	R^1	R^2
178	H	H
179	Me	H
180	H	SO_3Na
181	Me	SO_3Na

6.3.2 Sphinxolides and Reidispongiolides. Six macrolides from three sources enter discussion here. They are sphinxolide (**182**), sphinxolides B-D (**183-185**), and reidispongiolides A (**186**) and B (**187**), having the same carbon skeleton (C_{45}) and a ring system consisting of a 26-membered macrocyclic lactone and an additional δ-lactone. Sphinxolide (**182**) was first reported in 1989 by Pietra *et al.* as a potent cytotoxin isolated from an unidentified Hawaiian nudibranch (218). The name was derived from the mysterious Egyptian Sphinx, reflecting the authors' difficulty in defining the source and, for some time, the structure of the compound. Like all other compounds containing the terminal N-alkenyl-N-methylformamide, sphinxolide existed as an inseparable 2:1 mixture of rotational isomers as evidenced by the observation of doubled ^1H and ^{13}C NMR signals for the affected region. Such phenomenon is due to the restricted rotation around the C-N amide bond. The gross structure of sphinxolide (**182**) was secured by extensive 2D NMR analysis.

In 1993 Minale *et al.* reported the isolation and structure elucidation of three new congeners, sphinxolides B-D (**183-185**) together with **182** from the New Caledonian deep sea sponge *Neosiphonia superstes* (219). This sponge was also the source of

another class of macrolides, superstolides as described above (Section 6.1.3). The gross structures of the new compounds were determined by interpretation of NMR data as well as by comparison of spectral data with those of **182**. Except for the relative configurations at C10 and C11, no stereochemistries of the sphinxolides have been determined. These compounds exhibited potent cytotoxic activity against several tumor cell lines (Table 6).



		R1	R2	R3
Sphinxolide	**182**	OMe	OH	H
Sphinxolide B	**183**	H	OH	H
C	**184**	OMe	OH	Me
D	**185**	H	OH	Me
Reidispongiolide A	**186**	H	H	Me
B	**187**	H	H	H

Table 6. Cytotoxicity of sphinxolides (**182-185**) and reidispongiolides (**186, 187**)

Compound	IC_{50} (μg/mL)				
No.	P388	P388/Dox	KB	HT29	NSCLC-N6
182	0.0041	0.00033	0.007	0.115	0.027
183	0.0031	0.00002	0.00003	0.0024	0.016
184	0.04	0.03	0.04	0.03	0.03
185	0.003	0.008	0.003	0.022	0.06
186	0.16	0.01	0.1	0.04	0.07
187	0.06	0.02	0.06	0.04	0.05

P388: Murine lymphocytic leukemia. P388/Dox: P388 expressing the multi-drug resistance gene mdr, especially towards dexorubicine. KB: Human nasopharyngeal carcinoma. HT29: Human colon carcinoma. NSCLC-N6: Human bronchopulmonary non-small-cell lung carcinoma.

Subsequently the Italian group has found another New Caledonian deep sea sponge as a source of related compounds (220). Separation of

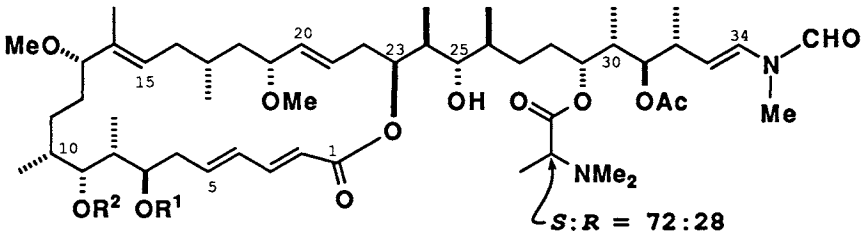
the cytotoxic extract of the sponge *Reidispongia coerulea* gave rise to two new compounds, reidispongiolides A (**186**) and B (**187**) along with sphinxolides B (**183**) and D (**185**) as the active constituents. The gross structures of the new macrolides were secured by comparison of spectral data with those of the sphinxolides. The new congeners, which lack a hydroxyl group at C10, also showed strongly cytotoxic activity but not as potent as the sphinxolides (Table 6).

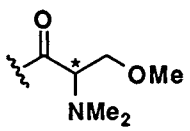
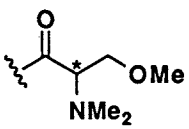
6.3.3 Aplyronines. The sea hare *Aplysia kurodai* has received much attention in the early stages of the development of marine natural product chemistry, as it was the source of the first brominated sesquiterpenes, e.g. aplysin, as reported by Yamamura and Hirata in 1963 (221). Thirty years later, interest in the animal was renewed by the discovery of exceedingly potent antitumor compounds. Thus, in 1993 Yamada *et al.* have reported the isolation of minute constituents termed aplyronines A-C (**188-190**) from *A. kurodai* and their structure elucidation (222). The isolation was guided by a cytotoxicity assay using HeLa-S_3 cells, and the yields of aplyronines ranged from 2.5 X 10^{-5}% (**188**) to 3 X 10^{-7}% (**190**). The gross structures were determined mainly with aplyronine A by spectroscopic analysis. All three compounds were 24-membered ring macrolides, differing from each other by the presence and/or by the position of an N,N,O-trimethylserine ester function. This amino acid and N,N-dimethylalanine moieties were found to be scalemic in the ratios of *S*:*R* = 52:48 and 72:28, respectively. Thus, each of the aplyronines are a mixture of not only rotational isomers caused by the formamide moiety, but also diastereomers due to the presence of the scalemic amino acids.

Aplyronines A (**188**), B (**189**), and C (**190**) showed potent cytotoxicity against HeLa-S_3 cells with IC_{50} 0.039, 4.39, and 159 ng/mL, respectively. Even more impressive were the *in vivo* results with aplyronine A, displaying T/C = 545 (dose: 0.08 mg/kg), 556 (0.04 mg/kg), 398 (0.04 mg/kg), 255 (0.08 mg/kg), and 201% (0.04 mg/kg) against P388, Lewis lung carcinoma, Ehrlich carcinoma, colon 26 carcinoma, and B16 melanoma, respectively (222).

Yamada's group have subsequently determined the absolute stereochemistry of aplyronine A as shown in **188** by a combination of degradation study and enantioselective synthesis of all of the chiral fragments (223-225). These synthetic efforts finally led them to the first total synthesis of aplyronine A (226, 227). Syntheses of aplyronines B (**189**) and C (**190**) and thus of their

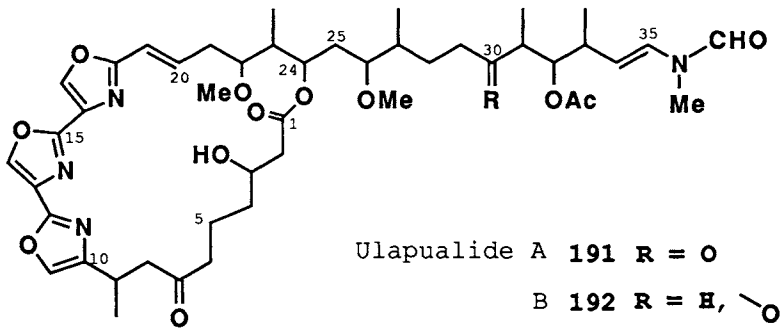
absolute stereostructures have also been described by the same group (228).



	R^1	R^2	
188		H	
189	H		* S:R = 52:48
190	H	H	

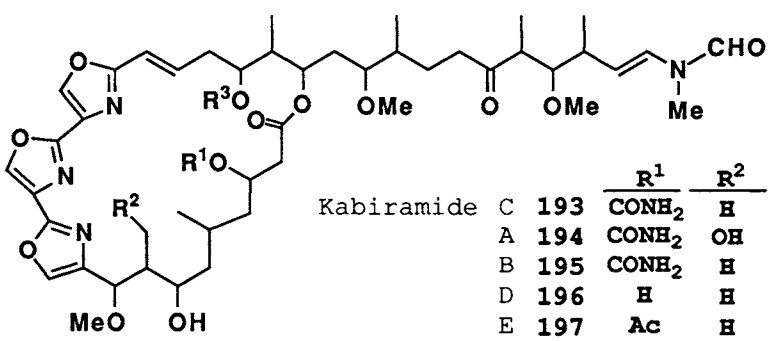
It should be noted that the substitution pattern and the absolute configuration of the side chain portion of the aplyronines are almost identical with those of scytophycin C (**140**). Furthermore, the striking resemblance of the side chain portion to those of the sphynxolides and of the ulapualides and related compounds suggests the possibility of the same stereochemistry for all of them.

6.3.4 Ulapualides and Related compounds. *Hexabranchus sanguineus* is a large, colorful nudibranch. It is sometimes called the Spanish dancer. It deposits red egg masses which resemble rose flowers. Although the egg masses are exposed and vulnerable, they seem to be rarely preyed on by predators. This observation led Roesener and Scheuer to investigate the organic constituents of the eggs. They found two extraordinary macrolides named ulapualides A (191) and B (192) which exhibited potent cytotoxicity (IC_{50} 0.01-0.03 μg/mL) against L1210 cells and antifungal activity against *Candida albicans* (229). The gross structures of ulapualides were determined mainly by spectoscopic analysis and partly by degradation which allowed the authors to resolve the linkage of an unprecedented tris-oxazole moiety.



A closely related macrolide, kabiramide C (**193**) was reported simultaneously by Fusetani *et al.* from the egg masses of *Hexabranchus* sp., collected at Kabira Bay, Ishigaki, Okinawa (230). The gross structure of **193** was solved mainly by 2D NMR study. It was reported to show marked antifungal activity against several fungi including *C. albicans* and *Aspergillus niger*.

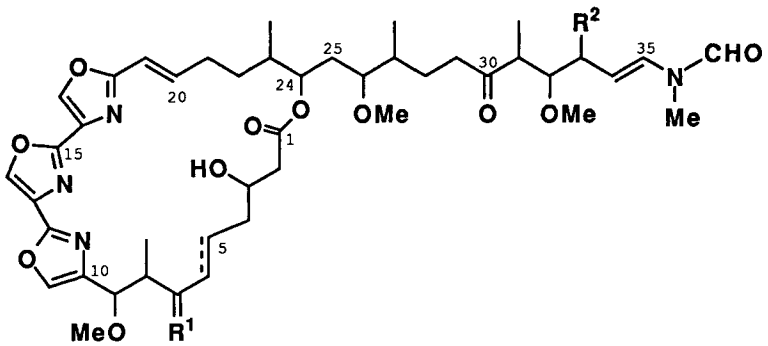
Since the discoveries of the ulapualides and kabiramide C in 1986, 22 closely related macrolides and one seco-derivative have been reported from sponges, nudibranchs, and recently from a coral. Other than ulapualides and kabiramides, related macrolides have been described under the names of halichondramides, mycalolides, and jaspisamides.



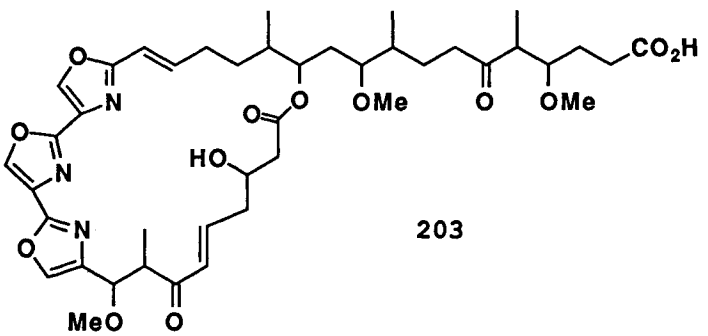
			R^1	R^2	R^3
Kabiramide	C	**193**	$CONH_2$	H	Me
	A	**194**	$CONH_2$	OH	Me
	B	**195**	$CONH_2$	H	H
	D	**196**	H	H	Me
	E	**197**	Ac	H	Me

From the *Hexabranchus* egg masses collected at two locations in Japan, Fusetani's group isolated six additional compounds, kabiramides A (**194**), B (**195**), D (**196**), and E (**197**), dihydrohalichondramide (**198**), and 33-methyldihydrohalichondramide (**199**). All of them including **193** showed potent cytotoxic activity

against L1210 with IC_{50} values ranging from 10 to 50 ng/mL (231). Meanwhile, Faulkner's group also encountered related compounds in their studies of antifungal metabolites from the nudibranch *H. sanguineus* and two species of the sponge genus *Halichondria* (232, 233). They were kabiramide C (**193**), halichondramide (**200**), dihydrohalichondramide (**198**), tetrahydrohalichondramide (**201**), isohalichondramide (**202**), acid **203**, imide **204**, and seco-derivative **205**. Compounds **198**, **200**, **201**, and **202** showed antifungal activity and inhibition of the cell division of fertilized sea urchin eggs (233).



		R^1	R^2	Δ^5
Dihydrohalichondramide	**198**	O	H	
33-methyldihydrohalichondramide	**199**	O	Me	
Halichondramide	**200**	O	H	*E*
Tetrahydrohalichondramide	**201**	H,OH	H	
Isohalichondramide	**202**	O	H	*Z*

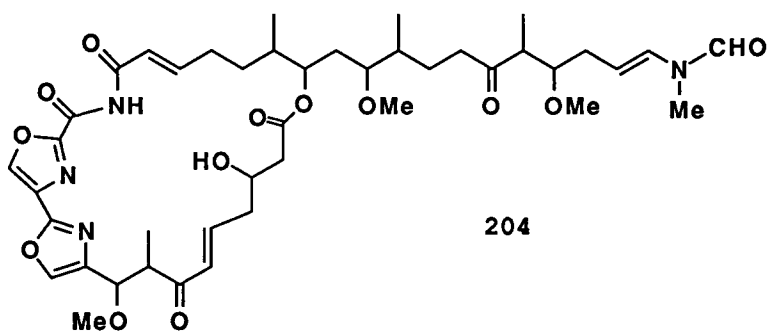


203

Mycalolides A-C (**206-208**) have been reported by Fusetani *et al.* from a sponge *Mycale* sp. and they were shown to have hybrid structures of ulapualides and halichondramides (234). Mycalolides exhibited antifungal activity against many pathogenic fungi and

cytotoxic activity against B16 melanoma cells with IC_{50}s of 0.5-1.0 ng/mL. Due to high toxicity mycalolides showed no efficacy in the *in vivo* antitumor assay. Mycalolides A and B have also been isolated by the same group from *Mycale adhaerens* (29). Quite unexpectedly, mycalolide C (**208**) and two new related compounds designated as mycalolides D (**209**) and E (**210**) have recently been described by Boyd *et al.* as cytotoxic constituents of the stony coral *Tubastrea faulkneri* (235). Other species of the genus *Tubastrea* have been described as sources of bioactive alkaloids (171). Finally, jaspisamides A-C (**211-213**) have been reported by Kobayashi *et al.* as cytotoxic macrolides from a sponge, *Jaspis* sp. collected in Okinawa (236). The structures of these compounds are closely related to the halichondramides. All three showed potent cytotoxicities against L1210 (IC_{50}s <1 ng/mL) and KB cells (IC_{50} 6-15 ng/mL).

In summary, all the compounds **191-213** (except for **205**) have the same 25-membered macrocyclic ring constituted on the same carbon backbone. Besides an oxazole moiety, they have in common an N-vinyl-N-methyl formamide; methyl substituents at C23, C27, and C31; oxygenation at C3, C7, C24, C26, C30, and C32 of which C26 substituent is a methoxyl; and a double bond at C19. Most of them contain ketonic functions at C7 and C30.



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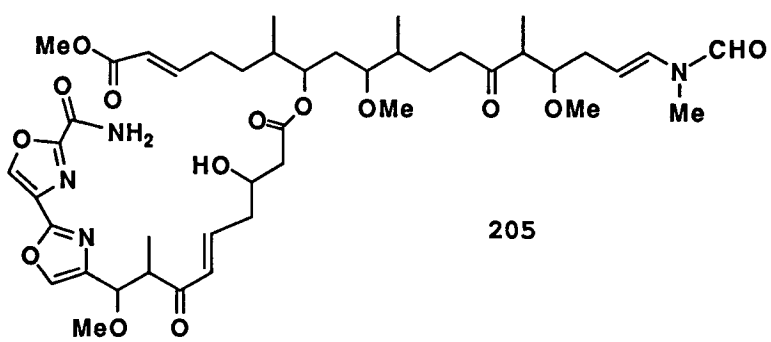

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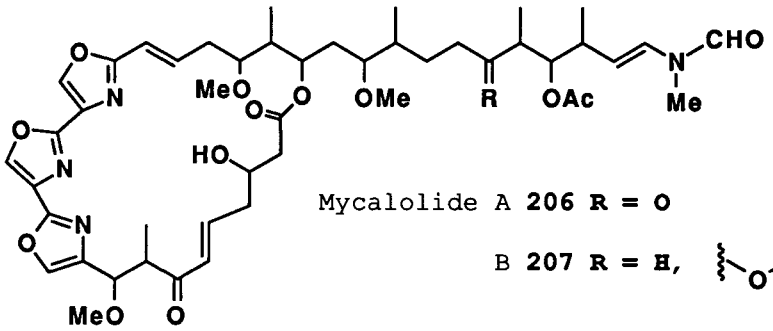
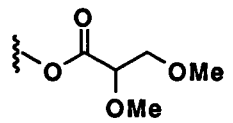
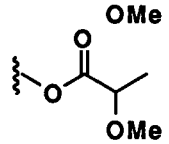


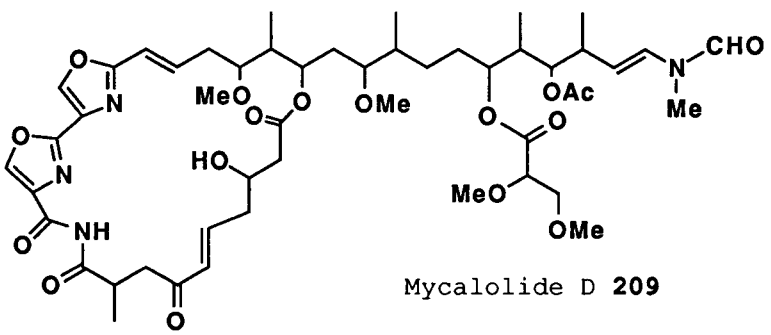
Mycalolide A **206** R = O

B **207** R = H,

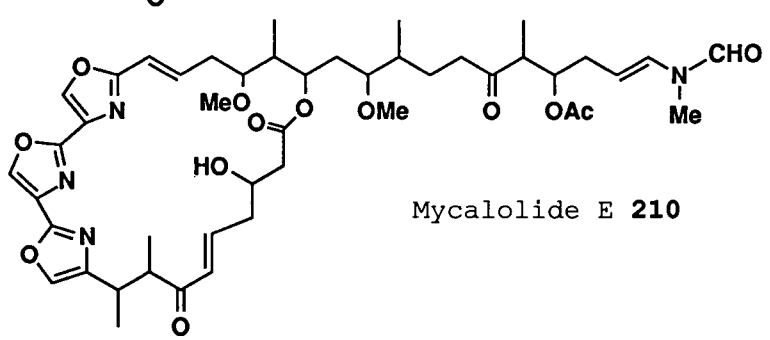


C **208** R = H,

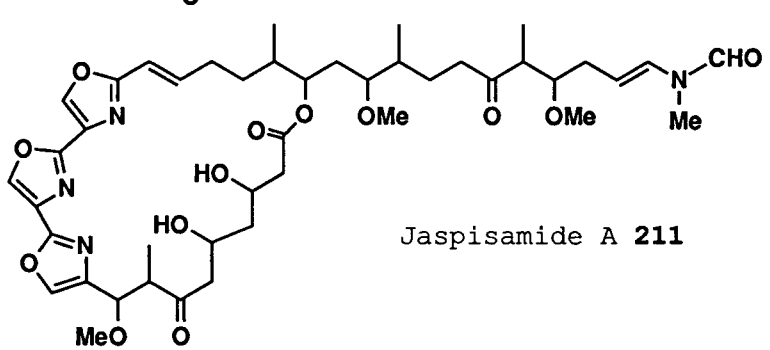




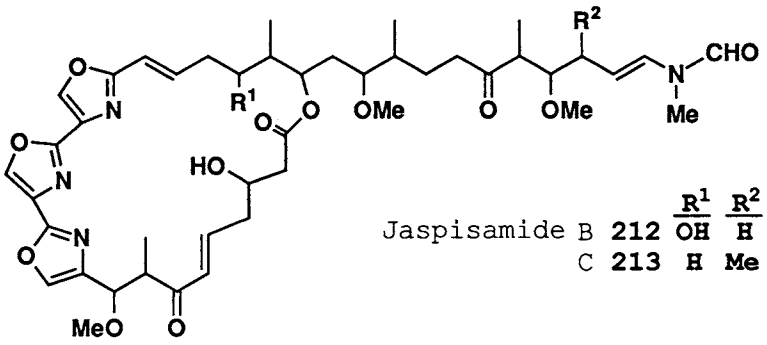
Mycalolide D **209**



Mycalolide E **210**

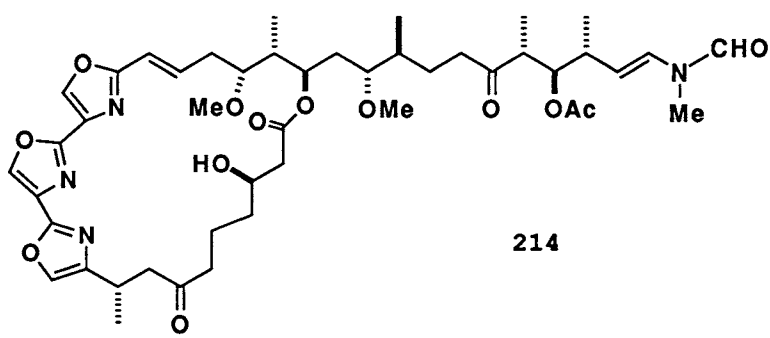


Jaspisamide A **211**



	R^1	R^2
Jaspisamide B **212**	OH	H
C **213**	H	Me

No stereochemistry of these macrolides has yet been determined. However, in conjunction with synthetic studies, Pattenden *et al.* published a predicted stereostructure (**214**) for ulapualide A (**191**) based on computer modeling studies (237). By assuming ulapualide A to be an ionophore, molecular mechanics calculations were made on metal chelated complexes to predict the stereochemistry. The results revealed good agreement with the stereochemistries reported for the side chain of scytophycin C (140) and aplyronine A (188). Synthetic studies of ulapualides and related compounds have been reviewed (93). No total synthesis has yet been reported.



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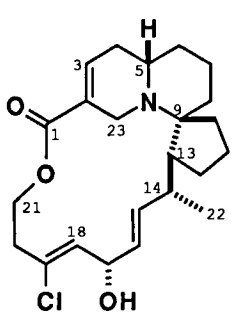
6.4 Other Alkaloidal Macrolides

The remaining alkaloidal macrolides can be classified into three distinctive structural types as described below. However, only one compound each has so far been reported for two of them, halichlorine and prorocentrolide class. The third class, aplidite has seven compounds.

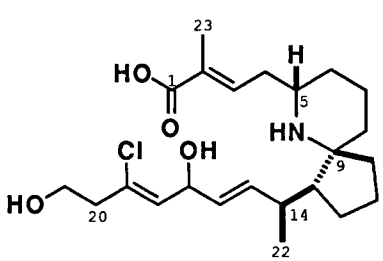
6.4.1 Halichlorine. The sponge *Halichondria okadai* was a source of the polyether macrolide halichondrins as described in Section 4.9. Further investigation on bioactive compounds of the sponge led Uemura *et al.* (238) to discover a new macrolide belonging to another class. From 200 kg (wet weight) of the sponge collected at a Pacific coast in the southwest of Tokyo, they obtained a compound named halichlorine (**215**) in a yield of 70 mg. The gross structure of halichlorine was deduced from spectroscopic data and the relative configuration from coupling constants and NOESY data. Halichlorine (**215**) is a 15-membered macrolide containing a quinolizidine ring attached with a cyclopentane in the spiro-junction and a chlorine atom. The quinolizidine ring system is not new in marine compounds as it has been known in such macrocyclic alkaloids as petrosins (239), but halichlorine is the first example having a quinolizidine as a part of a macrolide.

Halichlorine inhibited the induction of VCAM-1 (vascular cell adhesion molecule-1). A drug that blocks induced expression of VCAM-1 may be useful for the treatment of atherosclerosis, coronary artery diseases, angina, and noncardiovascular inflammatory diseases.

Interestingly, closely related compounds, pinnaic acid (**216**) and its taurine conjugate have also been reported by Uemura's group from a biologically unrelated and geographically distant specimen, the bivalve *Pinna muricata* which was collected in Okinawa. Obviously, there must be biogenetic relationship between halichlorine and pinnaic acid, but the configuration at C_{14} is opposite. The Shizuoka group has earlier reported from the bivalve pinnatoxin, a polyether macrocycle which was believed to be responsible for the human intoxication caused by the ingestion of the bivalve (241).

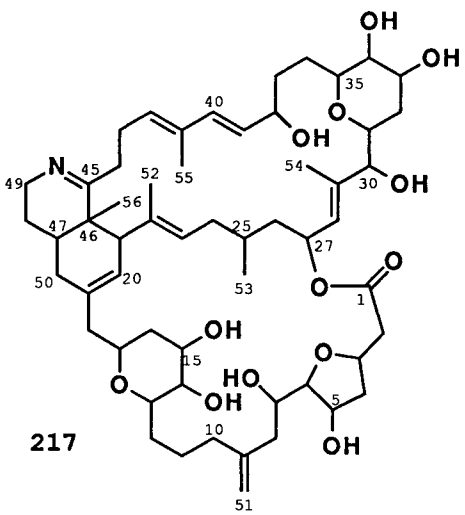


215



216

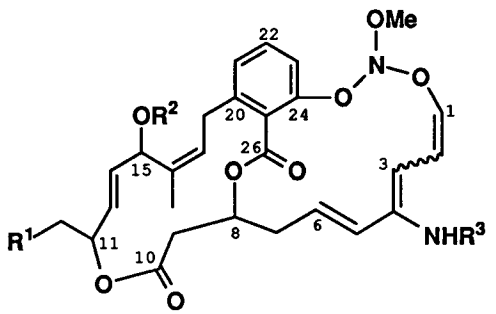
6.4.2 Prorocentrolide. Yasumoto *et al.* had earlier reported the isolation of okadaic acid (**80**) and its esters from the laboratory-cultured dinoflagellate *Prorocentrum lima* (242). In their continued research on the metabolites of the alga, they have discoverd a toxic macrolide termed prorocentrolide (**217**). The macrolide which showed mouse lethality of 0.4 mg/kg (ip) was isolated in a 70 mg yield from an extract of 2.7 X 10^{10} cells harvested from 1000 L of the culture (243). The gross structure of prorocentrolide was secured by NMR analysis. The stereochemistry remains to be elucidated. Besides a 28-membered macrocyclic lactone ring, the structure incorporates a 26-membered carbocycle, a hexahydroisoquinoline, two tetrahydropyran rings and one hydrofuran on a C_{56} backbone. Except for the 14-membered ring of cembranes, higher-membered carbocyclic rings such as the one in **217** are rare in marine natural products.



217

6.4.3 Aplidites. The last group of macrolides to be discussed here are unusual diolides named aplidites. Aplidites A-G (**218-224**) have recently been described by Capon *et al.* as constituents of an Australian tunicate, *Aplidium* sp (244). Gross structures of the aplidites were deduced mainly from spectroscopic data and also from derivatization and degradation. Aplidite A (**218**) and B (**219**), C (**220**) and D (**221**), and E (**222**) and F (**223**) are each geometric isomers of the C1 double bond. The geometries, however, remain to be assigned. Although no direct proof is available, the presence of an unprecedented orthonitrite function is supported by a number of

circumstantial evidences such as H1 chemical shift at δ 8.95 (for **218**), NOE between H27 and H1, and the formation of a phenolic product by $LiAlH_4$ reduction. No biological activity has been reported.



			R^1	R^2	R^3	$\Delta^{1,2}$
Aplidite	A	**218**	**H**	**H**	**H**	***E*** or ***Z***
	B	**219**	**H**	**H**	**H**	***Z*** or ***E***
	C	**220**	**OH**	**H**	**H**	***E*** or ***Z***
	D	**221**	**OH**	**H**	**H**	***Z*** or ***E***
	E	**222**	**OH**	**Ac**	**H**	***E*** or ***Z***
	F	**223**	**OH**	**Ac**	**H**	***Z*** or ***E***
	G	**224**	**H**	**Ac**	**Ac**	***E*** or ***z***

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Hormones in the Red Swamp Crayfish

A. Yasuda and Y. Naya

1. INTRODUCTION

Crustacea is a general term for a large class of Arthropoda comprising the majority of the freshwater and marine arthropodes and some terrestrial forms, such as lobsters, shrimps, crabs, hermit crabs, water fleas, barnacles, and the wood lice etc. Among them, shrimp, prawn, lobster, and crab are the most valuable food in many countries. Particularly, some species of them involve in a seasonable delicacy, i.e., "soft-shell crabs" in USA, "crayfish party" in Sweden, and "Nabemono with a crab" in Japan. The soft-shell crabs mean to be a particular phase of its life, just after molting. The crayfish, like the other members of Crustacea, have an exoskeleton which plays a role for the protection of the internal organs. It is, however, including a problem that the crayfish have to shed the shell as they are growing. Our scientific interest focuses on the chemistry of endocrine regulation for molting.

The red swamp crayfish, *Procambarus clarkii*, is one of American freshwater species. The history of expansion in habitat of the red swamp crayfish has begun in 1927, when a hundred of the red swamp crayfish had sent out from New Orleans, Louisiana to Japan to feed bullfrogs. After that, the crayfish is widely distributed throughout Japan. After hatching, red swamp crayfish molts 7 to 17 times (average 9 times) in the first year. The number of molting decreases 2 to 5 times (average 4 times) in the second year, and then becomes 2 times in the third years. The red swamp crayfish followed some 11 times of molting, if its shell increase about 6 centimeters long, arrives at maturity.

In crustaceans, there is a current knowledge about endocrine control of molting with two types of hormones, molting hormone and molt-inhibiting hormone. The first step toward the discovery of the eyestalk hormone came in 1905, when Zeleny (1) found that removal of eyestalks from crustaceans promotes molting. In 1939 Brown Jr. and Cunningham (2) advanced the presence of "molt-inhibiting hormone". They showed that the interval between molts in the crayfish is shortened by excision of eyestalks including sinus gland and reimplication of eyestalks returns to the normal molt cycle again. On the other hand, in 1966 a steroid hormone called "crustecdysone" was isolated from Y-organ located in the thorax by Hampshire and Horn (3). The first discovery of molting hormone in crustacean provided the great impact on the molting process.

Krishnakumaran and Schneiderman (4) have shown that 20-hydroxyecdysone (ecdysterone, β-ecdysone, crustecdysone) promotes molting in crustaceans as well as in a wide variety of arthropods, and ecdysone is effective in inducing molting of the crayfish. After then, in 1982 Soumoff and O'Connor (5) suggested that a molt-inhibiting hormone from X organ-sinus gland complex in the eyestalk suppress the biosynthesis and/or secretion of molting hormone by Y organs. Although a few materials have been a candidate for molt-inhibiting hormone in the past 15 years, the many questions still be present in the physiology of molting.

The crayfish have been utilized extensively for studies of internal anatomy, physiological regulation, and hormonal processes because of its relatively easy breeding compared to other crustaceans. In the following section, we will attempt to give an overview of the unique hormone structures in the red swamp crayfish. Namely, we discuss several aspects of molting hormones and the possible biosynthetic significance involved in the molting process. Next, we will turn our attention to the evolution of the peptide hormone, and structure-function relationship will be discussed in some detail.

2. ENDOCRINE CONTROL OF MOLTING AND GROWTH

Molting hormone, derived from dietary sterols, is a general term for several polyhydroxylated steroid hormones with A/B *cis* ring juncture (ecdysteroids) that induce premolt and the physiological and biochemical changes that lead to molt. It has long been believed that the titer of biologically active ecdysteroids such as ecdysone and 20-hydroxyecdysone merely regulate developmental events of insects and crustaceans. These studies have largely used radioimmuno-assay assays (RIA) of whole body titers. RIA, unfortunately, detects biologically inactive as well as active ecdysteroids on the base of different antisera which detect slightly different ranges of ecdysteroid molecules (section 3.1). 3-Dehydroecdysone is found (6) as a major biosynthetic product by the molting gland, Y-organ of *Procambarus clarkii*, where 3-dehydroecdysone is neither the descendant nor the precursor of ecdysone (section 3.1). 3-Dehydroecdysone functions as the molting hormone *via* biotransformations, in the peripheral area of the Y-organ, into ecdysone and 20-hydroxyecdysone together with their conjugates (7, 8) (3.2 and 3.4).

The crab *Cancer antennarius* and the white shrimp *Penaeus vannamei* also secrete 3-dehydroecdysone as a major product (9, 10). Recent study using the Y-organ of crayfish *Orconectes limosus*, confirmed that 3-dehydroecdysone is a conversion product of 5β-diketol and ecdysone is that of 5β-ketodiol, which is also derived from 5β-diketol (11). It has also been reported that the predominant products of the Y-organs are 25-deoxyecdysone in the portunid crab *Carcinus maenas* (12), both 3-dehydroecdysone and 25-deoxyecdysone in the xanthid crab *Menippe mercenaria* (13), together with ecdysone in the fiddler crab *Uca pugilator* (14) and also in the striped rock crab *Pachygrapsus crassiples* (15). Additionally, 3-dehydroecdysone, 20-hydroxy-25-deoxyecdysone or ponasterone A, and 24-methyl-20-hydroxyecdysone or makisterone A have been found in the circulation fluid and are thought to be involved in hormonal

function in the insect kingdom (16-19). Thus, the sequence of ecdysteroidogenesis by the molting gland is rather species-specific in both crustaceans and insects. Using the third larval of *Dorosophila*, it has been reported that 3-dehydro-20-hydroxyecdysone is the most efficient ecdysteroid in the initiation of steroid inducible P1 gene transcription *in vivo*, where 3-dehydroecdysone is the major ecdysteroid (20). Although the ecdysteroids present at some stages in molt cycle has been analyzed by more sophisticated techniques (21) (section 3.3), we really do not know at the molecular level what is happening during molt of the crayfish, To understand periodic molt event one has to sacrifice some of the detailed staging that is ideal, by using mimic stages that may well reflect in the biological event. Further investigations are required to elucidate the mode of action for ecdysteroid hormones involved in molting.

The model for molting control in crustacean is now found to be oversimplified and the view of a multi-hormonal regulation is recently accepted (22-24); neuropeptide hyperglycemic CHH (section 4.1), molt-inhibiting "MIH" (section 4.3), the ecdysone biosynthesis inhibitor EBI (section 3.5), and the juvenile hormone-like terpenoid methyl farnesoate MF, are so far known to be involved in the regulation of crustacean molting process. It is acceptable that the crustacean X-organ-sinus gland complex in eyestalk plays a central role in the regulation of metabolism, on the basis of the ability of neurosecretory neurons to both receive information from sensory neurons and transmit information to the endocrine system in the form of neurosecretory hormones, such as CHH, "MIH" and probably EBI etc. The crab mandibular organ secreted MF (24), which stimulated ecdysteroid secretion of the Y-organs (*C. maister*) at the much higher concentration than the actual hormone concentration. Eyestalk removal caused dramatic increase in MF levels in the hemolymph, while injections of sinus-gland extracts decreased MF to undetectable levels (25). Hemolymph MF binding proteins have been characterized (26) that has variable activity over the molt cycle, suggesting that MF may be a positive regulator of the Y-organs (23, 24). From the above results, MF may be speculated to coordinate with transportation of the sterol substrate to/in the molting glands; ecdysteroids are thus synthesized and doing so, to regulate the magnitude of the glands' response to "MIH", in contrast with insect PTTH (27). The coordination of EBI, 3-hydroxy-L-kynurenine and/or xanthurenic acid to cytochrome P450 monooxygenase(s) (28-30) is demonstrated to suppress ecdysteroidogenesis *in vitro* (31), though the former is responsible *in vivo* (32). The secretion of EBI from the X-organ/sinus gland complex in the eyestalks is fluctuated and probably related to that of neuro hormones such as 5-hydroxy-L-tryptophan (33), serotonin and melatonin. This expectation is based on strong indications that light influences molting rhythms. Indeed, in *P. clarkii*, presence of serotonin is reported (34).

3. MOLTING HORMONES

3.1 BIOSYNTHESIS OF ECDYSTEROIDS

Hormonal studies may have been complicated by the use of whole animals rather than isolated tissues *in vitro*. However, these studies will be completed by a combination of both experiments *in vivo* and *in vitro*. 3-Dehydroecdysone (ca. 85%) together with ecdysone, have been identified as the products by the molting glands (Y-organs *in vitro*) of crayfish (*Procambarus clarkii*). 3-Dehydroecdysone was reduced to ecdysone resulting in 20-hydroxyecdysone rapidly after leaving the Y-organ *in vivo*. Neither 3-dehydroecdysone nor ecdysone was the respective ultimate precursor synthesized by the Y-organ; the branch point of the biosynthetic pathway in the Y-organ was earlier than that previously thought.

3.1.1 MATERIALS

Animals. The fresh crayfish, *Procambarus clarkii*, were collected in Akashi and Okayama (Japan), and maintained in large outdoor aquarium. They were fed an artificial diet few days. Males at the intermolt stage, carapace width 20-23 mm and length 75-85 mm, were mainly used for our experiments. To stimulate the Y-organ biosynthesis and release ecdysteroids, eyestalks were cut off bilaterally and the chelae were forced to autotomize at the same time (29, 35); these treatments usually resulted in precocious molting.

Chemicals. Authentic ecdysone, 20-hydroxyecdysone and 2-deoxy-20-hydroxy-ecdysone were purchased from Sigma. [23,24-^{3}H]ecdysone ([^{3}H]ecdysone, 3064 GBq/mmol) was provided by DuPont/NEN Research Products. Authentic 3-dehydro-ecdysone was prepared according to the reported procedure (36). [^{3}H]3-Dehydroecdysone (851 MBq/mmol) was prepared from [^{3}H]ecdysone (3064 GBq/mmol) in dilution with ecdysone (1.17 mmol/GBq). Unless otherwise noted, all other chemicals were provided from Nacalai Tesque, Japan.

Antisera (S-3 and H-22). Antiserum S-3 was given from Dr. S. Sakurai of Kanagawa University (Japan) and H-22 from Dr. L.I. Gilbert of the University of North Carolina (USA); serum S-3 binding is relatively unaffected by substitution in the A-ring, and serum H-22 binding is not much affected by side-chain substitutions (18, 37).

3.1.2 METHODS & RESULTS

Culture of Y-organs. Excision of Y-organs was examined for 2-9 days after eyestalk removal and determined the optimum timing for ecdysteroid productions. Thus, the 6th day Y-organs after eyestalk removal were employed for the subsequent experiments. The excised Y-organ (day 6) was rinsed three times with sterile culture medium, transferred into a small glass dish containing culture medium (5-organs in 1 ml), and incubated on a rotary shaker (Taiyo, R-11) at 25°C for 6 h; ecdysteroid secretion by Y-organ increased for 6 h and appeared to level off within 9 h. The production (ca. 60 ng/organ) was the same whether the medium was replaced every 3 h or the same medium was left. The culture medium was prepared according to Keller and Schmid (38) with minor modifications: the medium 199 (Flow Laboratories) was buffered with 20 mM Hepes, containing NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ (424, 39.4 and 180 mg/100 ml, respectively), and adjusted to pH 7.5 with NaOH. The antibiotics' penicillin and streptomycin were added to give concentrations of 5000 U/100 ml and 5 mg/100 ml,

respectively. The medium thus prepared was sterilized by filtration through a 0.22 μm filter (Millipore, Millex-GV) just before use. In a parallel experiment, hemolymph was collected by hypodermic needle from the base of the fifth pereiopod, just before the Y-organ dissection. Each hemolymph sample was extracted with methanol, passed through a Bond Elut cartridge C_{18} (Analytichem International) for the determination of the total ecdysteroid content.

Radioimmunoassay. Serum S-3 and H-22 were diluted 2400- and 3200-fold, respectively, with 0.1 M borate buffer (pH 8.4) containing 0.05% rabbit gamma globulin and 0.2% bovine serum albumin (BSA), and used for the following experiments. The RIA was performed according to Carrow et al. (39) with minor modifications. To 10 μl of the samples to be assayed, were added 100 μl of antiserum solution (see above) and 100 μl (10,000 dpm) of [23,24-^{3}H]ecdysone in the borate buffer. The mixture was vigorously stirred and allowed to stand overnight at 4°C; then an equal volume of saturated ammonium sulfate was added. The mixture was kept at 4°C for 1 h, and then centrifuged at 5000 x g for 40 min. After the precipitate had been washed with 50% ammonium sulfate solution, its radioactivity was determined; to the precipitate dissolved in water (50 μl), 450 μl of scintillation fluid (ACS-II, Amersham) was added, and then the mixture was placed in glass vials and radioactivity was measured using a liquid scintillation counter (Aloka, LSC-700). The values were expressed as ecdysone equivalents. The useful range of the standard curve was between 15 and 1000 pg in linear logit-log plot.

Immunological identification of ecdysteroids. A small portion of the cultured Y-organ medium was directly subjected to radioimmunoassay (RIA) to determine the ecdysteroids produced by the Y-organs. The maximum RIA activity was ca. 60 ng/Y-organ, while detectable immuno-reactive ecdysteroids in the methanol-extract of the contralateral Y-organs (without incubation) were 1.2 ng/Y-organ. Each cultured Y-organ medium and a methanol extract of hemolymph was loaded onto a C_{18} cartridge, respectively. The cartridge was washed successively with 25 and 60% aqueous methanol. The latter eluate was concentrated and applied to analytical RP-HPLC (Wako, Wakosil 5C_{18}, 4.6 x 250 mm, or Tosoh, TSK gel ODS-120T, 4.6 x 250 mm). Elution was isocratic with 45% aqueous methanol and a flow rate was 1 ml/min at 40°C. The immunoreactive ecdysteroids in each fraction were tentatively identified by comparison of the retention times to those of the authentic samples (detection at 254 nm). Fractions were collected every 0.5 min for further studies.

In the cultured Y-organ medium, the RIA after RP-HPLC revealed four immunoactive fractions with S-3 (fraction Nos. 9, 31, 34, 42 in Fig. 1a), whereas only one fraction (No. 31) reacted with H-22. The cross-reactivity of authentic ecdysteroids to the antisera (TABLE 1), where the cross-reactivity was calculated according to the reported method (40), indicated that the major (No. 34) and the minor products (Nos. 9, 42) were modified in the A-ring. Retention times of the ecdysteroids (Nos. 31 and 34) on HPLC were coincident with those of the authentic ecdysone and 3-dehydroecdysone, respectively. The major peak (No. 34) on the RP-HPLC was confirmed to be a single

compound, using a diode-array detector (Yokogawa Hewlett Packard HP 1090 II/M); exhibiting >99% purity as 3-dehydroecdysone; Cosmosil $5C_{18}$, 4.6 x 250 mm, 1 ml/min, isocratic, **A** ($MeOH/CH_3CN$; 3 : 1); **B** (H_2O) in the ratio 2 : 3. This major ecdysteroid, produced by the incubation of 70 Y-organs, was isolated by repeated RP-HPLC (Cosmosil $5C_{18}$, gradient, 1%/min, 40-50% of **A** : **B** for 20 min, 40°C) and confirmed to be 3-dehydroecdysone using mass spectrometry (JEOL HX 110) in the mode of negative-ion FAB (first-atom bombardment, Xe gas of 6 kV energy) with glycerol as a matrix; *m/z* 461, $[C_{27}H_{42}O_6\text{-}H]^-$. After repeated experiments, 3-dehydroecdysone was found to be approximately 85% of the total RIA-reactive ecdysteroids.

TABLE 1. Cross-reactivities of authentic ecdysteroids to the S-2 and H-22 antisera.

Ecdysteroids	S-3	H-22
Ecdysone	1.0	1.0
20-hydroxyecdysone	2.0	1.5
3-dehydroecdysone	1.0	116.7
2-deoxyecdysone	1.5	83.3
2-deoxy-20-hydroxyecdysone	3.1	125.0

The isolated 3-dehydroecdysone was further confirmed by $NaBH_4$ reduction to give ecdysone and 3-*epi*-ecdysone in the same ratio. The resulted compounds were identified by comparison of retention times to those of authentic samples on RP-HPLC using a diode-array detector. 3-Dehydroecdysone was unstable in methanol but stable enough to be concentrated in $MeOH\text{-}H_2O$ (50-70%). In the purification process, a less polar compound was newly produced, which exhibited the same molecular ion with 3-dehydroecdysone, *m/z* 461, $[C_{27}H_{42}O_6\text{-}H]^-$. It is therefore assumed that the less polar compound was a methanol adduct (41) and reverted to the non-adduct under the condition of mass spectroscopic measurement. By the same technique being used, 20-hydroxyecdysone together with a little amount of ecdysone, 3-dehydroecdysone and 2-deoxy-20-hydroxyecdysone were identified in the hemolymph (day 6) (Fig. 1b).

Biosynthetic pathway in Y-organ. Six Y-organs (day 6) or their homogenates per 1 ml of incubation medium were incubated under the standard conditions for 6 h, in the presence or absence of hemolymph (50% v/v), with [^{3}H]ecdysone (4990 Bq/ml). After incubation, radioactivity of the above experiments were confined to ecdysone. In the similar experiments with [^{3}H]3-dehydroecdysone (5920 Bq/ml), radioactivity was not dispersed by incubation; RP-HPLC analyses were carried out under the conditions already mentioned. Present experiments indicated that interconversion between radio-labeled ecdysteroids (ecdysone and 3-dehydroecdysone) did not take place by the Y-organ. Under the separate experiments with homogenized Y-organs, it is worth noting that ecdysone was a major product instead of 3-dehydroecdysone. It can be assumed that 3-keto-(β)-reductase in the Y-organ was activated by homogenization (22), and hence biosynthetic pathway *via* 5β-ketodiol to ecdysone was prompted. Consequently, the pathway from 5β-diketol to 3-dehydroecdysone was affected and made it possible to convert all the available substrate (in the Y-organ) into ecdysone. Thus, proposed pathway for *Orconectes* and *Cancer* (22) was strongly supported by the Y-organ

ecdysteroidogenesis of *P. clarkii* (Fig. 2). It is also presumed that biosynthetic sites of ecdysone and 3-dehydroecdysone are independent at the sub-cell level.

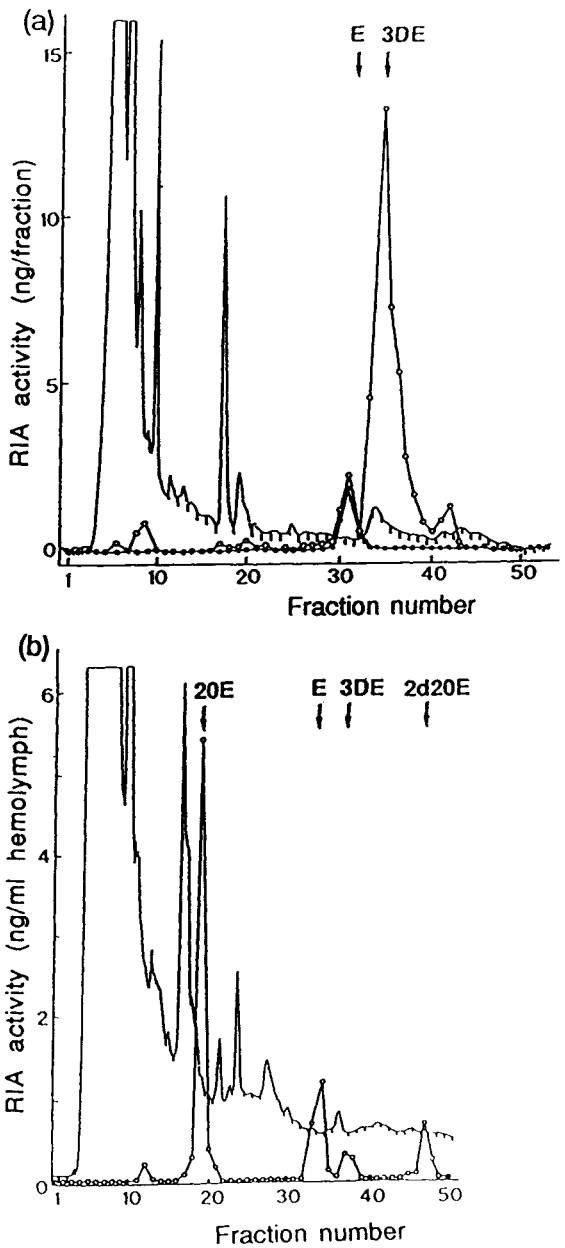


Fig. 1.
(**a**): RP-HPLC profile of ecdysteroids secreted by Y-organs in vitro (day 6).
(**b**): RP-HPLC profile of ecdysteroids in the hemolymph.

Each fraction was quantified by RIA using antisera S-3 (open circles) and H-22 (closed circles). RIA activities were expressed as ecdysone equivalents. The eluate was automatically detected by the UV absorption at 254 nm. Arrows indicate the retention volumes of the authentic ecdysteroids. E: ecdysone, 3DE: 3-dehydroecdysone, 20E: 20-hydroxyecdysone, 2d20E: 2-deoxy-20-hydroxyecdysone.

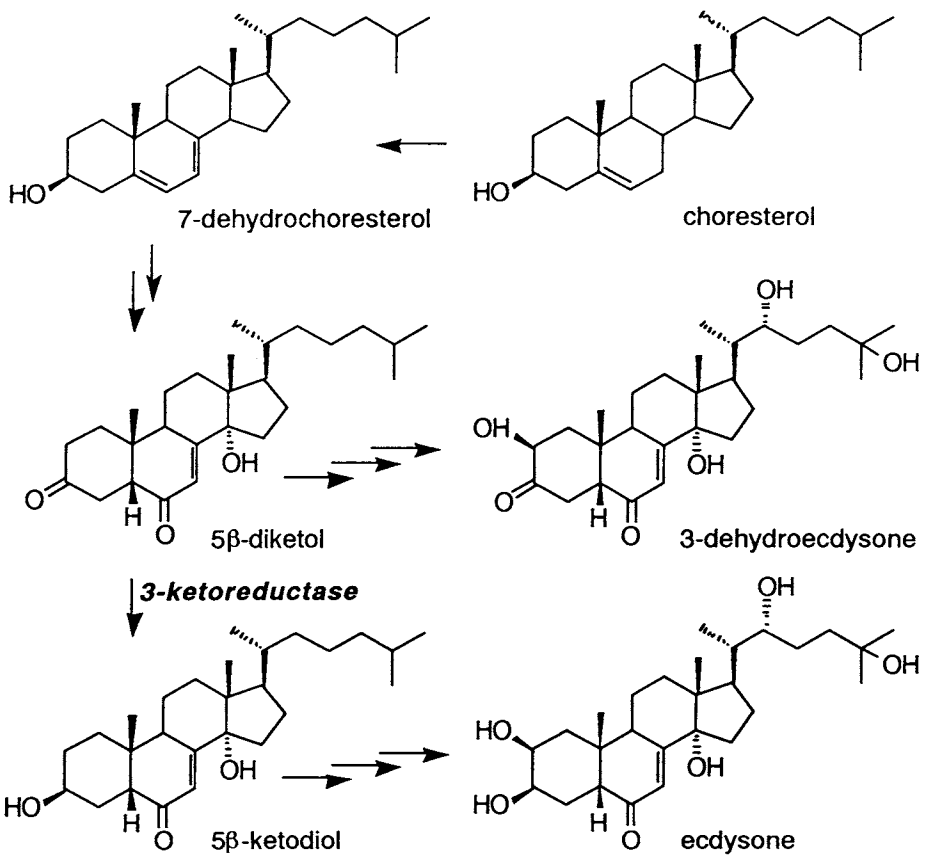


Fig. 2. Proposed biosynthetic pathway from cholesterol to 3-dehydroecdysone and ecdysone in the Y-organ, *P. clarkii*.

3.2 METABOLIC FATE OF 3-DEHYDROECDYSONE

To understand the physiological function and control mechanism of hormone release, the hormonal function and the metabolic fate of 3-dehydroecdysone were investigated by the use of *P. clarkii*. Injection of 3-dehydroecdysone induced molting in which 3-dehydroecdysone played its role as a probable precursor of ecdysone, in the peripheral of the Y-organ *in vivo*. Majority of 20-Hydroxyecdysone was found at the epidermis after injection of 3-dehydroecdysone.

3.2.1 MATERIALS

The animals and chemicals adopted in the following experiments were already described (section 3.1), unless otherwise noted. Authentic 3-*epi*-ecdysteroids (3α-

ecdysone, 3α-,20-hydroxyecdysone) were pre-pared by $NaBH_4$ reduction from the corresponding 3-dehydroecdysteroids (36). [^{3}H]3-Dehydroecdysone (851 MBq/mmol) was prepared from [^{3}H]-ecdysone (3063.6 GBq/mmol) diluting with ecdysone (1.17 mmol/1GBq).

3.2.2 METHODS & RESULTS

Hormonal response of ecdysteroids. Each ecdysteroid (3-dehydroecdysone, ecdysone and 20-hydroxyecdysone), dissolved in the medium 199 (300 μl) containing 10% ethanol, was injected into the hemocoel at the base of the first pereiopod; three experimental groups of crayfish (n=7-12, 70-90 mm, ca. 20 g) received at the dose (42) of 5 μg/g live weight, respectively. Controls received either 300 μl of the medium or cholesterol (5 and 20 μg) in the medium. After injection, the animals were exposed to air for 1 h and then returned to the aquarium at 25°C on a photo-phase 16 : 8 (L : D) to observe hormonal response for several days. All experimental animals receiving injection of ecdysteroids (day 0) underwent apolysis over 3-5 days. Although all died before completion of ecdysis, separations of the epidermis from the old cuticle and formation of gastroliths in the stomach were observed in accord with previous reports (43). Krishnakumaran et al. postulated (42) that the abnormal molt probably due to a large amount of hormone in one dose; hormone-secretion *in situ* is normally gradual. In our experiment, effects of ecdysteroids (5 μg/g each) were apparent by day 3 with 20-hydroxyecdysone, day 4 with ecdysone, and day 3-5 with 3-dehydroecdysone. However, doses up to 20 μg/g of cholesterol, 3-*epi*-ecdysone and 3-*epi*-20-hydroxyecdysone showed no effect. The time required for response to 3-dehydroecdysone was almost comparable with that of 20-hydroxyecdysone and ecdysone; a statistical study is necessary to determine the lag phase that may be due to enzymatic activities *in situ*. Thus, we postulated that 3-dehydroecdysone was involved in the molting process (*in vivo*) as a precursor of ecdysone and 20-hydroxyecdysone. It is also possible that 3-dehydroecdysone has a hormonal function of its own (9, 16) as that of ecdysone (44).

Biotransformation. Above observation led us to investigate whether 3-dehydroecdysone in the peripheral of Y-organs (*in vivo*) is an ultimate precursor of ecdysone and eventually of 20-hydroxyecdysone or not of those. It has been already proved that excised Y-organ carried no potency of interconversion between the ecdysteroids in question (section 3.1).

Each crayfish was injected (100 μg per animal: ca. 5 μg/g body weight) with [^{3}H]3-dehydroecdysone (851 MBq/mmol) in the protocol as already described. The epidermis with carapace, muscle, hepatopancreas, hemolymph and others (the all remaining tissues) were surgically separated with the passage of time and extracted with methanol. Each extract was subjected to a preparative RP-HPLC, and all radio-labeled fractions were counted by liquid scintillation counter to yield the profile of radioactivity. About 70% of the total radio-label were excreted within 1 h as unchanged 3-dehydroecdysone, and the rest 30% was retained in the body for 3 h. After repeated purification with NP- and RP-HPLC, each major constituent was identified by co-chromatography on HPLC with the corresponding authentic compound. The distribution patterns of the radioactivity in the body changed with time and indicated by a diagram (Fig. 3). The majority of injected radio-label was excreted shortly after injection; 66% (3h) and 89% (72 h). The converted ecdysteroids were found mainly in the epidermal tissues within 1-3 h, while unchanged tritiated 3-dehydroecdysone alone was

found in the hemolymph. The result indicated the presence of ecdysteroid-reception system at epidermis of the crayfish; in accord with the report on crabs (45). Rapid reduction of 3-dehydroecdysone with a short half-life (Ca. 30 min) into ecdysone *in vivo* was in agreement with the results from insects *Calliphora vicina* (16), *Manduca sexta* (46) etc. A typical profile of radio-active ecdysteroids from an extract of epidermis (with carapace) was depicted in (Fig. 4). Treatment (37°C over night) of the polar fraction **A** (Fig. 4) with *Helix pomatia* juice (2400 units of ß-glucuronidase, Type H-2) in sodium acetate buffer (pH 5.2, 100 mmol, 2 ml), revealed that fraction **A** was a mixture of conjugated ecdysteroid (3-dehydroecdysone-, ecdysone-, 3-*epi*-ecdysone, 20-hydroxyecdysone-, and 3-*epi*-20-hydroxyecdysone-).

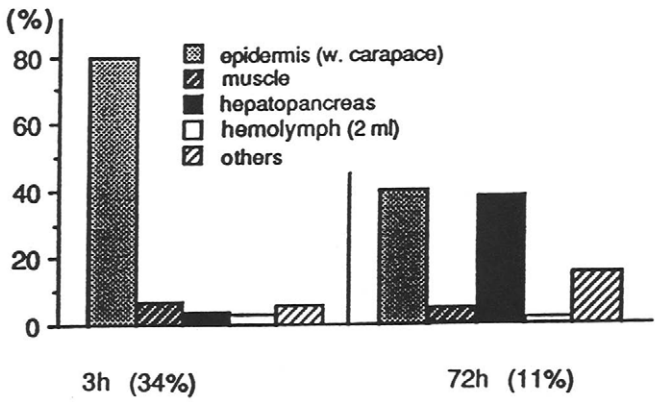


Fig. 3. Distribution of the radio-activity in the body after injection of [^{3}H]3-dehydroecdysone. The radio-isotopes in the target tissues were compared 3 and 72 h after injection. The height of each diagram is based on scintillation counting of the extracts from different tissues as indicated. The amount of hemolymph per animal was estimated as 2 ml. The majority of injected radio-label was excreted shortly after injection; 66% (3h) and 89% (72 h).

Even though our experiments did not represent normal physiological conditions, it might be suggested that there is a common mechanism and/or significant function of 3-dehydroecdysone in controlling the molting hormone titer. It has been shown that the presence of 3-oxo-ecdysteroid reductase activity was not restricted to 3β-reductase, and that 3α-reductase was active in releasing *epi*-ecdysteroids, which were selectively excreted.

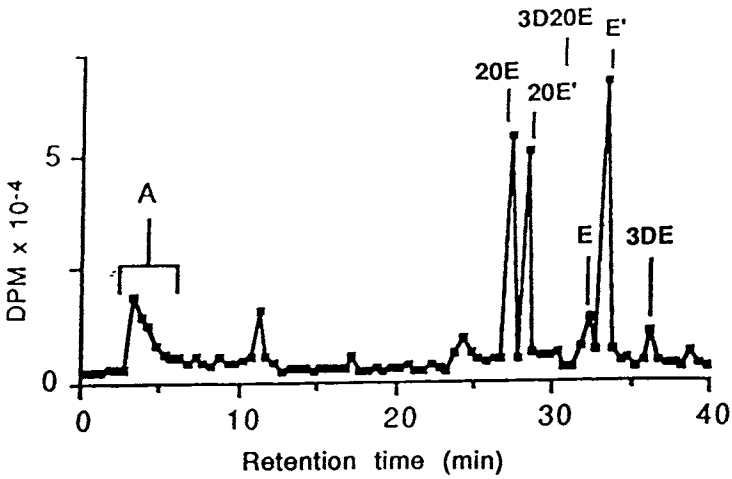


Fig. 4. Typical chromatogram of radio-labels derived from [^{3}H]3-dehydroecdysone. The extraction of epidermis with carapace, 3 h after injection, gave the profile of radioactivity counted by liquid scintillation counter. Arrows show retention times of the authentic compounds . E: ecdysone, E': 3-*epi*-ecdysone, 20E: 20-hydroxyecdysone, 20E': 3-*epi*-20-hydroxyecdysone, 3DE: 3-dehydroecdysone, 3D20E: 3-dehydro-20-hydroxyecdysone, **A**: a mixture of conjugate.

3.3 TITER FLUCTUATION (HPCE analysis)

The comparative time-course study (2-9 days after eyestalk removal), between the ecdysteroids produced by Y-organs (*in vitro*) and those in the hemolymph (*in vivo*), has been demonstrated by the use of HPLC. It was shown that 3-dehydroecdysone (major) and ecdysone (minor) were the products of Y-organs, and that they were released into hemolymph to result in 20-hydroxyecdysone (section 3.1). Injection experiment with [^{3}H]3-dehydroecdysone has revealed that the titer of ecdysteroids in hemolymph changed due to enzymatic activities involved in the metabolic pathway (section 3.2). This may be true to a certain extent under the physiological condition of crayfish. The titer change of ecdysteroids in hemolymph (crab, *Charybdis japonica*) was also obtained throughout a year and exhibited the staggered correlation (47) with the titer of EBI (ecdysone biosynthesis inhibitor). To provide further information on hormonal regulation, a handy HPCE analysis (high performance capillary electrophoresis) with less than ng levels was investigated using Jasco CE-800 system (21). Here, especially mode of micellar electrokinetic chromatography, MEKC (48, 49) was paid attention, because hormonal studies require high resolution and a short separation time. The utilization of inclusion complexing agents such as cyclodextrins (CD), their derivatives, and crown ethers provided new possibility for separating positional isomers and enantiomeric pairs (50).

3.3.1 MATERIALS

The crayfish, *P. clarkii* were purchased and de-eyestalked as already described. Unless otherwise noted, all chemicals were purchased from Nacalai Tesque (Kyoto, Japan). Authentic ecdysteroids were obtained as described above (section 3.1 and 3.2).

3.3.2 METHODS & RESULTS

Optimum resolution of ecdysteroids by HPCE. Micellar solutions were prepared by dissolving γ-CD in 100 mM sodium dodecyl sulfate (SDS)-100 mM borate buffer (pH 8.9). Authentic ecdysteroids were dissolved in a micellar solution containing 50% (v/v) methanol, and introduced by electromigration at 15 kV for 5 seconds. Analysis was performed with an applied voltage of 25 kV at room temperature and with UV detection at 245 nm. Electrophoretic optimization was accomplished by starting with a micellar solution, 100 mM SDS in 100 mM borate (pH 8.9). The interference between 3-*epi*-ecdysone and 3-dehydroecdysone was solved by the addition of γ-CD. The concentration of γ-CD, ranging from 5 to 25 mM, effected the migration time of ecdysone (Fig. 5). In a pseudo-phase with 100 mM borate (pH 8.9) containing 100 mM SDS and 15 mM γ-CD, five authentic ecdysteroids were well resolved on an electropherogram as shown in (Fig. 6a).

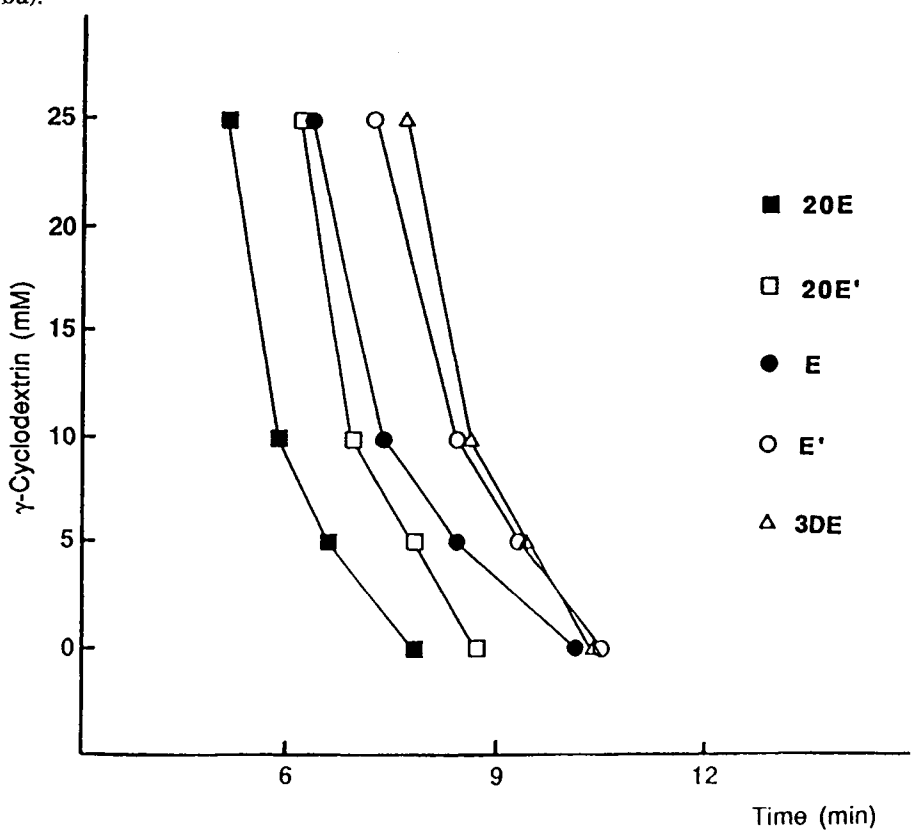


Fig. 5. Effects of γ-CD concentrations on the migration times of ecdysteroids. 20E: 20-hydroxyecdysone, 20E': 3-*epi*-20-hydroxyecdysone, E: ecdysone, E': 3-*epi*-ecdysone, 3DE: 3-dehydroecdysone.

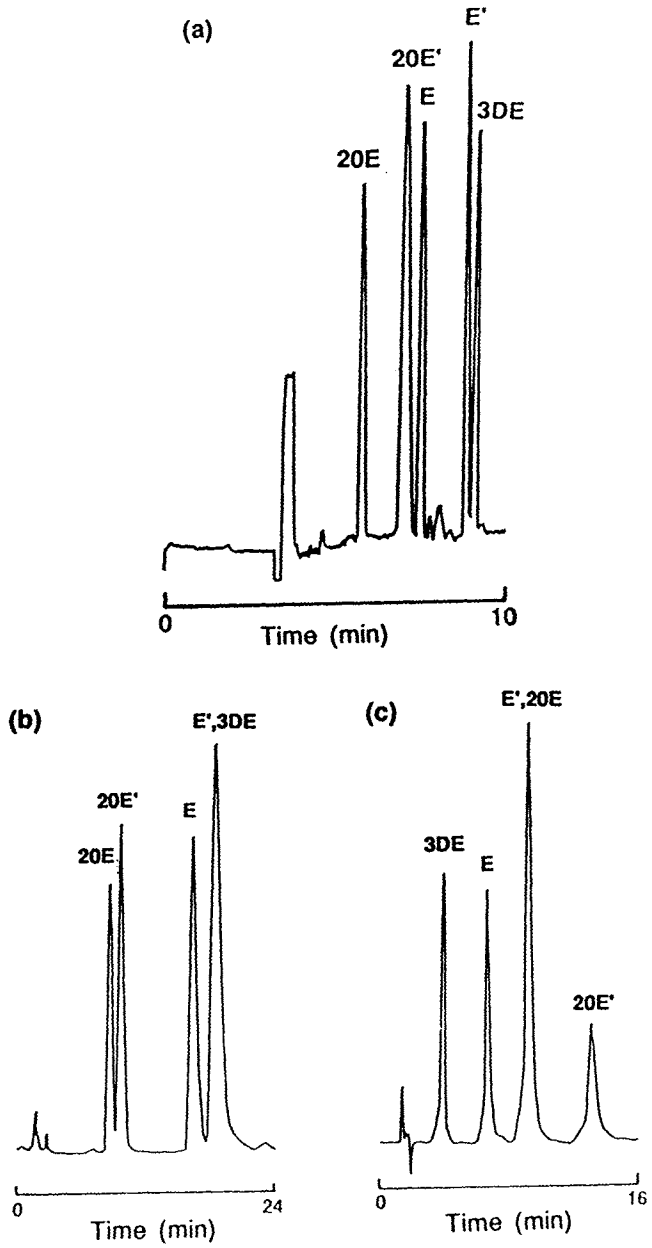


Fig. 6. Comparison of the separation of ecdysteroids. **a**) γ-CD modified MEKC with 100 mM borate buffer of pH 8.9 containing 100 mM SDS and 15 mM γ-CD. The injected amount of compounds was 500 fmol. **b**) RP-HPLC on a Capcell pak C18 AG 120 column. **c**) NP-HPLC on an APS-Hypersil column. The loaded compounds were 250 pmol for **b** and **c**. 20E: 20-hydroxy-ecdysone, 20E': 3-*epi*-20-hydroxyecdysone, E: ecdysone, E': 3-*epi*-ecdysone, 3DE: 3-dehydroecdysone.

For comparison, the elution profiles of the ecdysteroids on RP- and NP-HPLCs were also shown in (Fig. 6b, c). Using Jasco 800 series, RP-HPLC was carried out on a Capcell pak C18 AG 120 column (Shiseido; 0.46 x 25 cm, particle size 5 μm) with an isocratic elution of 40% methanol at 42°C in a flow rate of 1 ml/min. NP-HPLC was performed on an APS-Hypersil column (Shadon Southern Products; 0.46 x 25 cm, particle size 5 μm) with an elution of dichloromethane/methanol/isopropanol (95 : 4 : 1) containing 0.05% acetic acid and triethylamine, at 40°C and a flow rate of 2 ml/min. The effluents were monitored by absorption at 245 or 254 nm.

Analysis of ecdysteroids in the hemolymph by HPCE. Each hemolymph sample (1 ml) was collected by a syringe from the de-eyestalked crayfish (days' 3-6, 8 and 10, after eyestalk removal), diluted with 5 volumes of water, and centrifuged. The supernatants were subjected to Sep-Pak C18 cartridge (waters) equilibrated with water. The column was rinsed with 10 ml of water and 10 ml of 20% methanol, successively. The absorbed materials were eluted with 5 ml of methanol, and then dried. The residue was dissolved in 2 μl of the 50% methanol-micellar solution and subjected to HPCE. Representative electropherograms were shown in (Fig. 7). In each electropherogram on days 6, 8 and 10, was identified 20-hydroxyecdysone as a major circulating ecdysteroid. The relative standard deviation of migration time of 20-hydroxyecdysone was below 1%. The changes of 20-hydroxyecdysone levels in hemolymph, from the crayfish removing eyestalks, were illustrated in (Fig. 8). Ecdysteroids were at basal levels (<3 ng/ml) until day 5, begun to increase from day 6 and reached a peak on day 8 (about 200 ng/ml), then rapidly decreased. After a surge of 20-hydroxyecdysone, the animal started molting.

In the present study, any other ecdysteroids were not detected; probably due to their relatively lower concentration to the major ecdysteroid. The concentration sensitivity was over 10^{-5} M in spite of the low absolute detection limit (pg-range). The detection at 254 nm with ecdysteroid(s) was limited to 50 fmol, as determined by injected amounts of the authentic samples. However, the high polarity products (HPPs) were not detected; possibly due to an elimination from a Sep-Pak column by 20% methanol. Even if the applied samples contain HPPs, they will be pulled strongly into a positive pole based on their anionic property in HPCE analysis.

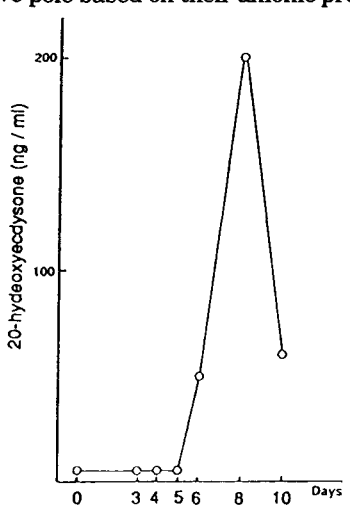


Fig 8. Changes in 20-hydroxyecdysone in hemolymph. Each hemolymph was obtained from an individual crayfish on the days after eyestalk removal (abscissa).

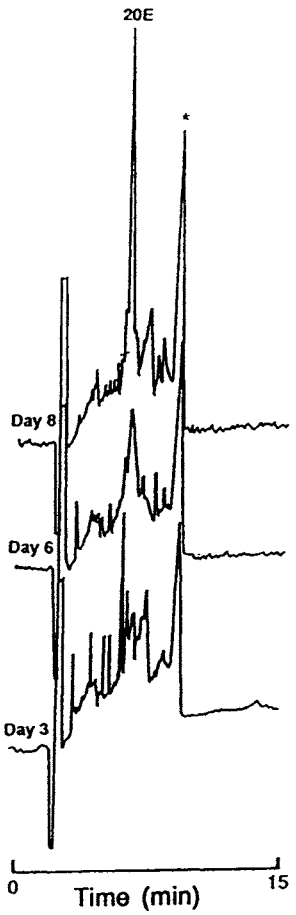


Fig. 7. Representative HPCE of ecdysteroids in hemolymph. Profiles of three samples (3, 6, and 8 days after eyestalk removal) are shown. The asterisk is an unknown peak.

3.4 CONJUGATED ECDYSTEROIDS (MS / MS analysis)

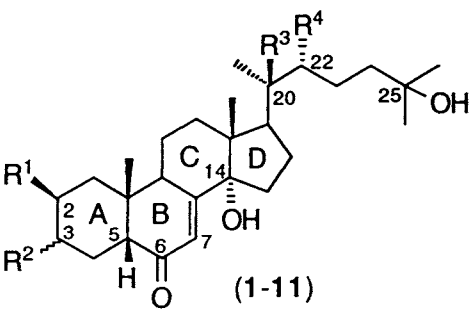
20-Hydroxyecdysone **1** was found as a major ecdysteroid in the hemolymph of *P. clarkii* and as a descendant of ecdysone **2**, which was derived from 3-dehydroecdysone produced by the Y-organ *in vivo*. However, conjugated ecdysteroids cannot be eliminated as the precursors of 20-hydroxyecdysone in the hemolymph (51-53). Identification of free ecdysteroids after hydrolysis of the conjugates (section 3.2) was not satisfactory to understand their roles. Since hormonal ecdysteroids and their derivatives are generally found only in very small amounts, it is required to establish a simple and effective method for their characterization. Thus, the tandem mass spectrometry in a series of ecdysteroid phosphates (ecdysteroids conjugated with phosphoric acid) was investigated.

3.4.1 MATERIALS

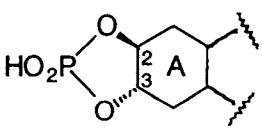
Ecdysteroid phosphates. Ecdysteroid monophosphates, ecdysone 22-phosphate (E-22P) **3**, 20-hydroxyecdysone 22-phosphate (20E-22P) **4**, 2-deoxyecdysone 22-

phosphate (2dE-22P) **5**, 2-deoxy 20-hydroxyecdysone 22-phosphate (2d20E-22P) **6**, 22-deoxy 3-*epi*-20-hydroxyecdysone 2-phosphate (22d20E'-2P) **7**, 22-deoxy 20-hydroxyecdysone 3-phosphate (22d20E-3P) **8**, and 2,22-dideoxy 20-hydroxyecdysone 3-phosphate (2,22d20E-3P) **9** were obtained from silkworm ovaries (54, 55) and provided by Dr. H. Sonebe (Konan University, Japan). Their structures were characterized by a combination of immunological and spectroscopic method. Additional authentic samples, ecdysone 2-phosphate (E-2P) **10**, 20-hydroxyecdysone 2-phosphate (20E-2P) **11**, 3-*epi*-ecdysone 2,3-phosphate (secondary) (E'-2,3P) **12**, and 3-*epi*-20-hydroxyecdysone 2,3-phosphate (secondary) (20E'-2,3P) **13**, were prepared from ecdysone (E) **2**, and 20-hydroxyecdysone (20E) **1** (56, 57). Structures (**1-13**) are summarized in (Fig. 9). Other reagents (guaranteed, analytical and chromatographic grades) were all supplied from Nacalai Tesque (Kyoto, Japan).

Instruments. HPLC system used in this study was a HP 1090 series (Hewlett-Packard, Waldboon, Germany) with UV detection (220-600 mm) (photo-diode array). A spectrometer JNM-EX400 (JEOL, Tokyo, Japan) was used for ^{1}H-NMR studies. Mass spectra were taken by a JMS-HX 110A/HX 110A tandem spectrometer (JEOL, Tokyo, Japan), which has EBEB geometry, with a JMS MP-7000 data system. The detector was an MS-ADS11 variable dispersion array detector (JEOL, Tokyo Japan).



(1-11)



(12, 13)

	R1	R2	R3	R4
20E (**1**)	OH	OH	OH	OH
E (**2**)	OH	OH (β)	H	OH
E-22P (**3**)	OH	OH (β)	H	OPO_3H_2
20E-22P (**4**)	OH	OH (β)	OH	OPO_3H_2
2dE-22P (**5**)	H	OH (β)	H	OPO_3H_2
2d20E-22P (**6**)	H	OH (β)	OH	OPO_3H_2
22d20E'-2P (**7**)	OPO_3H_2	OH (α)	OH	H
22d20E-3P (**8**)	OH	OPO_3H_2 (β)	OH	H
2,22d20E-3P (**9**)	H	OPO_3H_2 (β)	OH	H
E-2P (**10**)	OPO_3H_2	OH (β)	H	OH
20E-2P (**11**)	OPO_3H_2	OH (β)	OH	OH
E'-2,3P (**12**)	-	-	H	OH
20E'-2,3P (**13**)	-	-	OH	OH

Fig. 9. List of abbreviations and their structures (**1-13**)

3.4.2 METHOD & RESULTS

Sample preparation. All chemicals were completely dried before use. Each ecdysteroid (E, 20E, E' and 20E') (3 mg) was dissolved in tetrahydrofuran (0.2 ml) in a flask. Phosphorus oxychloride in pyridine (1/50 v/v) (0.1 ml for E or 20E, 0.025 ml for their 3-epimers) was added into the solution of ecdysteroid and stirred at room temperature for 2 h. Aqueous solution of sodium hydroxide was then added under ice cooling to make the reaction mixture slightly basic. It was extracted with ethyl acetate to remove pyridine. The aqueous phase was adjusted to slightly acidic with the use of 1-N hydrochloric acid. The solution was concentrated under reduced pressure and applied to preparative HPLC separation; a RP-column (Cosmosil-5 C18, 10 x 250 mm) was eluted with a linear gradient (70 min) of methanol in 10 mM phosphate buffer (pH 5.56) changing from 10% to 70% (v/v) at a flow rate of 2 ml/min at 40°C. Each eluate (20E-P, 45 min; E-2P, 52 min; 20E-2,3-P and E-2,3-P, 56 min) was desalted using Sep-Pak C18 (Waters-Millipore Corp., Milford, MA, USA) and characterized by ^{1}H-NMR and mass spectrometry.

Mass spectrometry. All the negative-ion FAB (fast-atom bombardment) mass spectra were recorded at 10 kV accelerating voltage using the first mass spectrometer (MS1 of a tandem spectrometer). Xenon gas as a source of Xe^+ ions of 6 kV energy was used to bombard the sample. Each phosphate sample was dissolved in methanol, and mixed with either 2,2'-dithiodiethanol or glycerol as a matrix for taking FAB-MS/MS; significant [M-H]$^-$ pseudomolecular ions with either a 2,2'-dithiodiethanol or a glycerol matrix were obtained; spectra of E-22P with different matrix were given in (Fig. 10).

Negative-ion FAB CID (collision-induced dissociation) spectra were generated using helium as the collision gas. The collision cell located in the third field-free region was maintained at 8 kV floating voltage. Helium pressure was sufficient to reduce the intensity of precursor ions to 30% of their original intensity. The products ions were detected with an MS-ADS11 variable dispersion array detector using a 10% dispersion.

Intense peak at m/z 79 and 97 due to PO_3^- and $H_2PO_4^-$, respectively, together with other product ions were obtained from fragmentation of [M-H]$^-$ ions as precursor; the fragmentation pattern of E-22P is shown in (Fig. 11). The characteristic fragments in the region above m/z 100 (CID MS/MS), fragment types and their relative abundance (based on the most intense peak) were given in the Tables. The CRF (charge-remote fragmentation) patterns due to phosphate moiety were characteristic to differentiate between the side chain 22-phosphates (TABLE 2) and the A-ring phosphates (2-, 3-P and 2,3-P) (TABLE 3 and TABLE 4). As shown in each master structural formula, cleavages along the side-chain from terminus to the C-22 position are indicated as **A**, **B**, **C**, **D** and **E**. Cleavages within the ring and between C-22 and the ring are indicated as **a** to **g**. Each 1 carbon unit, 2 carbon unit and 3 carbon unit attached to the phosphate group is indicated as **h**, **i** and **j** or **k**.

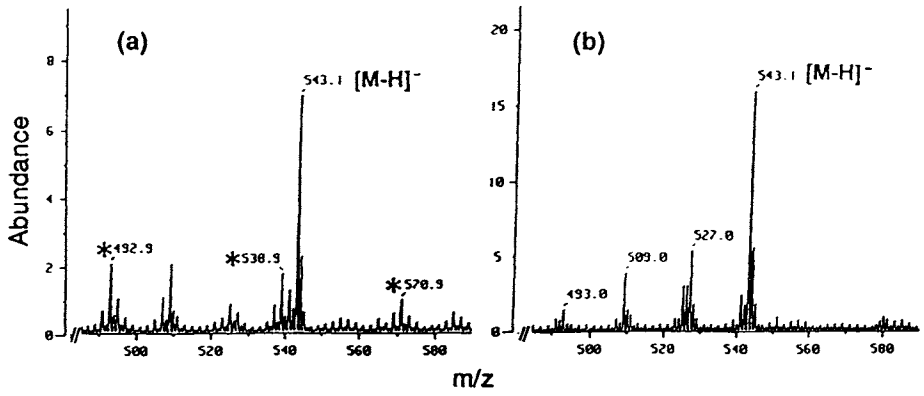


Fig. 10. Pseudomolecular ion [M-H]- (m/z 543) of E-22P **3**. (**a**) 2,2'-dithiodiethanol matrix. The marked peaks (*) were derived from the matrix. (**b**) glycerol matrix.

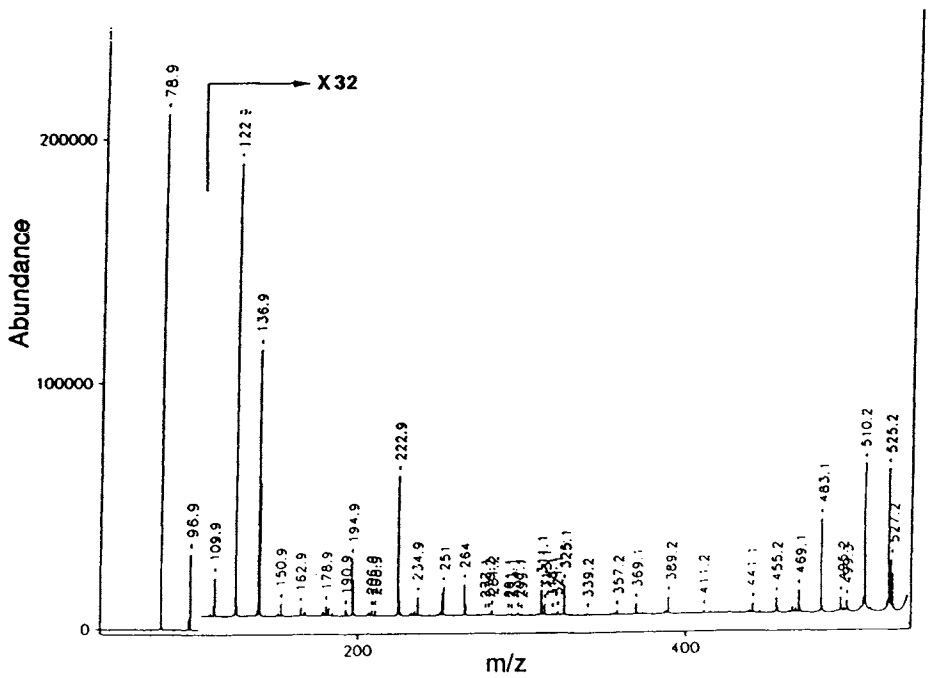
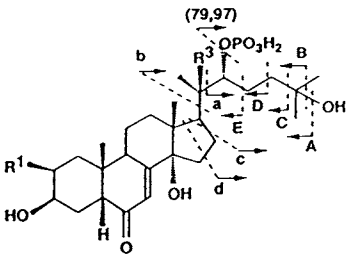
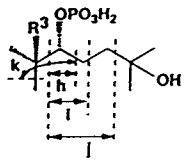


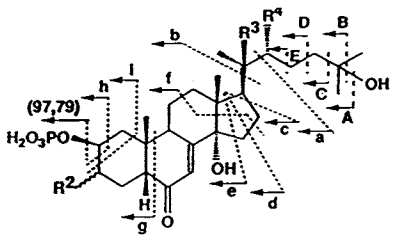
Fig. 11. The CID-MS/MS spectrum of ecdysone 22-phosphate **3**.



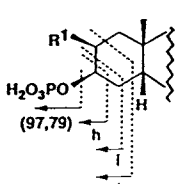


Fragment	Comp. (3 - 6) / m/z (%)			
[M-H]⁻	(3) 543	(4) 559	(5) 527	(6) 543
A	525 (30)	541 (50)	509 (21)	525 (47)
B	511 (12)	527 (12)	495 (9)	511 (7)
C	483 (20)	499 (6)	467 (16)	483 (5)
D	469 (5)	485 (4)	453 (4)	469 (2)
E	455 (4)	471 (4)	439 (1)	-
a	195 (12)	195 (18)	195 (8)	195 (23)
b	223 (30)	239 (17)	223 (23)	239 (14)
c	235 (4)	251 (4)	235 (2)	-
d	251 (7)	267 (4)	251 (3)	267 (4)
-				
-				
-				
h	110 (9)	110 (4)	110 (5)	110 (6)
i	123 (100)	123 (100)	123 (100)	123 (100)
j	138 (29)	138 (14)	138 (19)	138 (22)
k	137 (59)	-	137 (40)	-

TABLE 2. The characteristic CRF ions (above m/z 100, intensity %) derived from the 22-phosphates (**3-6**).



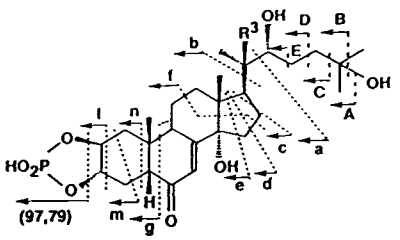
(7, 10, 11)



(8, 9)

Fragment	Comp. (7 - 11) / m/z (%)				
[M-H]⁻	(7) 543	(8) 543	(9) 527	(10) 543	(11) 559
A	525 (50)	525 (17)	509 (23)	525 (46)	541 (37)
B	511 (18)	511 (4)	495 (5)	511 (24)	527 (14)
C	483 (7)	483 (4)	467 (3)	483 (13)	499 (4)
D	469 (9)	469 (13)	453 (3)	469 (23)	485 (7)
E	455 (1)	455 (3)	439 (1)	455 (13)	471 (2)
a	441 (42)	441 (36)	425 (18)	425 (100)	441 (89)
b	397 (49)	397 (52)	381 (19)	397 (36)	397 (30)
c	383 (26)	383 (18)	367 (13)	383 (26)	383 (18)
d	371 (37)	371 (23)	355 (3)	371 (47)	371 (49)
e	357 (14)	357 (14)	341 (2)	357 (19)	357 (20)
f	329 (100)	329 (100)	313 (100)	329 (88)	329 (100)
g	207 (13)	207 (14)	191 (7)	207 (21)	207 (9)
h	-	110 (3)	110 (1)	110 (1)	-
i	123 (13)	123 (34)	123 (15)	123 (18)	123 (17)
j	-	-	138 (5)	-	-

TABLE 3. The characteristic CRF ions (above m/z 100, intensity %) derived from the 2- (**7**, **10**, **11**) and 3-phosphates (**8**, **9**).



Fragment	Comp. (12, 13) / m/z (%)	
$[M-H]^-$	(12) 525	(13) 541
A	507 (38)	523 (19)
B	493 (24)	509 (7)
C	465 (11)	481 (2)
D	451 (22)	467 (6)
E	437 (8)	453 (2)
a	407 (100)	423 (70)
b	379 (36)	379 (18)
c	365 (50)	365 (30)
d	353 (21)	353 (11)
e	339 (21)	339 (25)
f	311 (68)	311 (100)
g	189 (69)	189 (41)
-		
-		
-		
-		
l	121 (34)	121 (26)
m	134 (26)	134 (18)
n	149 (12)	149 (10)

TABLE 4. The characteristic CRF ions (above m/z 100, intensity %) derived from the 2,3-phosphates (**12, 13**).

The most intense peak of the 22-P fragment type appears at m/z 123 (fragment **i**). Comparing E-22P (**3**) to 20E-22P (**4**), CRF patterns were different by 16 mass units for **A-E** and for **b-d**, but not the fragment **a** at m/z 195. In the case of (**3**), the remarkable peak **k** appears at m/z 137 due to the lack of hydroxyl group at the C-20 position. The difference between 2dE-22P (**5**) and 2d20E-22P (**6**) was also observed in agreement with the above conclusion. Comparing (**3**) or (**4**) to (**5**) or (**6**), the patterns differed by 16 mass units for **A-E**, while the fragments' **a-d** were identical. These fragmentations confirmed the deoxy-ring skeletons in (**5**) and (**6**).

In the case of the A-ring phosphates (TABLE 3), the cleavage between C and D rings was obvious and the intense fragment **f** was observed. The CRF patterns of 22d20E'-2P (**7**) and 22d20E-3P (**8**) were almost identical and therefore neither positional isomers in the A-ring (C-2 and C-3) nor C-3 configurational isomers (α and β) could be distinguished from each other; these illustrated the limitations of the mass spectrum. The patterns of **A-D** for (**7**) and (**8**) were similar to those of E-2P (**10**), although the fragments' **a-i** were identical among (**7**), (**8**) and 20E-2P (**11**). Thus, the presence of hydroxyl groups at the C-20 of (**7**) and (**8**) were confirmed. The characteristic cleavage between C-20 and C-22 in both (**10**) and (**11**) resulted in the intense fragment **a**. Comparing (**10**) and (**11**), CRF patterns differed by 16 mass units for **A-E** and **a**, although the fragment ions for **b-i** appeared at the same numbers. This indicated the presence of an additional hydroxyl group at the C-20 of (**11**). In 2,22d20E-3P (**9**),

dehydroxylated at C-2 (or possibly C-3), exhibited the fragment **j** (*m/z* 138) as seen in the 22-phosphate (**3-6**).

In the case of (**12**) and (**13**), the fragments' **l**, **m** and **n** were observed instead of **h**, **i** and **j** in (**3-6**). The presence of an additional hydroxyl group at the C-20 position of 20E'-2,3P (**13**) was also confirmed by comparison of the patterns to those of E'-2,3P (**12**); identical patterns for **b-g** and **l-n**, and differences of 16 mass units for **A-E** and **a**.

The phosphates, after the first mass analyzer, afforded somewhat poor parent ions and concluded that MS/MS is better suited to the free ecdysteroids (58). However, MS/MS-CID of the phosphates provided CRF ions due to the electronegative moiety that gave many structural informations. In addition, making phosphates from free ecdysteroids was a one-pot reaction, in which 3ß-ecdysteroids afforded predominantly the 2-monophosphates. In the same procedure, 3α-ecdysteroids were derived to the 2,3-phosphate (secondary). The 2- or 3-monophosphates were exclusively derived from the 2- or 3-deoxy ecdysteroids. Thus, even free ecdysteroids in low concentrations can be better characterized by derivatization using CID-MS/MS.

3.5 ECDYSONE BIOSYNTHESIS INHIBITOR (EBI)

Attempts to isolate the molt-inhibiting hormone "MIH" of crustaceans resulted in 3-hydroxy-L-kynurenine and xanthurenic acid (28, 29). Xanthurenic acid suppressed ecdysteroid synthesis by the Y-organ *in vitro*, and therefore classified as EBI (ecdysteroid biosynthesis inhibitor). Xanthurenic acid interfered the cytochrome P450-dependent ecdysteroidogenesis in either Y-organ or PG (prothoracic gland) of insect (30, 31). It was also found that 3-hydroxy-L-kynurenine, released from the X-organ-sinus gland complex in the eyestalk, was accumulated and transformed into xanthurenic acid in the Y-organ *in vivo* (29). The presence of chemo-reception system at Y-organ was shown by autoradiography (32).

3.5.1 MATERIALS

Animals. The crayfish *P. clarkii* were purchased and treated as described previously. The sea water crabs *Callinectes sapidus* (blue crab), *Geothelphusa dehaai* (fiddler crab), and *Protunus trituberculatus* (swimming crab) were collected off different coasts of USA and Japan (a random mixture of sex, maturity, and molting stage). The 5th instar larvae of silk warm, *Bombix mori* (day 7), were purchased and reared on an artificial diet at 26-28°C under a 16L : 8D photoperiod for 4 days.

Chemicals. Authentic 3-hydroxy-L-kynurenine and Tween 80 were purchased from Wako Pure Chemicals (Japan), and Penicillin G and streptomycin were from Sigma Chemical Co.. Grace's medium was the product of Gibco, A 23187 (Calbiochem Ind.). Oxygen-18 gas was purchased from MSD ISOTOPES (Canada, 97.6%) or CEA (France, 98.0%). $P450_{PB}$ (induced by phenobarbital in rats) was a gift from Dr. Y. Funae (Osaka City university). [$^2H_4$]-2-Deoxyecdysone (23,23, 24,24-$^2H_4$]-3β,14α,22R,25-tetrahydroxy-5β-cholest-7-en-6-one) was synthesized according to a published method (59). Unless otherwise stated, all other chemicals were obtained from Nacalai Tesque (Japan). 3-Hydroxy-L-[^{3}H]- kynurenine (529 GBq /mmol) was ordered to The Institute of Whole Body Metabolism (Japan) for the autoradiogram.

3.5.2 METHODS & RESULTS

Bioassay for EBI. The protocol of conventional homogenized Y-organ culture was as follows. Dissected Y-organs were rinsed once with chilled 0.17M KH_2PO_4/$NaHCO_3$ buffer, (pH 7.1, 1.0 ml/Y-organ) to remove the "MIH" accumulated on the tissues, and homogenized in the chilled buffer (100 μl/Y-organ); this rinsing had a tendency to increase the ecdysteroid production by Y-organ. The suspension was transferred into centrifuge tubes (final volume 1.2 ml/Y-organ) and centrifuged below 5°C at 7700 x g for 20 min. The precipitate was centrifuged again with the same volume of buffer. To the combined supernatant containing the enzymatic system and its substrate, were added 0.2 mg streptomycin and 200 IU penicillin per 1 ml medium, and aliquots of 2.5 ml/Y-organ were incubated at 37°C with shaking for 20 h. These conditions prevented organ decay and allowed the Y-organ homogenate to biosynthesize ecdysone. Upon termination of incubation, each aliquot was lyophilized and extracted with methanol. The extract was passed through Sep-Pak C18 to submit ecdysone analysis (section 3.1). Ecdysone synthesis by the Y-organ was activated by homogenization of the organ, whereas 3-dehydroecdysone synthesis was inactivated; due to the activation of 3-ketoreductase. The combined Y-organ homogenates (e.g., prepared from 20 organs or 10 animals) rather than an individual Y-organ led to the leveling of variations among animals and reduced the number of control experiments. The protocol for Y-organ (whole) culture was already described (section 3.1).

EBI effect (in vitro) of eyestalk extracts. After immobilization of animals by chilling on ice, their eyestalks were uprooted from their bases with tweezers. The excised eyestalks were frozen with dry-ice and lyophilized to prevent deterioration of the tissue for storage in the cold-room (<5°C) as the EBI pool. The stored 660 eyestalks weighting 36 g, were extracted 6 times with 200 ml each of 0.1 M acetic acid, 100°C for 10 min and centrifuged (1580 x g) for 15 min. The supernatant was lyophilized and the residual powder (16 g) was stored as the reference, ES-X (Fig. 12). The inhibitory action of ES-X (in KH_2PO_4/$NaHCO_3$ pH 7.1 buffer) was assayed by co-incubation with the Y-organ homogenates for 20 h, 37°C in an ES-X/Y-organ ratio of 1 : 1, and percentage inhibition of ecdysone synthesis was calculated from the reduction in the ecdysone HPLC peak area as compared to that of the controls containing no ES-X. The inhibitory activity manifested itself even when tested among different crab species and silk worms (molting gland, prothoracic gland from *B. mori*). The inhibitor thus appeared to be species-nonspecific between ES donors and test animals. It was also found that the inhibitory activity of the ES-X was not destroyed upon heating at 100°C for 30 min.

Isolation of 3-Hydroxy-L-kynurenine. Attempts to isolate the EBI from the ES-X, following the inhibitory assay, led to the characterization of 3-hydroxy-L-kynurenine **1**. The 0.1 M acetic acid elution profile from the G-25 column was similar to those reported earlier which followed an *in vivo* assay in the decapod ES/amphipod system (60). Electrophoresis of fraction V on cellulose, 0.4 M acetic acid/0.12 M formic acid (1 : 1 v/v), pH 2.5 gave a pattern also closely resembling that published as "MIH" (60). Fraction V was further purified by Sephadex G-10, 0.1 M acetic acid, and cation exchange MCI gel, CK 10V (Mitsubishi), 0.8 M acetic acid/0.4-2 M ammonia, linear gradient elution, to yield 700 μg (ca. 1 μg/ES) of pure inhibitor, which was confirmed by co-culture with Y-organ homogenate. The structure was identical with **1** in all aspects: ultraviolet absorption (UV), electron impact mass spectrum (EI-MS), circular dichroic spectrum (CD), nuclear magnetic resonance spectrum (NMR), electrophoresis and

amino acid analysis. EI-MS m/z 322 (as diacetyl methylester); UV (in 0.005 M HCl) 224 nm (ε 18400), 266 nm (ε 7600), 370 nm (ε 4000); CD (in 0.005 M HCl) 321 nm (Δε +1.0), 273 nm (Δε +0.5), 370 (Δε -0.2). Incubation with authentic 3-hydroxy-L-kynurenine indeed exhibited inhibitory activity although the potency was less than that of the crude ES-X.

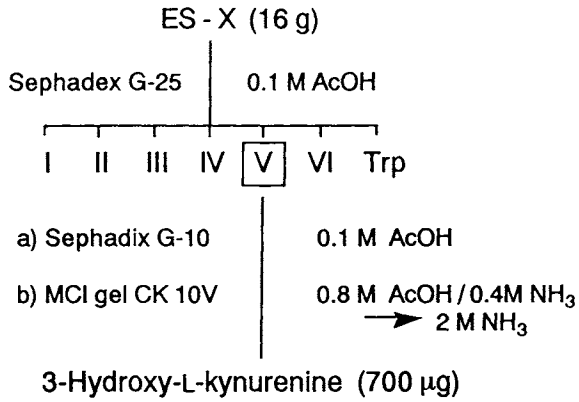
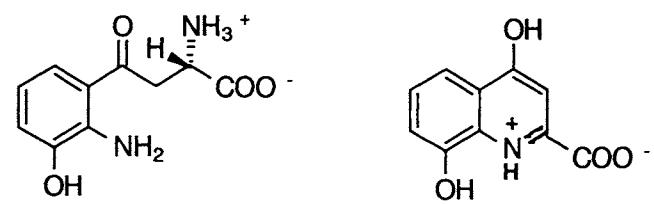


Fig. 12. Isolation procedure of 3-hydroxy-L-kynurenine **1**.



3-hydroxy-L-kynurenine (**1**) xanthurenic acid (**2**)

Isolation of xanthurenic acid. The most striking aspect of the finding is that the EBI activity *in vitro* was strongly exhibited by xanthurenic acid **2**, a key metabolite of **1**. An active search for **2** led to its detection in ES-X. Thus ES-X was dissolved in aqueous ammonium acetate (0.17 M, 250 μl/ES, pH 5, a solvent system found to be efficient for the extraction of **2**) and centrifuged at 1580 x g for 15 min. The supernatant was placed on a Asahipak GS 320 column (Asahi Chem. Ind.) and treated with aqueous ammonium acetate to elute **1**, and then with 20 % acetonitrile in 0.17 M ammonium acetate to elute **2** that was co-chromatographed with an authentic specimen on HPLC for its identification. The amounts of **1** and **2** as estimated from 920 ES were 3.4 μg and 2.0 μg/ES, respectively (Microsorb C_{18} 7.6 mm x 500 mm, 0.17M CH_3COONH_4 pH 5, 1 ml/min for **1** and 20% CH_3CN/CH_3COONH_4 pH 5, 2 ml/min for **2**, 240 nm detection). Paralleled to the above experiment, eight X-organ/sinus gland complexes were carefully removed from the fresh eyestalks in ice-cold physiological saline, extracted with the

ammonium acetate buffer, and centrifuged; HPLC indicated the amounts of **1** and **2** per X-organ/sinus gland complex to be 28 ng and 128 ng, respectively.

Aminotransferase activity. An equal volume of hemolymph was taken from de-eyestalked animals at 24-h intervals beginning of day 1. The total 6 ml was diluted with an equal volume of buffer solution (0.17 M KH_2PO_4/$NaHCO_3$, pH 7.1) containing 0.06 g of casein. The mixture was gently stirred at 0°C for 1 h and then centrifuged at 1580 x g for 20 min. The supernatant was divided into 6 aliquots (2 ml each), which were incubated at 37°C for 3 h with stirring in the presence or absence of exogenous **1** (50 μg/150 μl buffer). Upon termination of the incubation, each aliquot was lyophilized and dissolved in 2 ml of 0.1 M ammonium hydroxide, then dialyzed overnight against 40 ml of 0.05 M ammonium hydride (cellulose dialyzer tubing, VT-801). The dialyzable solution was lyophilized, and the quantity of **2** in the residue was determined by HPLC as described above. The transformation of **1** into **2** was L-enantiospecific and accomplished in 61% yield. The crude enzyme preparation obtained from their eyestalks and Y-organs were also capable of transforming **1** into **2**.

EBI effect (in vivo) of 3-hydroxy-L-kynurenine. The crayfish in the intermolt stage, 7-8 cm in length and weighing ca. 30 g after towel drying, were randomly divided into several entry groups that consisted of 10-15 animals each of both sexes. They were bilaterally de-eyestalked and forced to autotomize their chelae on the same day. Beginning on the next day, **1** (30 or 100 ng/per animal) in 100 μl of general crustacean physiological saline was injected daily (control animals were injected with 100 μl of saline), and the number of animals that had shed their exoskeletons was tallied each day for two successive molting cycles; eyed animals that received the same treatment did not molt for 22 days. The injection amount of **1** was determined on the basis of the maximum physiological level over one year (150 ng/ml hemolymph); each animals received daily injection of **1** (30 ng/100 μl saline) delayed the onset of the first molt by 2 days, and lengthened the interval between the first and second molts by 3 days (Fig. 2). On the other hand, daily injection of **2** in saline (10 and 30 ng/100 μl) induced no significant effects regarding the molt inhibiting properties before the animals died. Quantitative analysis revealed that injection of **1** suppressed the normal increase of 20-hydroxyecdysone in hemolymph. The increased titer preceded ecdysis in control animals; 20-hydroxyecdysone in hemolymph peaked at a value >30 ng/ml (average of 10 animals) in eyestalk-ablated, autotomized control animals. The 20-hydroxyecdysone concentration remained low (5 ng/ml) for about 13 days by injection of **1** and increased to about 40 ng/ml just before molting. Injection of **2** did not produce any hormonal change detectable by the quantitative analysis (Fig.13). These results indicated that the circulating **1** was primarily responsible for EBI effect *in vivo*. Indeed the presence of the chemo-reception system for **1** in the Y-organ (*in vitro*) was shown by the specific incorporation of [^{3}H]-**1** as followed by radioautography (Fig. 14).

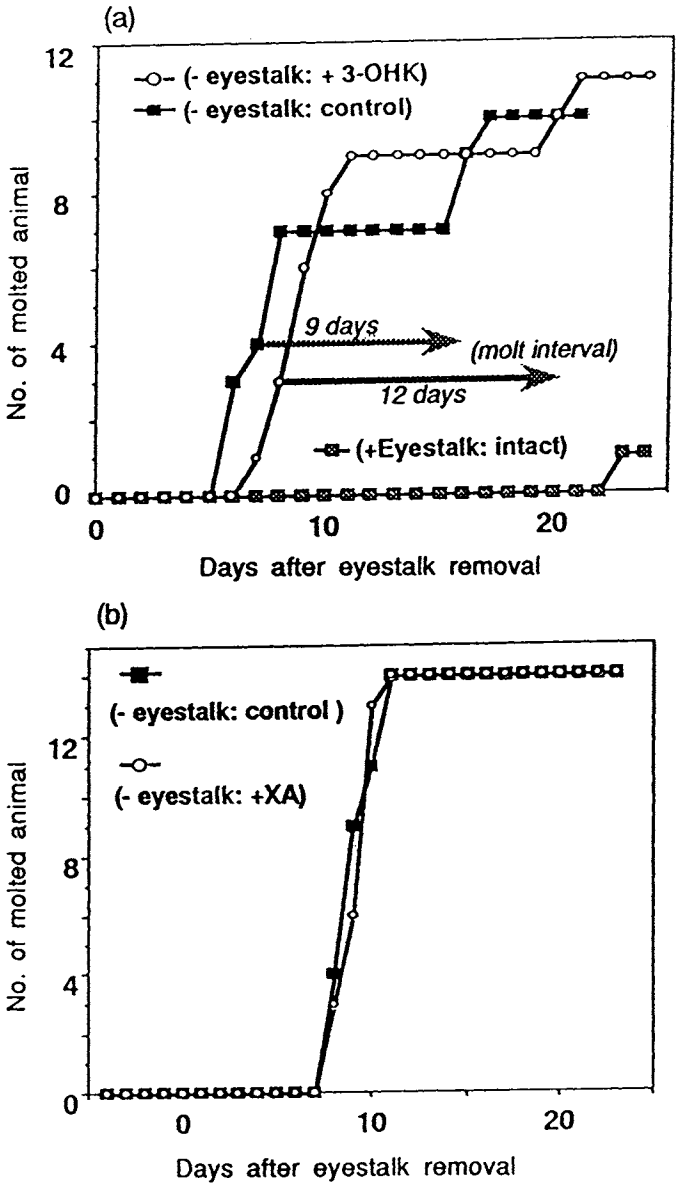


Fig. 13. Injection effects of 3-hydroxy-L-kynurenine and xanthurenic acid on crayfish (*P. clarkii*). Animals were removed their eyestalks (day 0). Daily injections were administrated for two molt cycles, beginning on day 1. (**a**): 3-hydroxy-L-kynurenine **1**. (**b**): xanthurenic acid **2**.

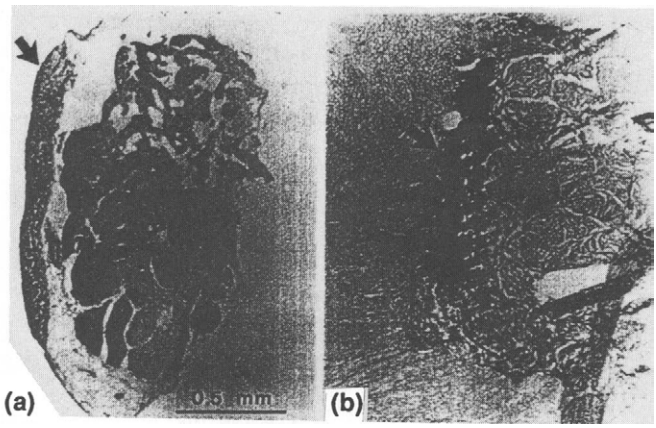


Fig. 14. (**a**): Y-organ of *P. clarkii* (vertical section); (**b**): [^{3}H]-3-hydroxy-L-kynurenine incorporated. The dark position on the resulting radioautography coincides with the Y-organ. (photographs taken by H. Sonobe and Inst. of Whole Body Metabolism, respectively)

Effect of xanthurenic acid on P450-dependent biotransformations. To proof the involvement of cytochrome P450 in the ecdysone biosynthesis by Y-organ, we next focused on C-2 hydroxylation of 2-deoxyecdysone (2dE); 2-deoxy-20-hydroxyecdysone (2d20E) was found in the hemolymph (6). The comparative experiments using Y-organs from *P. clarkii* and PGs from *B. mori* (*in vitro*) were performed with 23,24-[$^{2}H_4$]-2-deoxyecdysone under an $^{18}O_2$ atmosphere (61) in the presence or absence of **2** as EBI; the hydroxylation process of PG products is known to be mediated by P450 monooxygenases (62), which are localized in mitochondria and microsomes. In these experiments, the commercial culture media were somewhat modified before use in the incubation of the Y-organs (a) and the PGs (b), respectively: (a) Medium 199 containing Hank's salt (Gibco) was modified according to Keller's method (63), and prior to use the pH was adjusted to 7.5 with NaOH; (b) Tween 80 (0.002%) was added to Grace's insect medium (supplemented with lactalbumin hydrolysate, yeastolate, L-glutamate and methionine, without insect hemolymph, pH 6.5, Gibco).

Using 1 ml of the specified medium (a or b), each batch of glands (3-Y-organs, n=3 or 10 PGs, n=4) was incubated at 25°C in a stream of oxygen ($^{16}O_2$) for 18 h in the presence of [$^{2}H_4$]-2-deoxyecdysone dissolved in 5 μl of ethanol (45 μg for Y-organs and 90 μg for PGs: much higher than physiological concentration). On the other hand, only ethanol (5 μl) was added to the medium for each control experiment with the paired molting glands. The productions of the deuterated ecdysone either by Y-organs or PGs were significant; Y-organs produced ecdysone more than 1000 times, even when the controls produced only undetectable amount under our experimental conditions (<0.5 ng). Under the separate experiments, Y-organs from the animals in different sizes and sexes converted deuterated 2-deoxyecdysone (2dE) into deuterated ecdysone (E) but not into deuterated 3-dehydroecdysone (3dhE), although the latter was shown to metabolize

into 20-hydroxyecdysone (20E) *via* ecdysone (E) at peripheral of the Y-organ *in vivo* (7). In the similar experiment, PGs produced ecdysone more than 200 times compared to the controls (8.4 ng/PG). These biotransformations under $^{16}O_2$ were confirmed using mass spectrometry: the $[M+H]^+$ (m/z 469) corresponding to $[^2H_4]$-ecdysone was identified (Fig. 15). The yield of the bio-transformation was about 20% (calcd. from the starting material).

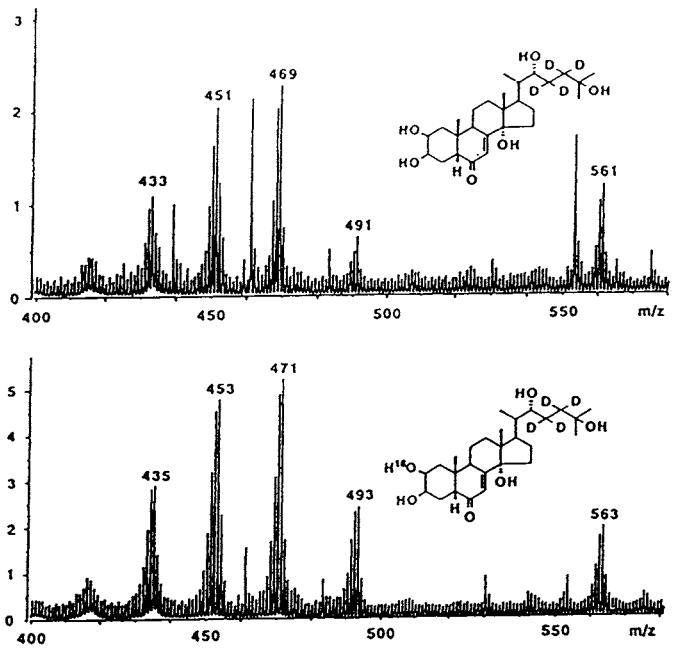


Fig. 15. FAB mass spectrum of deuterated ecdysone transformed from $[^2H_4]$-2-deoxyecdysone. (above): The protonated molecular ion containing four deuterium atoms, produced under $^{16}O_2$, can be seen at m/z 469. Fragment ions at m/z 451 and 433 due to sequential losses of water are also observed. The pseudomolecular ions $[M + Na]^+$ and $[M + glycerol + H]^+$ are present at m/z 491 and 561, respectively. In addition to those major peaks, presence of the isotopes attributable to the incorporation of d_0-d_4 was indicated (see text). The residual intense peaks come from the glycerol matrix. (below): Using $^{18}O_2$, all important peaks are shifted by two mass units higher.

In the presence or absence of heavy molecular oxygen ($^{18}O_2$), bio-transformations were demonstrated; gas composition was adjusted to the proportion of $^{18}O_2/N_2 = 1 : 4$. The nitrogen gas was purified by passing through Fieser's solution to eliminate contamination by atmospheric oxygen. Hydrogen sulfide generated by Fieser's solution was removed by a saturated lead acetate solution (64). Each incubation medium was degassed by sonication under vacuum for 10 s, then saturated with helium gas at 4°C. The incorporation of molecular oxygen was confirmed by MS analysis; the $[M+H]^+$ ion peak at m/z 471 (Fig. 15) indicated the incorporation of an ^{18}O atom into ecdysone, in Y-organs or PGs. The contamination with ^{16}O-ecdysone can be explained as

follows: Under EI and FAB ionization modes, the MS spectra of our synthetic deuterated 2-deoxyecdysone revealed that the relative abundance of $d_0/d_1/d_2/d_3/d_4$ was almost 1 : 2 : 6 : 10 : 10. The distribution of the isotopes was attributable to the contamination by H_2 in the $^2H_2$ gas used for catalytic reduction of the synthetic intermediate with an acetylenic function. A similar isotope distribution was observed with a model compound having a long $-(CH_2)_2-$ sidechain. The ratio of ^{16}O-ecdysone *m/z* 469 to ^{18}O-ecdysone *m/z* 471 was estimated to be 12 : 88.

Xanthurenic acid **2** (100 μM for Y-organs and 10 mM for PGs) significantly inhibited (50-55%) the biotransformations of deuterated 2-deoxyecdysone (2dE) into ecdysone (E). The ED_{50} of ES-X, *i.e.* the effective dose of ES-X leading to 50% inhibition of ecdysone synthesis (Y-organ *in vitro*) was estimated to be ca. 1 μg ES-X, namely 136 ng of **1** and 78 ng of **2**. Even when assuming a 100% conversion from **1** to **2**, this combined amount of **1** and **2** appeared to account for most of but not the full potency of ES-X.

Molecular mechanism of EBI. The mode of action with EBI was first investigated under the working hypothesis (28, 29) that the ecdysteroidogenic cascade is mediated by the biocatalyst cytochrome P450s (62). Incubation of a model enzyme $P450_{PB}$ (induced by phenobarbital in rat) with **2** resulted in a deference UV spectrum exhibiting an absorption shift from 417 to 448 nm (30). An increase in absorbance at 448 nm, the Soret peak (γ) arising from the formation of the cytochrome $P450_{PB}$ ferrous-XA forms, was observed in a dose-dependent manner. Although changes in the α-band (569 nm) and β- band (535 nm) were obscure, the peak at 448 nm indicated that the hydroxyl group most likely constituted the sixth ligand of $P450_{PB}$ (Fig. 16). An analog, kynurenic acid, which bears no hydroxyl group at C-8, showed no spectroscopic evidence of binding to P450PB. No evidence of interaction between **1** and the biomolecules was also found. It was thus suggested that the 8-OH of **2** is involved in ligand exchange at the iron porphyrin of $P450_{PB}$. When cytochrome c was used as a model (28), equivalent absorption shifts in UV and the binding Fe^{3+}-O- in ESR (g=2.30, 2.12, 1.88, 77°K) spectra were observed. Although the inhibitory effects were increased in the presence of the detergent Tween 80, the inhibition was independent of changes in the intracellular calcium ion levels caused by addition of the calcium ionophore A23187.

As described above, the C-2 hydroxylation step was definitely mediated by a cytochrome P450 (Y-organ *in vitro*). Thus, the regulation mechanism of ecdysteroidogenesis by the Y-organ can be concluded as the result of binding of an active EBI **2**, onto the active site of the P450-monooxygenase. The EBI effect was stronger against ecdysone (94% inhibition) than against 3-dehydroecdysone (28% inhibition). Present results suggest that there are two types of C-2 hydroxylases in the different biosynthetic site of the Y-organ; one require 3-hydroxyl group as the substrate and another does not. It is also possible that different type of P450, with different interaction to xanthurenic acid, is involved in the corresponding biosynthesis. It was already described that the homogenized Y-organ produced only ecdysone distinct from a whole Y-organ. Xanthurenic acid did not suppress the 20-hydroxylation of ecdysone in the peripheral of Y-organ.

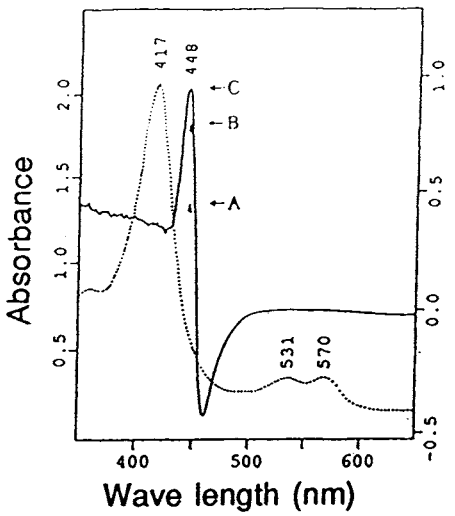


Fig. 16. Absorption spectrum of cytochrome $P450_{PB}$ and the difference spectra of XA-ligated states. Spectra were obtained at 20°C in 0.35 M sodium phosphate buffer (pH 7.4) containing 0.05% cholic acid and 20% glycerol. Dotted line: absorption of the enzyme (12 nM) in the ferric state (left axis); solid line: ferrous form, XA-ligated state (right axis); A: +XA (10 μM), B: +XA (20 μM), C: +XA (25 μM). +XA: xanthurenic acid added. The numerals indicate the positions of absorption maxima in nm.

In conclusion, the molting hormone regulation in the crayfish was protected by stepwise: 1) the production ratio of 3-dehydroecdysone and ecdysone is depending on an activity of 3-ketoreductase in the Y-organ; 2) overproduction of ecdysone by Y-organ is strongly suppressed by EBI, which is regulated by an activity of aminotransferase; 3) transformation of 3-dehydroecdysone into 20-hydroxyecdysone *via* ecdysone is regulated by the activity of 3α- and 3β-ketoreductases out of the Y-organ. Involvement of "MIH" is believed for many years, however, the mode of action of "MIH" remains to be elucidated (section 4.3).

4. EYESTALK HORMONE

In decapod crustacean, the X-organ-sinus gland complex in the eyestalks plays a significant role on a peptidergic neurosecretory system. As earlier studies, testing for an endocrine effect from the eyestalks has been performed by injection of the crude extract, in which the target responses were followed; concentrating/dispersing chromatophore, light-adapting/dark-adapting distal retinal pigment, hyper-/hypo-glycemia, inhibition/acceleration of molting, inhibition/growing of gonad etc. It is, however, unclear that whether such responses are evoked by the particular hormone or

by other factor in the extract. Thus, experimental attention should focus on purification of hormones, because definitive testing and determination of the structure can be done only with highly purified hormone.

Until recently, chemical information of four eyestalk peptidic hormones in the crayfishes is available for red-pigment concentration hormone (RPCH), pigment-dispersing hormone (PDH), hyperglycemic hormone (CHH), and molt-inhibiting hormone (MIH)

4.1 CRUSTACEAN HYPERGLYCEMIC HORMONE

4.1.1 ISOLATION PROCEDURE

Crustacean hyperglycemic hormone (CHH) is the most readily obtainable of the eyestalk hormones in crustaceans. However, in our preliminary experiments, no presumptive CHH fraction was found in the extract using HCl-acetone, acetic acid, and hot water. When the hot HCl extraction according to the isolation procedure of the lobster (*Homarus americanus*; Hoa-) CHH (65) was examined, the red swamp crayfish (*Procambarus clarkii*; Prc-) CHH was successfully extracted with higher yield.

The first isolation of Prc-CHH was as follows (66): The X-organ-sinus gland complexes (20 equivalents) were routinely homogenated in 1 ml of 0.1N HCl and extracted with continuous stirring for 3 min in a boiling water bath. After cooling under ice-water and stirring for another 60 min, the mixture was centrifuged for 30 min at 10,000 rpm at 4°C, and the supernatant was lyophilized. The extract dissolved with 0.05N acetic acid was subjected to gel filtration on a Sephadex G-75 column (superfine, 1.6 x 50 cm) equilibrated 0.05N acetic acid and eluted with the same buffer, as shown in Fig. 1.

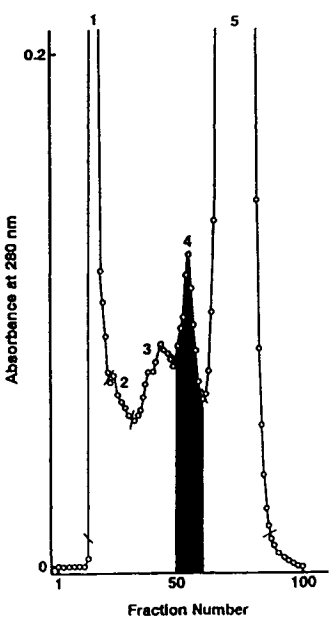


Fig. 1. Elution profile of an acetic extract of the crayfish *Procambarus clarkii* of X-organ-sinus gland complex (400 equivalents) on a Sephadex G-75 column (superfine, 1.6 x 50 cm) equilibrated with 0.05N acetic acid. Flow rate, 9 ml/hr, fraction size, 1.2 ml. Hyperglycemic activity was concentrated in fraction 4 and is marked with shading.

The fraction containing CHH was lyophilized. Measurement of hormonal response, the increasing glucose level, will be described later (section 4.1.3) in detail. The sample was dissolved in 0.1 ml of 0.1% trifluoroacetic acid (TFA) and applied to reversed-phase high-performance liquid chromatography (HPLC) on a TSK gel ODS 120T column (0.45 x 25 cm) with a gradient of 35% to 45% acetonitrile in 0.1% TFA for 40 min. The final step involving reversed-phase HPLC on a TSK gel ODS-120T column aided to separate into two components of the hormones, as shown in Fig. 2. We designated them as CHH-I and CHH-II according to the order of elution.

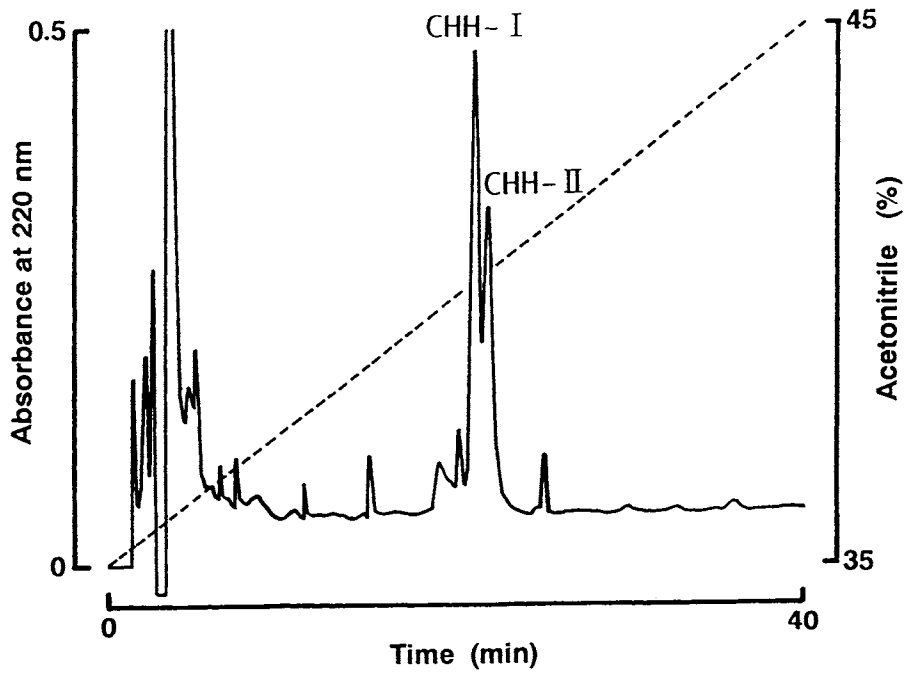


Fig. 2. High-performance liquid chromatography of Prc-CHHs on a TSK gel ODS 120T (0.46 x 25 cm, 5μm). The dotted line represents a gradient of acetonitrile in 0.1% TFA.

Although highly purified Prc-CHHs were obtained, its amino terminus of pyroglutamic acid (section 4.1.2) consequently arises the question of whether formation of pyroglutamic acid occurred during the extraction performed under the heated acidic condition or yielded by post-translational modification. Thus, we must examine to improve on extraction procedure again.

The improved method was briefly as follows: Sinus gland was dissected only from the eyestalks, and then extracted with saline solution containing 30% acetonitrile for 3 min at 4°C. After centrifugation, the supernatant applied to reversed-phase HPLC. Present isolation procedure yields 40 pmol from two sinus glands obtained from one red

swamp crayfish, and the purified hormones are identical to one obtained from the hot HCl extraction. The method also showed that the isoforms with a constant ratio are present in a single gland, either of the two sides (Fig. 3).

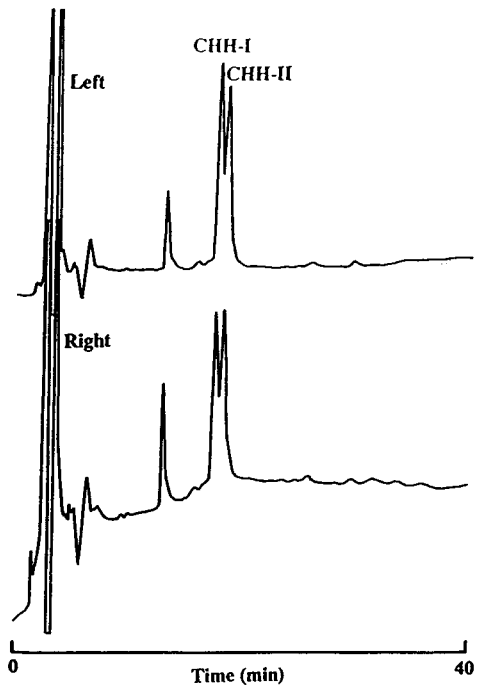


Fig. 3. High-performance liquid chromatography of Prc-CHHs extracted from a single gland on a TSK gel ODS 120T (0.46 x 25 cm, 5μm).

4.1.2 PRIMARY STRUCTURE

Molecular Weight. Investigation of the molecular weight of the hormones as obtained by mass spectrometry using electrospray indicated that they have identical molecular mass of 8,386 ± 1. The hormone was dissolved with 5% acetic acid being concentration of 10 pmol/μl. 2 μl of the solution was applied to a Hitachi M-1200H mass spectrometer interfaced with electrospray and flowed at 50% methanol with the rate of 25 μl/min.

Amino Acid Analysis. Amino acid composition of the hormones was determined by phenylthiocarbamyl (PTC) method (67). The hormones were hydrolyzed with 6N HCl containing 0.6% phenol at 110°C for 24 hr (68). The resulting amino acids were derivatized with phenylisothiocyanate in triethylamine-ethanol buffer (ethanol: triethylamine:water=7:1:1). The PTC amino acids were analyzed by reversed-phase HPLC on a TSK gel ODS 80 TM column at 40°C, using a linear gradient of 0.07M sodium acetate containing 5% acetonitrile and 60% acetonitrile in water for 20 min. UV detector was set at 254 nm, and the flow rate was 1 ml/min. In here, both hormones were exhibited very similar amino acid compositions, as shown in TABLE 1.

Amino-terminal Analysis. No amino-terminal residue of the hormones was

detectable for a gas-phase sequencer (Shimadzu, PPSQ-10). Thus, determination of amino terminus was carried out by tandem mass spectrometry, as described below.

Carboxy-terminal Analysis. Determination of carboxy-terminal residue of the hormones was as follows (69): The intact hormone (100 pmol) was digested with thermolysin (substrate:enzyme =100:1) in 0.1 ml of 0.1M ammonium acetate (pH 8.2) at 37°C for 4 hr, and further digested with carboxypeptidase Y (substrate:enzyme=40: 1) in 0.1 ml of 0.1M ammonium (pH 6.0) at 37°C for 24 hr. The digest was lyophilized, and coupled with phenylisothiocyanate, as described above. The derivatives were dissolved with 0.1 ml of ethyl acetate and 0.1M sodium hydroxide and mixed well. After centrifugation, the organic solvent was transferred into a sample tube (1.5 ml type). The resulting PTC-amino acid amide was identified by a reversed-phase HPLC on a TSK gel ODS 80TM column (0.46 x 25 cm, particle size 5 μm). The solvents were (**A**) 0.07M sodium acetate (pH 5.3) containing 2% acetonitrile and (**B**) 60% acetonitrile in water. The gradient % of **B** was as follows; 5-12% in 3min, 12-30% from 3 to 6 min, 30-60% from 6 to 10.5 min, 36-90% from 10.5 to 19 min, 90-100% from 19 to 20 min, and held at 100% from 20 to 25min. A single carboxy-terminal residue of Val-amide from each other was detectable in this analysis. The standard profile for the carboxy-terminal amide analysis was shown in Fig.4.

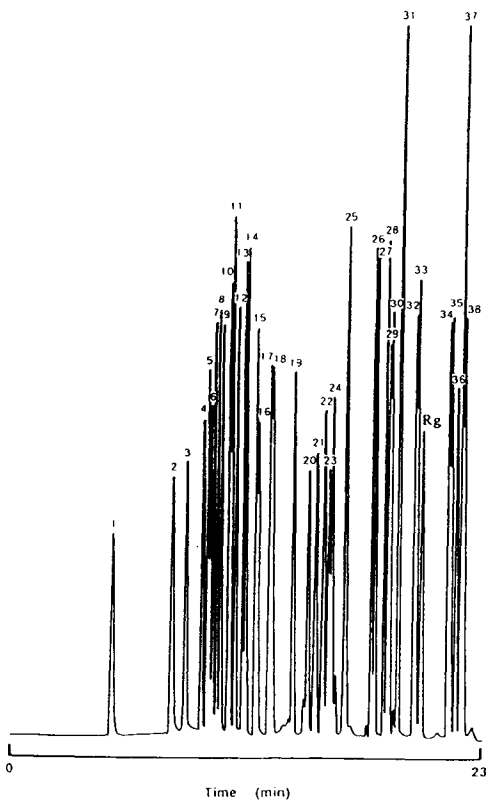


Fig. 4. Carboxy-terminal analysis. A separation of 250 pmol of the PTC-amino acids and the PTC-amino acid amides on a TSK gel ODS 80TM column. Peaks (1) cysteic acid, (2) Asp, (3) Glu, (4) Asn, (5) Ser, (6) Gln, (7) Asp-amide, (8) Gly, (9) His, (10) Glu-amide, (11) Arg, (12) Thr, (13) Ala, (14) Pro, (15) Asn-amide, (16) Arg-amide, (17) Ser-amide, (18) Gly-amide, (19) Tyr, (20) Pro-amide, (21) Thr-amide, (22) Val, (23) Ala-amide, (24) Met, (25) Cys, (26) Ile, (27) Leu, (28) Phe, (29) Tyr-amide, (30) Trp, (31) Lys, (32) Val-amide, (33) Met-amide, (34) Ile-amide, (35) Leu-amide, (36) Trp-amide, (37) Lys-amide, (38) Phe-amide. A peak designated Rg is present in the reagent blank.

In this carboxy-terminal amide analysis, a key is that peptide is fully cleaved with enzymes to liberate the carboxy-terminal residue. Thus, application of this system is limited it when carboxy-terminal residue are releasing. Recently, the mass spectrometry with high sensitivity becomes to cover the analysis of microsequencing of peptides. The strategy for determination of its carboxy-terminal residue in the most proteins and peptides is as follows: Carboxy-terminal fragment peptide with at least less of 15 residues is prepared by enzymatic and/or chemical cleavage and by HPLC separation. The resulting fragment peptide is subjected to analyses of its amino acid composition, sequence, and molecular weight. Based on these results, we make judgement whether its carboxy-terminus is amide or not.

Amino Acid Sequence Analysis. The complete amino acid sequences of the CHHs were determined by analyses of peptide fragments prepared by digestion with lysyl endopeptidase and by a combination of trypsin and endopeptidase Asp-N digestion. First, disulfide bonds in the hormone were oxidized by performic acid (70). A mixture of 19 vol of 98% formic acid and 1 vol of 30% hydrogen peroxide was allowed to stand in a tube at room temperature for 2 hr, and then 0.1 ml of performic acid was added in a tube containing 200 μg of hormone. The cleavage of disulfide bonds was performed at 4°C for 4 hr. The solution was diluted with water and lyophilized. Next, the oxidized hormone was digested with lysyl endopeptidase (substrate:enzyme=60:1, by weight) in 0.1M ammonium bicarbonate (pH 8.0) at 37°C for 15 hr. Fractionation of fragment peptides was performed by reversed-phase HPLC on a TSK gel ODS 120T column (0.46 x 25 cm) using a linear gradient of isopropanol in 0.1% TFA or on a TSK gel TMS column (0.46 x 25 cm) using a linear gradient of acetonitrile in 0.1 % TFA. the eluate was monitored by absorption at 220 nm. Peptide maps of lysyl endopeptidase digests of the oxidized CHH-I and CHH-II by HPLC using an ODS column showed separation of four fragments, in which a shift of the retention time between LE1 fragments was observed, as shown in Fig. 5. As the largest fragment became missing on an ODS column, the peptide named as LE5 recovered from a TMS column. Both LE5 showed the same elution time. Amino acid compositions of LE fragments are summarized in TABLE 1.

Although LE1 fragments exhibit the different retention time, they have the same amino acid composition. It seems that whether there is a different order of sequence in their peptides or a shift of retention time is caused by a conformational change between two peptides. When subjected to tandem mass spectrometry by a JMS-HX/HX110A (JEOL), the mass spectrometric data indicated the same sequential order of amino acids in both LE1 fragments, pGlu-Val-Phe-Asp-Gln-Ala-Cys(SO_3H)-Lys. Since the sequence involved in the blocked amino-terminal residue, LE1 is located in amino terminus in the hormone. Judging the results and structural feature of amphibian and mollusk peptides (section 5.1), we hit on the presence of D-amino acid in the CHH molecule. Accordingly, re-investigation of amino acid compositions by prederivative method using (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) (71) was performed. The methods is as follows: The sample was dissolved in 0.5 ml of 1M borate buffer (pH 6.85) and 0.5 ml of 18mM FLEC solution in acetone, and then allowed to

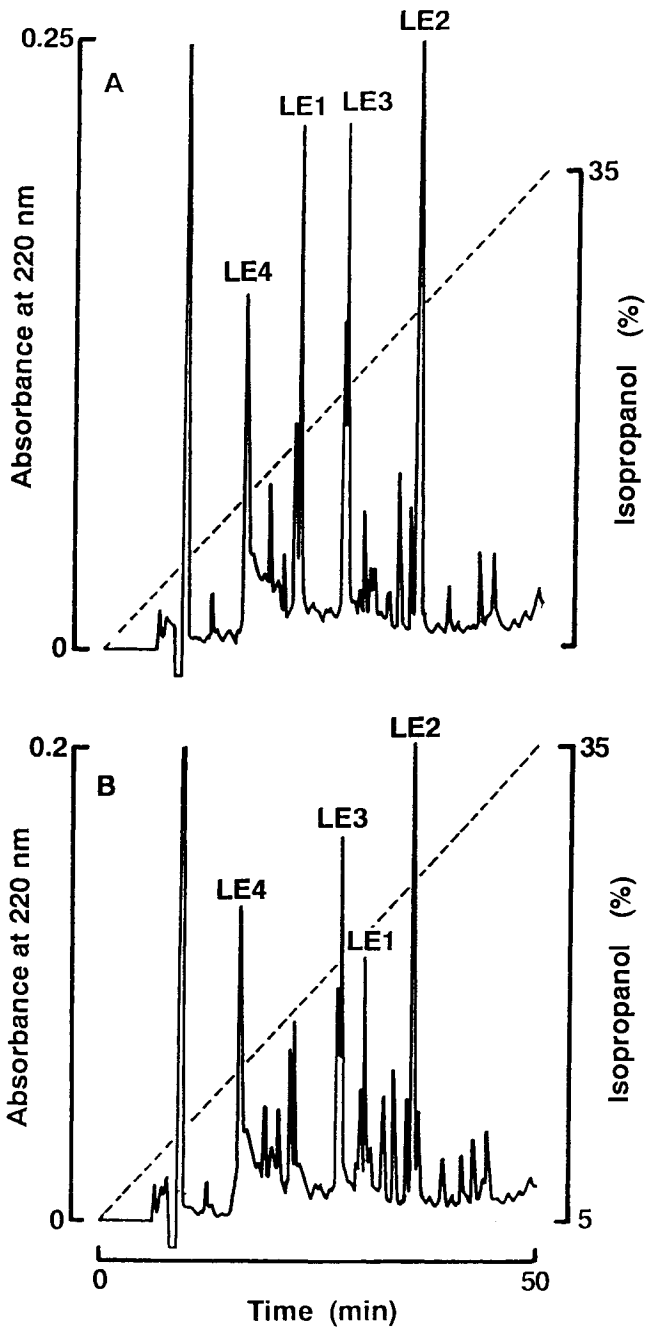


Fig. 5. High-performance liquid chromatography of the digests of performic acid oxidized Prc-CHH-I (A) and Prc-CHH-II (B) with lysyl endopeptidase on a TSK gel ODS-120T column. The dotted line represents a gradient of isopropanol in 0.1% TFA.

TABLE 1. Amino acid compositions of the red swamp crayfish CHHs and LE fragments obtained from lysyl endopeptidase digestion.

	Prb-CHH[a]	Prc-CHH-I	LE1	LE2	LE3	LE4	LE5	Sum of LEs
1/2Cys[b]	6	6.2(6)[c]	1.1		2.0	2.1	1.0	6.2
Asp	11	10.7(11)	0.9	0.9	2.7	1.9	3.8	10.2
Glu	7	6.8(7)	1.8		0.9	1.0	2.9	6.6
Ser	2	2.1(2)					1.9	1.9
Gly	2	2.2(2)		1.2			1.1	2.3
His	0	0 (0)						
Arg	5	5.1(5)		1.1	2.0	1.0	1.0	5.1
Thr	3	2.8(3)				1.9	0.9	2.8
Ala	4	3.9(4)	1.0	1.0		2.0		4.0
Pro	1	1.3(1)				1.2		1.2
Tyr	6	6.0(6)		1.1	2.1	1.9	0.9	6.0
Val	8	7.5(8)	1.0		1.0	1.0	4.6	7.6
Met	0	0 (0)						
Ile	4	3.8(4)		2.0			2.1	4.1
Leu	6	5.3(6)			2.1		3.5	5.6
Phe	3	3.1(3)	1.0	1.0			1.0	3.0
Trp	0	0 (0)						
Lys	4	3.9(4)	1.0	1.0	1.9			3.9
Total	72	70.7(72)	7.8	9.3	14.7	14.0	24.7	70.5

	Prc-CHH-II	LE1	LE2	LE3	LE4	LE5	Sum of LEs
1/2Cys[b]	6.2(6)[c]	1.1		2.1	2.1	1.0	6.3
Asp	10.8(11)	1.0	0.9	2.8	1.9	3.7	10.2
Glu	6.9(7)	1.9		0.9	0.9	3.1	6.8
Ser	1.9(2)					1.8	1.8
Gly	2.3(2)		1.1			1.2	2.3
His	0 (0)						
Arg	5.1(5)		1.0	2.0	1.0	1.0	5.0
Thr	2.7(3)				1.9	0.9	2.8
Ala	4.1(4)	0.9	1.0		2.1		4.0
Pro	1.1(1)				1.2		1.2
Tyr	6.0(6)		1.0	2.1	2.1	1.0	6.2
Val	7.7(8)	1.0		1.0	1.0	4.7	7.7
Met	0 (0)						
Ile	3.9(4)		2.0			2.0	4.0
Leu	5.2(6)			2.1		3.6	5.7
Phe	3.0(3)	1.0	1.0			1.0	3.0
Trp	0 (0)						
Lys	3.8(4)	1.0	1.0	2.0			4.0
Total	70.7(72)	7.9	9.1	14.9	14.2	25.0	71.1

[a]Taken from Huberman *et al.* (72). [b]Determined as cysteic acid. [c]Numbers in parentheses represent the number of residues determined by sequence analysis.

stand at room temperature for 5 min. After addition of 1 ml of pentane, shook it, and the pentane phase was discarded. The removal of excess reagent was carried out twice. The

resulting FLEC amino acids were separated by reversed-phase HPLC on a Wakosil 5C18 (Wako Chemicals) with a linear gradient of 25% tetrahydrofuran (THF) in acetate buffer (pH 4.0) to 50% THF in acetate buffer (pH 5.1) and monitoring by a fluorescence detector set at an excitation wavelength of 260 nm and an emission wavelength of 315 nm. Only Phe in LE1 derived from CHH-II was identified as D-amino acid, as shown in Fig. 6. From these results, it is clear that occurrence of D-amino acid is third position in the hormone.

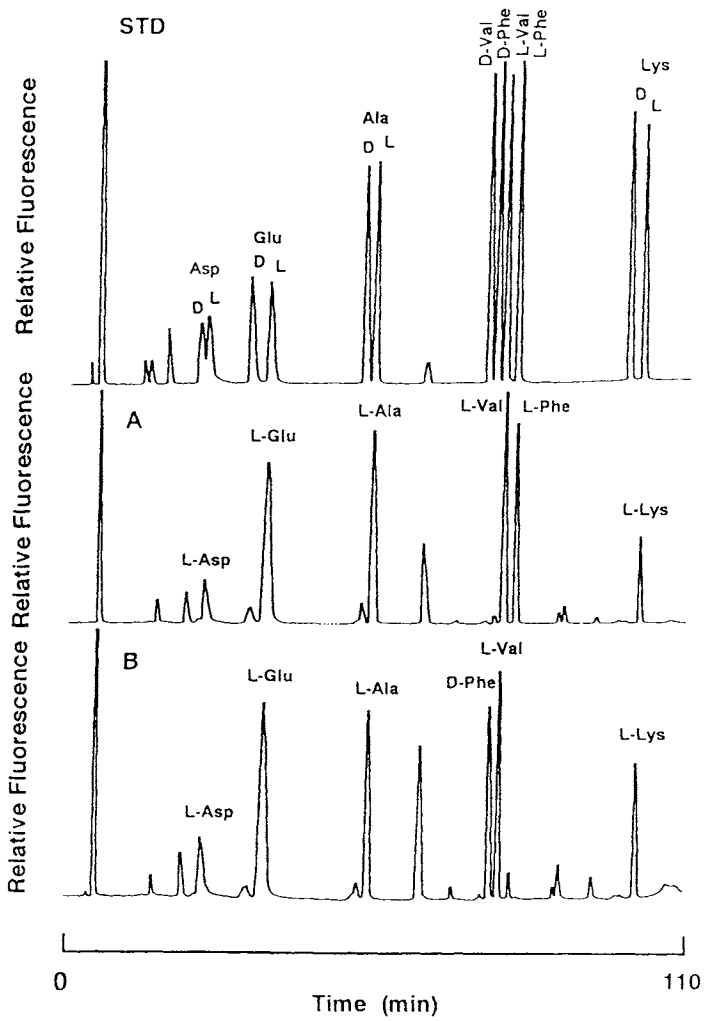


Fig. 6. HPLC chromatograms of amino acids generated after hydrolysis and derivatization with FLEC of LE1 derived from Prc-CHH-I (A) and CHH-II (B). STD is a chromatogram of authentic FLEC amino acids.

In order to determine the position of sulfide bonds, the intact hormone (200 μg, mixture of CHH-I and CHH-II) was also digested with trypsin (substrate:enzyme=60:1, by weight) in 0.2M ammonium acetate (pH 8.0) at 37°C for 4 hr. After lyophilization, the digest was further cleaved with endoproteinase Asp-N (substrate:enzyme=60:1, by weight) in 0.1M Tris-HCl (pH 8.0) at 37°C for 4 hr. The fragment peptides were separated by reversed-phase HPLC on a TSK gel ODS 120T column (0.46 x 25 cm) using a linear gradient of isopropanol in 0.1% TFA. The results of sequence analysis for three fractions containing disulfide bond are illustrated in Fig. 7. A fraction, TAN23, was used to determine the C-terminal portion of the hormone. The results of sequences LE fragments and TAN23 are summarized in TABLE 2.

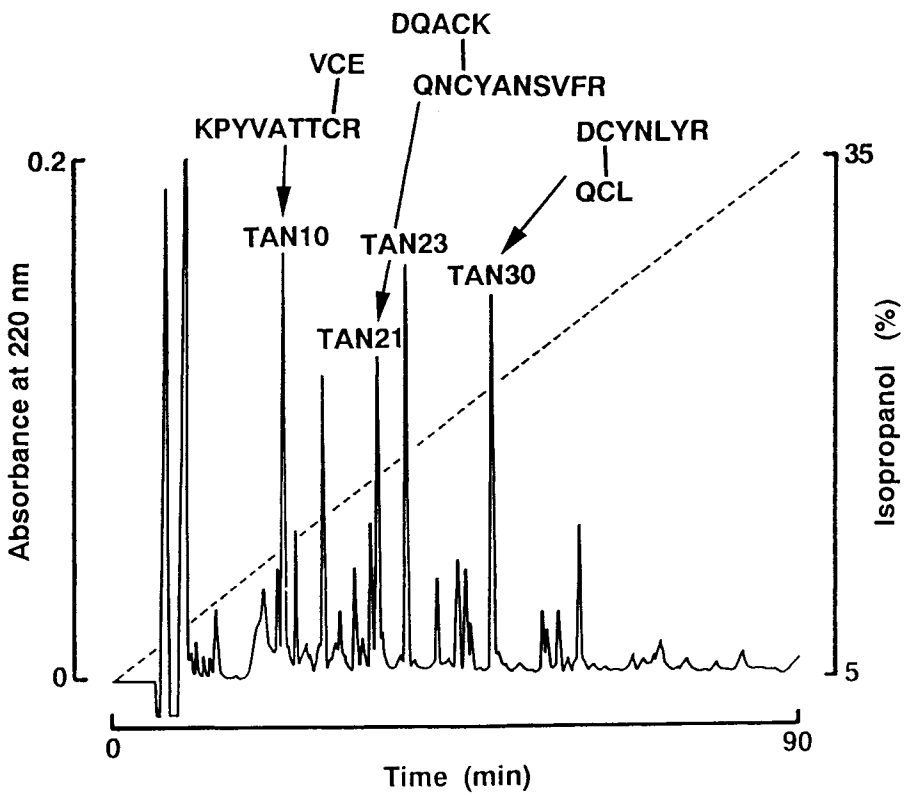


Fig. 7. High-performance liquid chromatography of the digest of intact CHH with trypsin and endoproteinase Asp-N on a TSK gel ODS-120T column. Peptides were collected and then subjected to sequence analysis.

TABLE 2. Amino acid sequences of fragment peptides obtained from Prc-CHH.

LE1:	pGlu-Val-Phe-Asp-Gln-Ala-Cys-Lys
LE2:	Gly-Ile-Tyr-Asp-Arg-Ala-Ile-Phe-Lys
LE3:	Lys-Leu-Asp-Arg-Val-Cys-Glu-Asp-Cys-Tyr-Asn-Leu-Tyr-Arg-Lys
LE4:	Pro-Tyr-Val-Ala-Thr-Thr-Cys-Arg-Gln-Asn-Cys-Tyr-Ala-Asn
LE5:	Ser-Val-Phe-Arg-Gln-Cys-Leu-Asp-Asp-Leu-Leu-Leu-Ile-Asp-Val-Val-Asp-Glu-Tyr-----
TAN23:	Asp-Glu-Tyr-Ile-Ser-Gly-Val-Gln-Thr-Val-amide

Arrangement of the fragment peptides was aided by sequence similarity to the Mexican crayfish (*Procambarus bouvieri*, Prb-) CHH which have determined by Huberman *et al.* (72). The structure of Prc-CHHs is that both isoforms contain 72 amino acid residues with three disulfide linkages, at positions 7-43, 23-39, and 26-52, and differ from each other by the D/L epimerization of phenylalanine at position 3 .

It is believed that proteins and peptides synthesized by living organisms are constituted L-amino acids, although the α-carbon atom of the amino acids is asymmetric and thus can exist at least two stereometric forms. Indeed, occurring D-amino acids within the biologically active proteins and peptides are rare, but it is observed in a wide variety of animal kingdom (section 5.1). Is it possible to isolate a peptide containing D-amino acid from natural sources? Our studies, as described above, will be worthy noticing; i.e. in the case of a "doublet" reversed-phase HPLC peak observed, if these two isoforms have identical amino acid compositions and molecular weights as well as the instance with identical amino acid sequences, there is a possibility that the purified isomer contains D-amino acid. Next, how to evidence the structure of the isomers? We think amino acid analysis is better than several methods according to use of synthetic peptides, CD, NMR and X-ray analysis etc, because amino acid analysis entails advantages with relatively easy operation and higher sensitivity. In the case of Prc-CHH, amino acid analysis utilizing FLEC derivatives was done, though it includes handicaps, that reproducibility for retention time is inferior and the separation of FLEC-amino acids is not readily accomplished using a buffer system. The reason is that hydrolysate amino acids obtained from the fragment peptide are restricted within Asx, Glx, Ala, Val, Phe, and Lys, as shown in Fig. 6. The lower reproducibility of retention time is caused by the bubbles occurred within the line from buffer's bottle to HPLC pump due to use of tetrahydrofuran. We now employ liquid chromatographic determination of D- and L- amino acids by prederivatization with o-phthaldealdehyde and N-isobutyryl-L-cysteine (73), in spite of no reaction with proline. Because its derivatization and subsequent applying to HPLC are automatically performed and permit the separation of the hydrolysate standard including DL-tryptophan and DL-cysteic acid. The standard profile of DL-amino acid analysis is shown in Fig. 9.

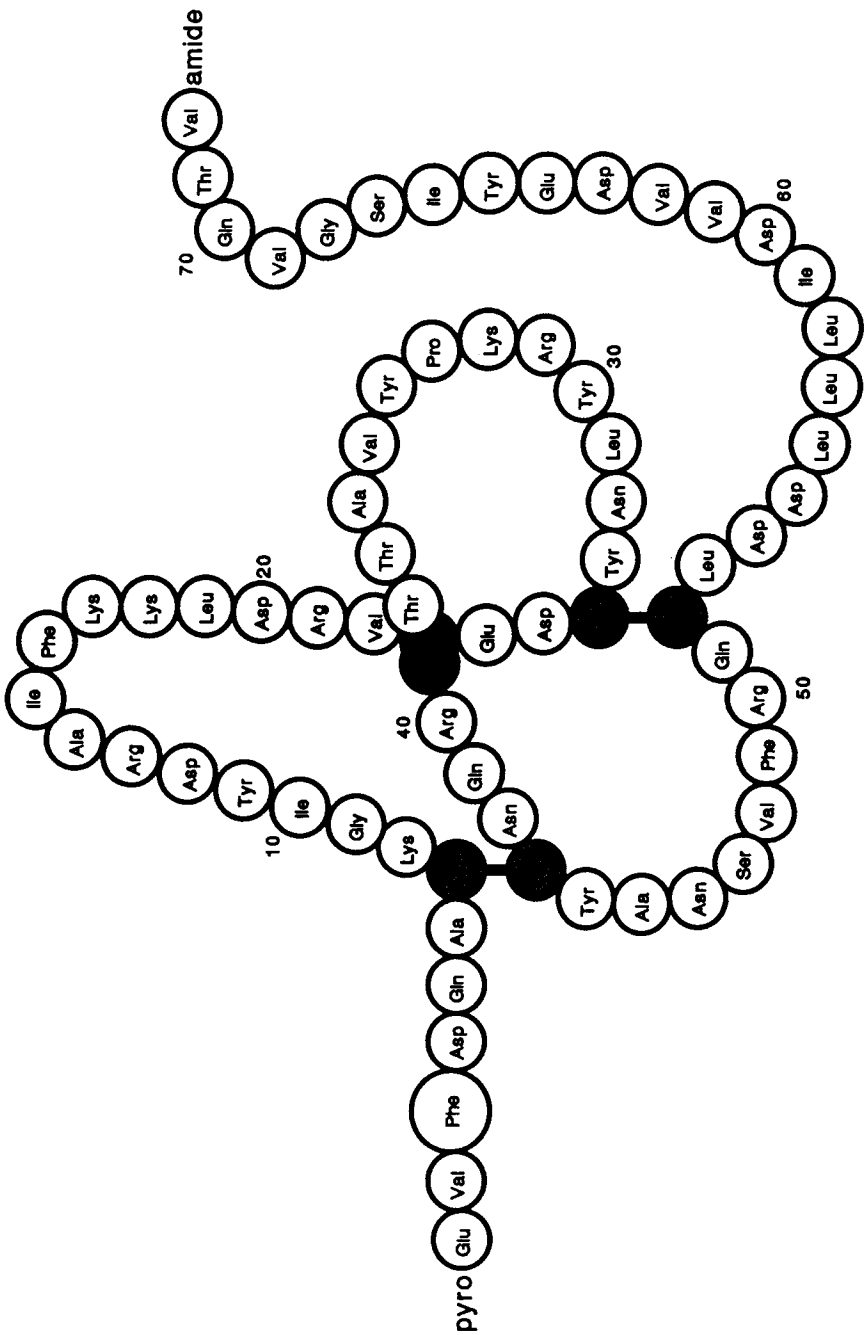


Fig. 8. Amino acid sequence of Prc-CHH. The residue at third position is L-Phe (CHH-I) and D-Phe (CHH-II).

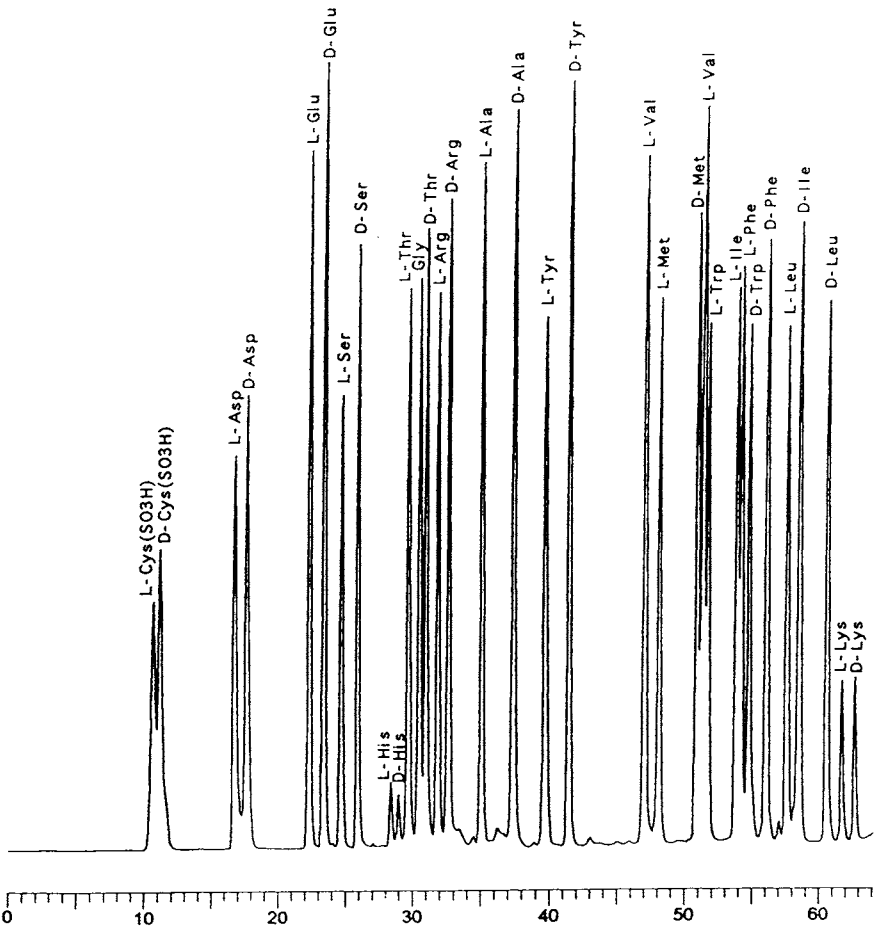


Fig. 9. Elution profile of standard of amino acids derivatized with OPA-IBLC. The amino acid derivatives (each 125 pmol) were separated by reversed-phase HPLC using a Capcell Pak C18 UG (0.46 x 25 cm, 5 μm) column. Conditions: A) 5% methanol/acetonitrile (6:4) in 0.02M phosphate buffer (pH 5.75); B) 60% methanol/acetonitrile (6:4) in 0.02M phosphate buffer (pH 5.75). Linear gradient, 0 to 75% B in 65 min. Fluorescence detection, Ex 340 nm; Em 450 nm. Flow rate, 1 ml/min. Column temperature, 30°C.

4.1.3 BIOLOGICAL ACTIVITIES

It has been well known that crustacean hyperglycemic hormone is involved in carbohydrate metabolism in most crustacean, and displays overlapping hyperglycemic and molt-inhibiting activity in several species. Therefore, we will need more investigations what two CHHs of red swamp crayfish possess the primary physiological function in its species.

Hyperglycemic Activity. After removal of eyestalks, the red swamp crayfish were kept for 48 hr at room temperature in container with a small amount of water. The purified hormone of 12.5 pmol (or fraction in gel filtration) was dissolved in crustacean saline (74) and then injected into the de-eyestalked crayfish. 20 μl of hemolymph was collected both prior to and 90 min following injection. The samples and saline (20 μl) were mixed to the dried TIDE control solution N, and subjected to a potable glucometer, TIDE (M.B. Saunders Co.) . TIDE is a portable glucometer for mammalian blood, based on the hexokinase method and its optical density. Thus, glucose in crustacean hemolymph can not be directly analyzed as there is no erythrocyte. TIDE control solution N contains erythrocyte like material and glucose with concentration of about 100 mg/100 ml. When 20 μl of the control solution was freeze-dried and re-constructed with 20 μl of hemolymph and/or saline, it is possible to analyze glucose level of crustacean hemolymph.

Hemolymph glucose level of de-eyestalked crayfish was about 15 mg/100 ml. When we examined a test for fractions obtained from gel filtration, an increased activity in hemolymph glucose level was observed in a fraction (Fig. 1). Investigation in response for the highly purified hormones indicated that both hormones, CHH-I and CHH-II seem to be equipotent in increasing hemolymph glucose levels, as shown in Fig. 10. In the case of the lobster *Homarus americanus* CHH-A (75), when injected into the crayfish *Orconectes limosus*, the two isomers have different effect on the time course of the elevation of hemolymph glucose level. This may suggest the different isoforms have different functions and possibly different targets.

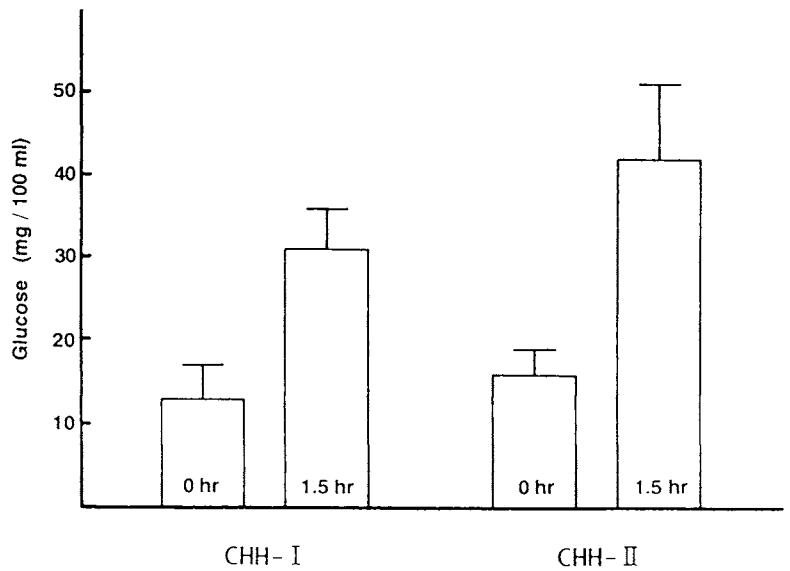


Fig. 10. Hyperglycemic activity of Prc-CHHs (each 12.5 pmol/animal injected) with the de-eyestalked crayfish. Data show means ± SEM, n = 3.

Molt-inhibiting activity. There are two viewpoints of "molt-inhibiting". One includes augmentation of molt cycle by hormonal effects in de-eyestalked crustacean, and other is inhibition of ecdysteroid synthesis by isolated Y-organs. Molt-inhibiting activity described here belongs to the later. Y-organs were excised from the de-eyestalked crayfish which kept for 48 hr at room temperature in container with a small amount of water before use. The incubation medium was consisted of 90% Medium 199 and 10% fetal bovine serum plus 4.8 g of N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid, 4.3 g of NaCl, 0.4 g of $MgCl_2$, and 1.8 g of $CaCl_2$ in 1 liter medium. The pH was adjusted to 7.5 with 5N NaOH. The isolated Y-organs were cultured at room temperature for 2 hr in 200 µl of the medium without CHH or with 50, 5, 0.5, and 0,05 pmol of CHHs. After incubation, the medium was subjected to Sep-Pak C18 1CC cartridge (Waters Ltd.) equilibrated with water. The absorbed materials were rinsed with 20% methanol and then eluted by methanol. Ecdysteroid content was analyzed by an enzyme immunoassay kit (Cayman Chemical Co.) according by a method by Porcheron *et al*. (76). When tested in concentration of 0.25 and 2.5 nM, both the CHHs were inactive. When tested in 25 nM, CHH-II caused a significant inhibition of ecdysteroid synthesis, although CHH-I was inactive. At the dose of 250 nM, CHH-I was less potent than CHH-II. Namely, as shown in Fig. 11, it is clear that CHH-II is more potent in repressing ecdysteroid synthesis than CHH-I.

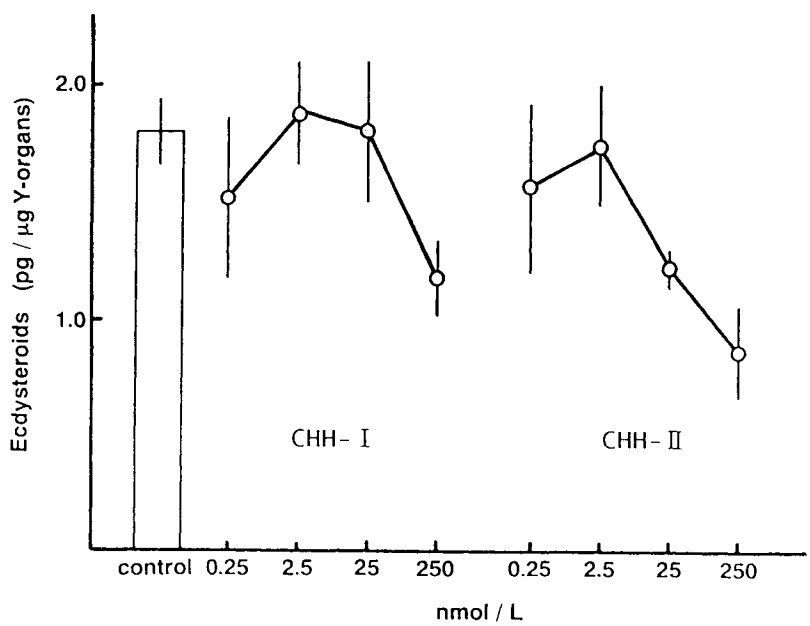


Fig. 11. Effects of Prc-CHHs on depressing ecdysteroid synthesis in Y-organ culture. Vertical lines represent one standard error of mean of the ecdysteroid production (n = 3).

In conclusion, CHH-II containing D-configuration gains a higher inhibitory effect in biosynthesis of ecdysteroids than that of CHH-I in the isolated Y-organs, although both hormones have a significant hyperglycemic activity. This observation might infer that in order to regulate developments in crayfish, involving metabolism and molting, the diversity of functions is dependent on structural change together with occurrence of its receptor in a specific organ and/or a limited period. The crayfish CHHs have both hyperglycemic and molt-inhibiting activities, suggesting that the designation of these hormone as crustacean hyperglycemic hormone or as molt-inhibiting hormone is premature at this time.

4.2 CRUSTACEAN HYPERGLYCEMIC HORMONE PRECURSOR RELATED PEPTIDE

During last few years, application of molecular biological techniques has piled up information for preprohormone structure in crustacean endocrine research. Knowledge of the preprohormone for CHH in the shore crab *Carcinus maenas* (77), the crayfish *Orconectes limosus* (78), and the lobster *Homarus americanus* (79) reveals that CHH prehormone consists of CHH and an additional peptide which is preceding than CHH in its sequence and called the CHH-precursor related peptide (CPRP).

When we examined to isolate CHH, CPRP was recovered either of acetic acid extraction and a hot HCl extraction. The CPRP and CHHs contents containing both CHH-I and CHH-II in the extract were almost equivalent with each yield of 1 nmol from 60 of X-organ-sinus gland complexes. Molecular mass of Prc-CPRP was estimated 3,545±1 by a Hitachi M-1200H mass spectrometer interfaced with electrospray. The CPRP sequence with 33 amino acid residues was reverted by a gas-phase sequencer (Shimadzu, PPSQ-10), and is to follows

H-Arg-Ser-Val-Glu-Gly-Ser-Ser-Arg-Met-Glu-Arg-Leu-Leu-Ser-Ser-Gly-Ser-
Ser-Ser-Ser-Glu-Pro-Leu-Ser-Phe-Leu-Ser-Gln-Asp-Gln-Ser-Val-Asn-OH

Fig. 12. Amino acid sequence of CPRP from the red swamp crayfish.

This sequence is identical to one of the crayfish *Orconectes limosus* CPRPs, but a sole replacement is present at position 33 between Orl-CPRP molecules, Asn and Ser (80). As shown in Fig. 13, CPRP exhibited a single peak on reversed-phase HPLC, although CHH molecule is separated into two peaks. The presence of D-amino acid in CPRP molecule was denied in D,L-amino acid analysis using FLEC. That is, a specific Phe residue corresponding to third position in CHH molecule is isomerized only.

The polymerase chain reaction (PCR) technique provided the partial sequence for prehormone of Prc-CHH. Two primers were designed on the basis of the obtained Prc-CPRP and CHH amino acid sequences and used for PCR-screening of the library

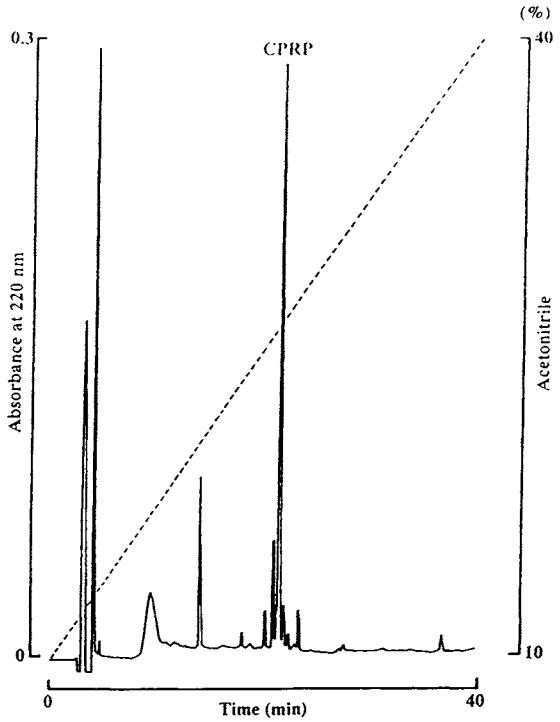


Fig. 13. High-performance liquid chromatography of Prc-CPRP on a TSK gel ODS-120T column using a gradient of acetonitrile from 10% to 40% in 0.1% TFA for 40 min.

prepared from X-organs to isolate a clone. A genomic DNA encoding the CHH precursor contains 6 amino acid residues for C-terminal portion of CPRP and 27 residues for N-terminal potion of CHH. CPRP and CHH are joined by dibasic amino acids, Lys-Arg. The partial nucleotide sequence also reveals that D-Phe present in the CHH molecule is encoded by the usual L-Phe codon (TTC). Based on these observations and coexistence of two isomers of CHH in the gland , we suppose CPRP and CHH are produced in X-organs by the processing of a ribosomally made precursor, and L-isomeric CHH further undergo epimerization to yield D-isomeric CHH. However, the mechanism of in-chain epimerization still remains unclear.

```
                CPRP ←←          →→ CHH
cag gac cag agc gtc aac aag cga cag gtg ttc gac cag gct tgt aaa gga ata
 Q   D   Q   S   V   N   K   R   Q   V   F   D   Q   A   C   K   G   I

tac gac aga gcc atc ttc aag aag ctt gac cga gtg tgc gaa gac tgc tac
 Y   D   R   A   I   S   K   K   L   D   R   V   C   E   D   C   Y
```

Fig. 14. Partial nucleotide sequence of Prc-prepro-CHH. This sequence has been submitted to the GenBank/EMBL/DDBJ Data Bank with accession no. D14610.

4.3 MOLT-INHIBITING HORMONE

Elucidation of phenomenon of molting in molecular level is a interesting of vital importance. Among Arthropoda, however, it has set forth two the hypotheses. Molting in insects is initiated by a tropic hormone called prothoracicotropic hormone which is synthesized in brain, whereas crustaceans is usually influenced by inhibiting hormone secreted from sinus gland and loss of a hormone triggers their molting.

Two materials, which are chemically different, are a candidate for molt-inhibiting hormone in crustaceans. One was reported it as "an indole alkylamine" isolated from the shrimp *Pandalus* sp. (60) and subsequently identified to be 3-hydroxy-L-kynurenine from blue crab *Callinectes sapidus* (28). The detail of low molecular compound in molt-inhibiting described in section 3.5. Other embraces a few compounds with closely related amino acid sequence. In red swamp crayfish, CHH-I and CHH-II having D-amino acid show an inhibitory effect of ecdysteroidogenesis in Y-organ *in vitro* (section 4.1.3). Under investigation of CHH in saline-30% acetonitrile extraction, we found a peptide with molecular mass of 8,646 ± 2 and with molt-inhibiting activity (81). The structural determination for the peptide in amino acid sequence has completed and being investigation of its biological activity, just as, primary structure of Prc-MIH reported from elsewhere (83). Prc-MIH consists of 75 amino acid residues with three disulfide bonds and has 25% sequence homology to Prc-CHH. The amino acid sequence is as follow:

H-Arg-Tyr-Val-Phe-Glu-Glu-Cys-Pro-Gly-Val-Met-Gly-Asn-Arg-Ala-Val-His-Gly-Lys-Val-Thr-Arg-Val-Cys-Glu-Asp-Cys-Tyr-Asn-Val-Phe-Arg-Asp-Thr-Asp-Val-Leu-Ala-Gly-Cys-Arg-Lys-Gly-Cys-Phe-Ser-Ser-Glu-Met-Phe-Lys-Leu-Cys-Leu-Leu-Ala-Met-Glu-Arg-Val-Glu-Glu-Phe-Pro-Asp-Phe-Lys-Arg-Trp-Ile-Gly-Ile-Leu-Asn-Ala-amide

Fig. 15. Amino acid sequence of red swamp crayfish MIH (From Nagasawa *et al.* (83)).

More recently, the inhibitory activity of Prc-MIH was tested in ecdysteroid secretion by cultured Y-organ in homologous experiments (83). Y-organs were dissected out from the crayfish which were kept for 3 days after eyestalk removal. They were placed on in glass dishes containing 0.5 ml of culture medium consisted of medium 199 with 20 mM Hepes containing NaCl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ (424, 39.4 and 180 mg/100ml, respectively) adjusted to pH 7.5 with NaOH. After incubation for 6 hr at 25°C, the culture medium was removed from each dish and stored at -20°C. The isolated peptides were dissolved in the culture medium and incubated with Y-organs. Peptide concentrations were expressed as X-organ-sinus gland (SG) equivalents per 0.5 ml of culture medium. 50 μl of the medium was used to determine the amounts of

ecdysteroids according to radioimmunoassay. When incubated 5 SG equivalents and Y-organ, approximately 20-30% inhibition of ecdysteroid secretion was observed for MIH and CHHs. At the dose of 0.5 SG equivalents, the inhibitory potency of MIH increased to about 50%, whereas that of CHHs held about 10-20%.

Although there are these observations, further study is needed to understand the relationship between the eyestalk hormones and physiological process. Because no putative MIH, as well as CHHs, fully inhibits secretion and/or synthesis of molting hormone. Moreover, as Y-organs in red swamp crayfish produce two different molting hormones, 3-dehydroecdysone and ecdysone, the interesting will center on the mode of action of candidates for molt-inhibiting hormone.

4.4 CHROMATOPHOROTROPINS

4.4.1 RED-PIGMENT-CONCENTRATING HORMONE

It has known that color changes in animals occur in response to relevant environmental condition, and in association with development, growth, and sexual maturity. Red-pigment-concentrating hormone (RPCH) was first isolated and sequenced from the eyestalks of the shrimp *Pandalus borealis* (84).

pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-amide

Fig. 16. Amino acid sequence of RPCH (From Fernlund and Josefsson (84)).

In 1990 Gaus and co-workers (85) have characterized RPCH from the crayfish *Orconectes limosus*, in which the peptide was isolated from suboesophageal ganglion (SOG). 30 SOG were extracted with 2N acetic acid, and directly subjected to HPLC. Because they considered that SOG is a rich source of the hormone than eyestalks based on immunocytochemical studies with RPCH-antiserum. A yield of RPCH was approximately 900 pmol from 30 SOG. The structure of crayfish RPCH was identical to that of shrimp RPCH. RPCH immunoreactivity dyed neurosecretory eyestalk neurons projecting to the neurohemal sinus gland (86). In another study (87), it is revealed that RPCH acts as an excitatory modulator of swimmeret activity rhythms in the crayfish.

Recently, molecular cloning of RPCH of the shore crab *Carcinus maenas* has been reported (88). The predicted preprohormone consists of a putative signal peptide with 25 amino acids, the eight amino acid RPCH sequence followed by a Gly as donor of amide, dibasic amino acids for processing site and a peptide of 74 residues with unknown function. Several peptides with RPCH like structure have been found in insects (89) and mollusc (90, 91). The insect peptides display adipokinetic and/or hyperglycemic activity, but some of them show myotropic and cardioexcitatory activity. The snail peptide has myotropic activity and inhibitory neurotropic effects (90, 91). When compared with the preprohormone structures between crustacean RPCH

and insect AKH (92), the structural organization of all preprohormone, signal peptide/ hormone/precursor related peptide, is similar. RPCH precursor shows a high identity in the region corresponding to RPCH and AKH, but a low identity for other regions.

4.4.2 PIGMENT-DISPERSING HORMONE

In 1988 McCallum and colleagues (93) isolated and sequenced pigment-dispersing hormone (PDH), an octadecapeptide from the red swamp crayfish *Procambarus clarkii*. Interestingly, PDHs have a marked specific activity depending upon species. For example, Prc-PDH is much less active on the melanophore of the fiddler crab *Uca pugilator* than its own hormone although a sole replacement is observed at position 17, Asp for the crab PDH (94).

Asn-Ser-Glu-Leu-Ile-Asn-Ser-Ile-Leu-Gly-Leu-Pro-Lys-Val-Met-Asn-Glu-Ala-amide

Fig. 17. Amino acid sequence of Prc-PDH (From McCallum *et al.* (93)).

Earlier immunocytochemical study contributed a suggestion that PDH probably plays a neuromodulator role, as the association of PDH-immunoreactivity of not only with secretory pathways but also with apparently non-secretory neurons throughout the nervous system in the crayfish *Orconectes limosus* (95).

In 1993 De Kleijin *et al.* (96) reported structure and localization of mRNA encoding PDH in the eyestalk of the crayfish *Orconectes limosus*. The structure of the PDH preprohormone consists of a putative signal peptide with 20 amino acids, a peptide with 33 residues, and PDH peptide at the carboxy-terminal end. In their report, three cell clusters in the optic ganglia of the eyestalk were stained both with the PDH cRNA probe and PDH antiserum. The clusters are situated dorso-laterally at the external side of the eyestalk, dorso-laterally at the internal side of the eyestalk, and the medulla terminalis ganglionic X-organ. In addition, the perikarya in the proximal part of the lamina ganglinaris were immunoreactive and negative stain when tested in situ hybridization, suggesting the presence of a PDH-like peptide in the eyestalk.

5. MOLECULAR EVOLUTION OF CHH/MIH FAMILY

In general, structure-function relationships of functionally active proteins and peptides have been investigated on chemical modification of amino acid residue, chemical and enzymatic fragmentation of them, or use of synthetic analog for peptides. These approaches have been successful in assignment of active site of enzyme and in identification of essential amino acid residue in small peptides, whereas there has been only limited success for hormonal proteins and relatively large peptides. Modern technique using molecular biology has leaded to elucidation of hormonal active sites due to preparing of several variants for hormones. However, this approach includes

disadvantages that the applications are confined within higher expression happened. Reconstruction of the hormone having some disulfide bonds is too difficult in its application. In contrast, knowledge of the comparative hormone structures from diverse species would provide correlative evidence for the relationships between structure and function. Namely, highly conserved domain obtained sequence comparison may be responsible for function of hormone.

5.1 OCCURRENCE OF BIOLOGICALLY ACTIVE PEPTIDES HAVING D-AMINO ACIDS

Crustacean hyperglycemic hormone is a unique structurally and biologically. In 1994 it has been reported that the CHHs from the crayfish *Procambarus clarkii* (66) and the American lobster *Homarus americanus* (75) occur in two variants, with either L- or D-phenylalanine as the third position. Accordingly, we introduce here secretory peptides containing D-amino acid from living organisms.

More than 50 years ago, two antibiotics called tyrocidine and gramicidine, the small peptides contained D-amino acids, were found from bacteria (97). The synthetic pathways of these peptides proposed that they are assembled in a stepwise fashion by multienzyme complexes where its peptide bonds are formed through amino acylthioesterers (98, 99). In 1971 Gross and Morell (100) demonstrated that the lantibiotics, D-amino acid-containing antibiotic peptides, consisted of many unusual amino acids, including lanthionine. These peptides are final products that yield from precursor peptides synthesized by the participation of messenger RNA and ribosomes, following the multitude of modifications.

In 1980s a biologically active peptide with D-amino acid was, the first time, isolated from skin of a South American tree frog *Phyllomedusa sauvagei* (101). This is called dermorphin and has a high affinity and selectivity for the μ-type of the opiate receptors. Until recently, several peptides having D-amino acids have been isolated from invertebrates. These include frog skin peptides and molluscan peptides, as following four dermorphin related peptides (102), four deltrophin related peptides (103), and antimicrobial peptide termed bombinins H (104) in the frogs; achatin (105), fulicin (106), and *Mytilus*-FFRFamide (107) in the mollusc. The common feature in these peptides from amphibian and mollusks is that the occurrence of D-amino acid in their sequences is at second position only.

The most recent topics are a crustacean hormone and a spider toxin. The former includes CHH from the crayfish and the later is P-type calcium channel toxin isolated from the venom of the funnel web spider *Agelenopsis aperta* (108, 109). The characters of CHH from the red swamp crayfish are that two peptides, named Prc-CHH-I and Prc-CHH-II, have identical amino acid sequences with 72 residues and three disulfide linkages and differ from each other by a stereoinversion of phenylalanine at position 3 (section 4.1.2); and they have similar hyperglycemic activity but more effect for repressing ecdysteroid synthesis in Y-organ culture, as described above (section 4.1.3). In addition, during isolation of eyestalk hormones from the red swamp crayfish, we have

TABLE 1. Some biologically active peptides containing D-amino acids in vertebrates and invertebrates.

Crayfish and Lobster	
CHHs	pGlu/Gln-Val-**D-Phe**-Asp-Gln-Ala-Cys-Lys-Gly- (72 residues)
Spider	
ω-Agatoxin IVB	Glu-Asp-Asn-Cys-Ile-Ala-Glu-Asp-Tyr-Gly-Lys-Cys-Thr-Trp-Gly-Gly-Thr-Lys-Cys-Cys-Arg-Gly-Arg-Pro-Cys-Arg-Cys-Ser-Met-Ile-Gly-Thr-Asn-Cys-Glu-Cys-Thr-Pro-Arg-Leu-Ile-Met-Glu-Gly-Leu-**D-Ser**-Phe-Ala
Mollusc	
Achatin-I	Gly-**D-Phe**-Ala-Asp
Fulicin	Phe-**D-Asn**-Glu-Phe-Val-amide
Mytilus-FFRFamide	Ala-**D-Leu**-Ala-Gly-Asp-His-Phe-Phe-Arg-Phe-amide
Frog	
Dermorphin	Tyr-**D-Ala**-Phe-Gly-Tyr-Pro-Ser-amide
[Hyp^6]Dermorphin	Tyr-**D-Ala**-Phe-Gly-Tyr-Hyp-Ser-amide
[Lys^7]Dermorphin	Tyr-**D-Ala**-Phe-Gly-Tyr-Pro-Lys
[Trp^4, Asn^7]Dermorphin	Tyr-**D-Ala**-Phe-Trp-Tyr-Pro-Asn
Met-Deltorphin	Tyr-**D-Met**-Phe-His-Leu-Met-Asp-amide
Ala-Deltorphin I	Tyr-**D-Ala**-Phe-Asp-Val-Val-Gly-amide
Ala-Deltorphin II	Tyr-**D-Ala**-Phe-Glu-Val-Val-Gly-amide
Leu-Deltorphin	Tyr-**D-Leu**-Phe-Ala-Asp-Val-Ala-Ser-Thr-Ile-Gly-Asp-Phe-Phe-His-Ser-Ile-amide
Bombinin-Hs	Ile/Leu-**D-allo-Ile**-Gly-Pro-Val-Leu-Gly-Leu/Met-Val-Gly-Ser-Ala-Leu-Gly-Gly-Leu-Leu-Lys-Lys-Ile-amide

found minor CHHs. Minor CHHs were extracted in crustacean saline containing 30% acetonitrile and purified by reversed phase HPLC on a TSK gel ODS 120T column with a gradient of acetonitrile in 0.1% TFA, and then were separated into two peaks, as shown in Fig. 1. The purified minor CHHs were subjected to mass spectrometry (Hitachi 1200H) interfaced electrospray, sequence analysis using gas-phase sequencer (Shimadzu PPSQ-10), amino acid analyses according to PTC method and OPA-IBLC method (section 4.12). Both minor CHHs have same molecular weight of 8,403 ± 2 which is 17 mass large than major CHHs, and identical partial amino acid sequence;
Gln-Val-Phe-Asp-Gln-Ala-Cys-Lys-Gly-Ile-Tyr-Asp-Arg-Ala-Ile-Phe-Lys-Lys-Leu-Asp-Arg-Val-Cys-Glu-Asp-Cys-Tyr-Asn-Leu-Tyr-Arg-Lys-Pro-Tyr-Val-Ala-Thr-Thr-Cys-Arg-Gln-Asn-Cys-Tyr-Ala-Asn---.

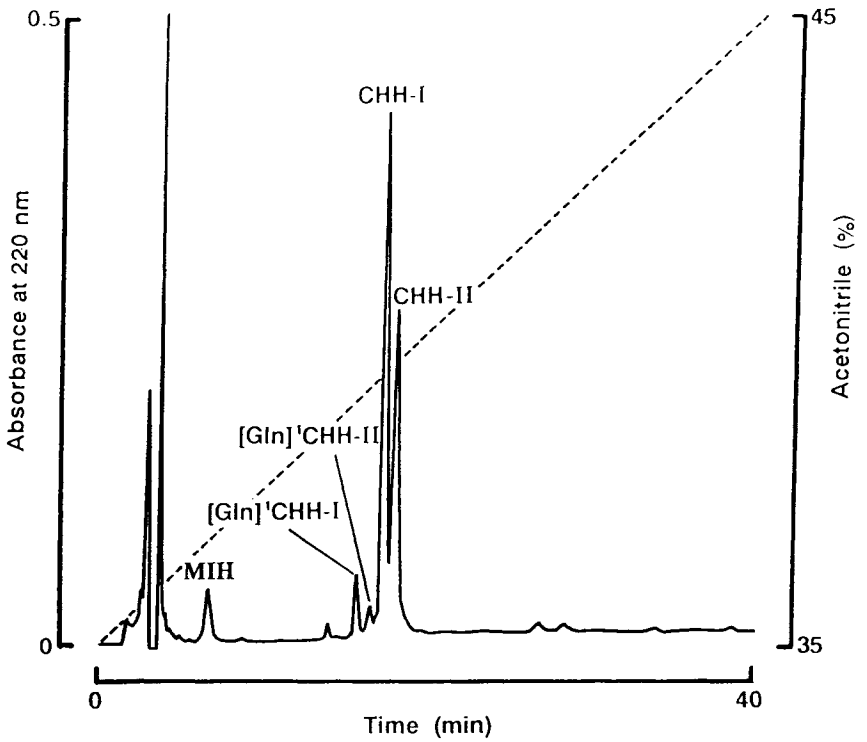


Fig. 1. High-performance liquid chromatography of Prc-CHHs and MIH on a TSK gel ODS-120T column. The dotted line represents a gradient of acetonitrile in 0.1 % TFA.

The same amino acid compositions by PTC method were observed with each other, but one residue of D-Phe were detected from minor CHH-II by the improved OPA method. These results suggest that structural difference between minor CHH and major CHH is probably limited at first position, Gln for minor CHH and pGlu for major CHH, and third position in minor CHH-II is changed to D-amino acid, like the case of major CHH.

Similarly, CHHs from the Mexican crayfish *Procambarus bouvieri* (110) and from the American crayfish *Orconectes limosus* (78) are separated into two peaks by reversed-phase HPLC, and one of them in each species contains D-phenylalanine at position 3. In the American lobster, two CHHs expressed from two genes are present, both CHHs show doublet peak on HPLC separation. Each have D-configuration of phenylalanine at position 3, respectively. On the other hand, the spider toxin is also present as two peptides, termed ω-Aga-IVB and ω-Aga-IVC. They have identical amino acid sequences with 48 residues and four disulfide bonds, yet have opposite absolute configurations of Serine at position 46; and similar selectivities for blocking voltage-

sensitive calcium channel subtypes but different potencies for blocking P-type voltage-sensitive calcium channels in rat cerebeller Purkinje cells (108, 109).

When we put a question concerning the occurrence of D-amino acid into peptides and its justification for existence, how can we answer about it. As shown Table 1, it seems that no general rule regarding the pattern of amino acid and position on the sequence occurred is permitted, but each case beginnings to assume a individual nature within own phyletic class. The occurrence of D-amino acids is, if anything, limited in amino- or carboxy-terminal portion. Biological activity for the peptides is also versatile such as hormone, neuromodulator and toxin. Deliberating evolutionary pathway for the CHH in the red swamp crayfish, since CHH-I and CHH-II have the similar potency of hyperglycemic activity and different effectiveness of molt-inhibiting activity, we believe that stereoinversion of amino acid is one of functional divergence and acquirement of such a mode is relatively recent event throughout its evolutionary period.

In the last year, a peptide isomerase for the agatoxin was purified from the venom of the funnel web spider, and sequenced (111). The isomerase consists of an 18 residues light chain and a 243 residues heavy chain connected by a single disulfide bond, and contains three intramolecular disulfide bonds and one N-linked oligosaccharide chain in the heavy chain. A speculation for the conversion of an L- to the D-amino acid in the peptide has been proposed that epimerization may proceed via a two-base mechanism, whereby the portion of the α-carbon of an amino acid is removed from one side and added to the other side (112). However, nothing is known it that in all instances the peptides undergo such post-translational modification during processing ways.

5.2 SEQUENCE COMPARISON OF CRUSTACEAN HYPERGLYCEMIC HORMONE FAMILY

The report for the structure of CHH family began in 1989, in which the hormone was from the shore crab *Carcinus maenas*, Cam- (113). More recently, a considerable volume of structural information on the CHH family and their genes from invertebrate species has become available: the crayfish CHHs, *Orconectes limosus*, Orl- (114), *Procambarus bouvieri*, Prb- (72), *Procambarus clarkii*, Prc- (66); the lobster CHH, *Homarus americanus*, Hoa- (115); the Kuruma prawn CHH, *Penaeus japonicus*, Pej- (116); the terrestrial isopod CHH, *Armadillidium vulgare*, Arv- (117); the shore crab MIH, *Carcinus maenus* (118); the crayfish MIHs, *Procambarus clarkii* (82), *Procambarus bouvieri* (119); the edible crab MIH, *Cancer pagurus*, Cap- (120); the Kuruma prawn MIH *Penaeus japonicus* (121), and the genes for the lobster CHH/MIH, *Homarus americanus* (122); for the white shrimp MIH, *Penaeus vannamei*, Pev- (123); for the shore crab MIH, *Carcinus maenas* (124), for the blue crab MIH, *Callinectes sapidus*, Cas- (125); for the shore crab CHH, *Carcinus maenas* (77); the crayfish CHH, *Orconectes limosus* (78). In the lobster *Homarus americanus*, a neuropeptide from X-organ-sinus gland complex, called vitellogenesis inhibiting hormone (VIH) or gonad-inhibiting hormone (GIH), has been isolated and sequenced (126). This hormone inhibits ovarian synthesis of yolk proteins *in vitro* (127), and occurs as two isoforms, only one

that has gonad-inhibiting activity (126). Hoa-preproGIH has been reported, recently (128). Moreover, two neuropeptides have been isolated from sinus gland of the edible crab *Cancer pagurus* (129). These peptides repressed methyl farnesoate synthesis by the mandibular organs, and named mandibular organ-inhibiting hormone (MOIH). The crab MOIHs consist of 78 amino acid residues with one replacement of Gln/Lys at position 33. The existence of CHH-like peptide being spreads in the field of invertebrates. The first report has been done in the field of insect peptide. Ion-transport peptide, isolated from the corpus cardiacum of the desert locust *Schistocerca gregaria*, is structurally related to CHH family. Considerable homology existed between the partial sequence of the locust peptide and the first 34 residues of Hoa-CHH, that is 59% identity. In addition, the low molecular weight peptide from the venom of the black widow spider *Latrodectus mactans tredecimguttatus* has been sequenced (130). The sequence homology search showed that the spider peptide and CHHs are structurally related as 22% amino acid identity and conservative localization of 6 cysteines.

Sequence comparison among CHH family, as shown in Fig. 2, reveals that the molecules are classified into two types structurally, what is called CHH-like and MIH-like group. The homology within CHH and MIH shows over 55%, but between CHH and MIH it is gathered in range from 20% to 30%. The spider toxin, LMWP, has only 20% identity to Prc-CHH and 13% identity to Prc-MIH, but the identical pattern of three disulfide bridges suggests the third group in CHH family as the critical importance of the tertiary structure for CHHs, MIHs, and LMWP is supported by the conservation of disulfide linkages. The homology also indicates that GIH from the lobster and MOIH from the edible crab belong to MIH-like group, whereas the Prv-hormone, reported as MIH, shows striking similarity to CHH-like group. Comparison of preprohormones for CHHs and MIHs reveals that the CHHs and the MIHs including GIH belong to two distinct group, as only the CHH preprohormones contain an additional peptide, CPRP. The lack of a CPRP-like peptide in the precursors of MIHs and GIH may be caused by a deletion in the ancestral CHH/MIH/GIH gene in an earlier of its evolutionary divergence (128).

In the case of CHH molecules, the carboxyl termini of the CHHs have a very low degree of common homology, but four regions are found as a highly conserved domain. These are located in aligned positions 7-14 (Ac), 23-32 (Bc), 40-45 (Cc), and 50-57 (Dc) as shown in Fig. 2. It should be noted that all domains are closely arranged in the tertiary structure, since they are bound together by the disulfide linkage. As described section 4.1.3, Prc-CHH-I and Prc-CHH-II exhibited a similar potency in hyperglycemic activity, although there is a significant difference at position 3. This strongly suggests that domain Ac, Bc, Cc and Dc are necessary for the activity. Similarly, five of the highly conserved regions were assigned in the MIH molecules, that are located in aligned positions 5-14 (Am), 23-32 (Bm), 40-45 (Cm), 50-56 (Dm), and 69-76 (Em). The location of four domains, A, B, C, and D, are common to both CHHs and MIHs, whereas domain E is specific to MIH molecules.

We, here, refer to the case of pituitary hormones in vertebrates. Growth

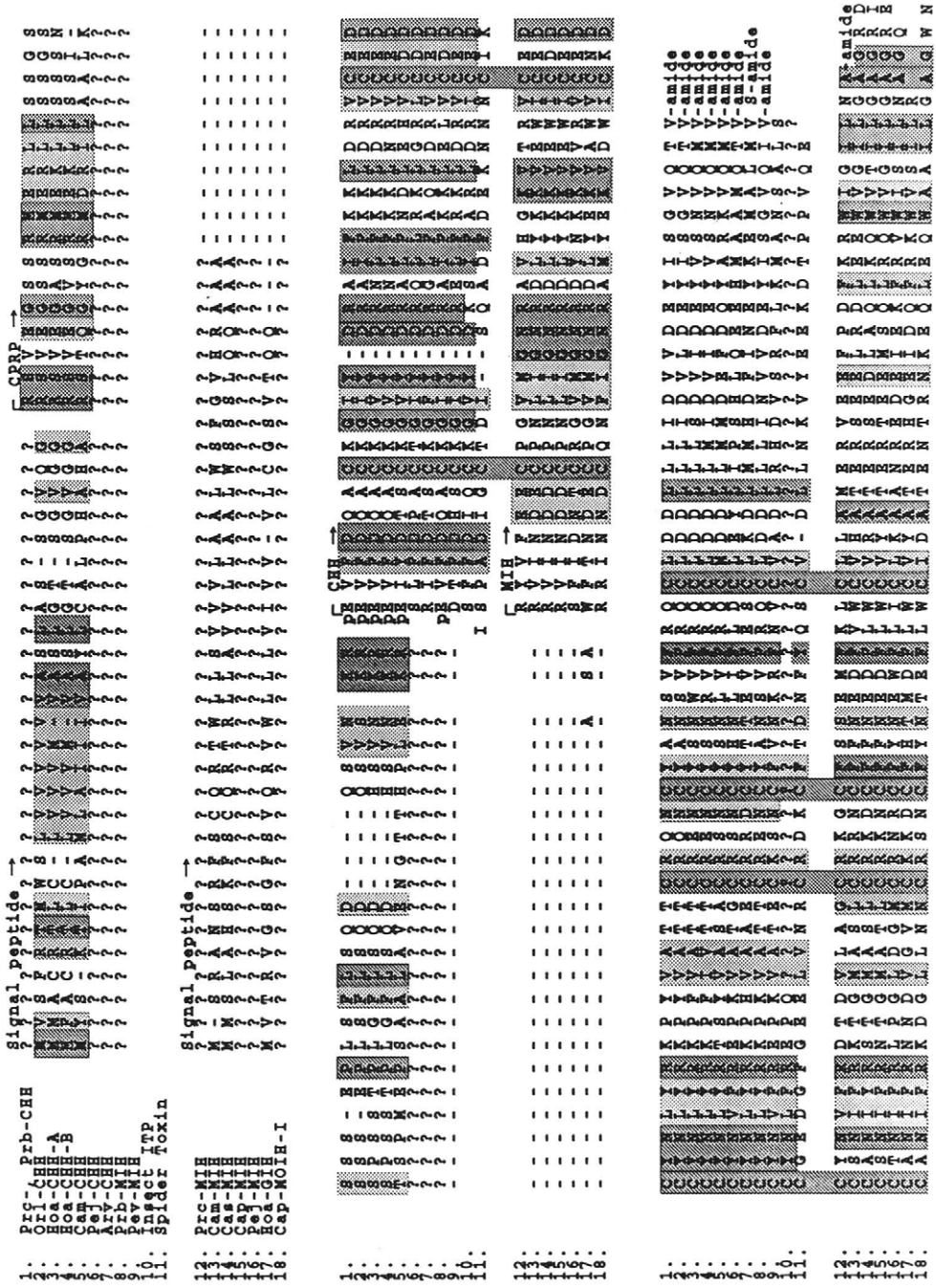


Fig. 2. Comparison of amino acid sequences among CHH family. "-" represents gaps and "?" is unknown residues.

hormone (GH) and prolactin (PRL) are a family of peptide hormones that share a number of common structural and biological characteristics. In human, GH is involved in growth through its effects on metabolism and PRL regulates mammary growth and lactogenesis. Human GH is also known to exhibit both somatic and lactogenic action. However, between both the hormones, there is 25% identity of amino acid sequences, and the identical residue is scattered all over their sequences. Nevertheless, overlapping activity observed in both pituitary hormones is realized from such a reason that the three dimensional architecture of GH and PRL molecules is conserved throughout their evolution as four cysteine residues are arranged at same position to form two intramolecular disulfide bridges. Likewise it described above, CHHs in the red swamp crayfish are unique which observed some physiological effects including hyperglycemia and inhibition of ecdysteroid synthesis. Although similarity between Prc-CHH and Prc-MIH is 25%, six cysteine residues present at CHHs and MIHs are arranged same positions on their sequences. This indicates that both hormone have a similar conformation. It is somewhat surprising that a domain with consecutive identical residues is present at positions 21-31 in both CHH and MIH molecules. This conserved portion is corresponding with domain B found when different CHHs and MIHs compared. These might infer that the unchangeable domain together with conserved tertiary structure is responsible for overlapping biological activity.

```
-Arg-Val-Cys-Glu-Asp-Cys-Tyr-Asn-Leu-Tyr-Arg-    Prc-CHH
  *   *   *   *   *   *   *   *   :   :   *
-Arg-Val-Cys-Glu-Asp-Cys-Tyr-Asn-Val-Phe-Arg-    Prc-MIH
```

Fig. 3. Amino acid sequences of the domain B between Prc-CHH and MIH.

CHH is also one of the most heterogenous hormone in the sinus gland in terms of molecular forms. It has been known that CHHs from several crustaceans give multiple peaks on advanced HPLC with a reversed phase column (65, 131, 132, 133, 134, 135). Why polymorphic forms occurred in crustaceans? From what little has been learned so far, a fascinating picture of the CHH molecules is emerging. It may not only be transcribed in more than one genetic isoform, but after translation, a few chemical modifications are introduced into the hormone. For examples, in the case Hoa-CHHs, two genes encoding structurally different precursors are expressing (122) and the precursors undergo stereoinversion during its processing (75). Thus, at least four CHH molecules are present in the lobster. In the case of Orl-CHHs, although two genes are expressed, as the portions corresponding to CHH are identical, only two isomers with/without D-amino acid are observed (78). In our investigations, four molecules of Prc-CHH were identified. This is caused by post-translationally modification, such as formation of pyroglutamic acid and stereoinversion at position 3. Accordingly,

structural variants of CHH in a given species are either genetic variants, posttranslationally modified variants, or mixture of both types. Therefore, it is necessary to take them into account when further investigation of crustacean peptidic hormones is beginning. Occurrence of the hormone having D configuration has been limited within Astacidea so far. This might assume that crayfish begins to use D-amino acid in their hormone, in which the modification has the potential to alter the conformation and its ability to interact/separate with other molecules in target tissues. On the other hand, if the stereoinversion observed is mutually exclusive in crustaceans, it seems that the animals have put D-amino acids to practical use from time immemorial.

6. CONCLUDING REMARKS

The chemical and molecular information for crustacean endocrinology has being gathered. The role of ecdysteroids as molting hormone has been substantiated from several approaches. The identification of two ecdysteroids that can lead to molting in the crayfish has opened up new lines for physiological research on hormonal regulation during its development. It has been clear that crustaceans are maintaining its life with recycling ecdysteroids, and thereby ecdysteroids are not it so-called "throwaway hormone". That's where it is convenient model system including a large number of chemical reactions. Recently, an interesting observation has been reported that ecdysteroids may not only play a role for molting but the materials from an archaic marine arthropod *Pycnogonum litorale* act as chemical defense against the shore crab *Carcinus maenus* (136). This finding has also indicated a novel feature for arthropods.

Some eyestalk hormones has often been discussed in term of the inhibitor of its physiological processes. This is reversible to insect hormones which are tropic hormone. The traditional name for crustacean hormones, molt-inhibiting hormone, gonad-inhibiting hormone, and mandibular organ-inhibiting hormone, as well as hyperglycemic hormone, seems to be loose in the information as it conveys its many effects. Investigations on eyestalk hormones have yet provided confusion to study the mechanism of action of each hormone, but have pointed to important areas of crustacean physiology related to the hormones.

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The Structure and Antigenicity of Novel Polysaccharides from Microorganisms and Plants

John H. Pazur

1. INTRODUCTION

With the advent of micro-methods for the methylation of polysaccharides (1) and for the analysis of the methylated products by gas liquid chromatography and mass spectrometry (2), the detailed structure of many antigenic and novel polysaccharides has been elucidated (3,4,5). As a result it has been possible to clarify the biological functions of polysaccharides in many types of cells. A significant development has been the elucidation of the structural units of polysaccharides responsible for the activation of the immune system ultimately resulting in the synthesis of anti-carbohydrate antibodies (6). Some of the anti-carbohydrate antibodies are being utilized for identifying abnormal glycoproteins in diseased tissue.

By far the most important function of polysaccharides in cells is providing a framework to maintain the integrity and shape of the cell. Polysaccharides together with the complex of glycosamino-peptides and teichoic acids form the cell wall of many microbial cells. The regulation and transport of nutrients and metabolites in and out of the cell is controlled by the nature of this wall. In plants the polysaccharide, cellulose, is the most abundant carbohydrate and is the main structural element of plant cells. However, starch is an important plant polysaccharide and is the major food reserve in plants.

There are many applications in the use of polysaccharides and anti-carbohydrate antibodies. Thus, microbial polysaccharides which generate antibodies are the basis of serological methods for the detection of pathogenic microorganisms. Further, polysaccharides have been used to produce unique antibodies and these have value as analytical and therapeutic agents in the detection and treatment of a number of diseases. Many plant polysaccharides with unusual physical properties are used to improve the quality of foods, the stability of pharmaceuticals and

the physical properties of personal care items. Antibodies induced by some plant polysaccharides have important uses for detecting and measuring additive constituents to food, confectionary and dairy products.

Polysaccharides are polymers of monosaccharides of different chemical structures arranged in specific sequence and joined together by different types of glycosidic linkages. The main types of monosaccharides which are structural units of microbial and plant polysaccharides are listed in Table 1. In polysaccharides these monosaccharides are held together by glycosidic bonds from the hemiacetyl hydroxyl group of one unit to a primary or secondary hydroxyl group of a contiguous monosaccharide unit. Polymers of long chains of monomers are thereby formed. The chain may possess numerous side chains of monosaccharide, oligosaccharide or other carbohydrate polymeric units. It has been observed in our studies that the side chains are important structural elements. They are the predominant immunodeterminant groups of polysaccharides which initiate the synthesis of antibodies with specificity for carbohydrate units.

TABLE 1

The monomeric residues which are common constituents of microbial and plant polysaccharides.

Polymer	Monomer
Microbial polysaccharides	D-glucose, D-galactose, D-mannose, D-glucuronic acid, L-rhamnose, L-fucose, D-glucosamine, D-galactosamine, 6-deoxy-L-talose
Microbial glycosaminopeptides	D-glucosamine, muramic acid, glutamic acid, glycine, lysine, alanine
Microbial teichoic acids	ribitol, glycerol
Plant polysaccharides	D-glucose, D-galactose, L-galactose, D-mannose, D-fructose, D-xylose, D-arabinose, L-arabinose, D-glucuronic acid, D-galacturonic acid, L-fucose, L-rhamnose

In this article, procedures for the isolation of polysaccharides, methods for the structural analysis, and the types of assays for antigenicity used or developed by the author are reviewed. However, the pioneering studies of Avery and Heidelberg (7) that established for the first time an immunological function for microbial cell wall polysaccharides are included along with some appropriate contributions of other notable scientists (8-12). Some applications of polysaccharide antigens and the anti-carbohydrate antibodies are considered. No attempt has been made to compile an all inclusive list of antigenic polysaccharides of microbial and plant origin.

2. MATERIAL AND METHODS

2.1 Antigenic polysaccharides

Microorganisms capable of undergoing division and growth possess certain common organelles and similar structures in the biochemical components. Variations in location and in micro structure do exist and account for the variability and diversity of microorganisms. The biochemical components on the surface of microbial cells are in distinct layers, the capsule, the cell wall and the protoplast membrane. Fig. 1, frame A shows a schematic diagram depicting the cell surface layers and a few types of biochemical compounds for a typical gram positive organism (13). Frame B of the figure shows a thin section of a portion of a bacterial cell as revealed by electron microscopy (14).

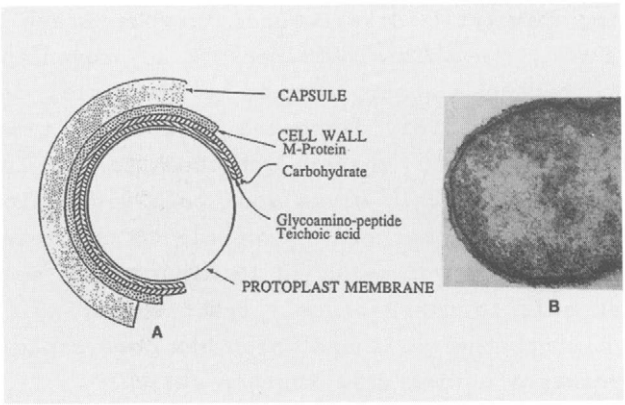


Fig. 1. A: Diagrammatic representation of sub-cellular components of gram-positive microbial cell (13). B: Electron microscope picture of a cell (14).

As for the function of the various layers, the capsules consisting of uronic acid and amino sugar polymers, though important in some groups of organisms, contribute little to the metabolic reactions of the cell. The cell wall is responsible for shape and rigidity of the cell. This layer consists of several types of polymers. The rigidity is due primarily to a polymeric glycoaminopeptide (15) which may be linked to teichoic acids (16,17) and polysaccharides of varied molecular structure. Some organisms have rare monosaccharide residues constituting the polysaccharide and an unusual sequence arrangement of residues (18,19). Some of the polysaccharides are highly immunogenic but in addition to polysaccharides, the M-protein in the cell wall is also immunogenic. The protoplast membrane is necessary for performing numerous cellular functions. For example, the membrane serves as an osmotic barrier and allows passage of nutrients into the cell and end products out of the cell.

2.1.1 Disrupting Microbial Cells. A variety of techniques are used for disrupting microbial cells from which cell walls can be isolated. The techniques are described in detail in reference (14). Widely used procedures are based on mechanical disintegration. A Mickle disintegrator is available commercially and is suitable for the preparation of small quantities of disrupted cells. In this unit a bacterial cell suspension in buffer is shaken in a cup with glass beads (14). The frequency and amplitude of the unit will determine the length of time for satisfactory rupture of the cells. Another unit used for disintegrating hemolytic *Streptococci* is the Braun homogenizer (20). The use of this instrument has proven particularly advantageous, because larger batches of bacterial cells can be efficiently and effectively disrupted in a short time. A third unit is the high speed shaker attached to an International Centrifuge (14). This unit gives good cell disruption and large quantities of cells can be used. Essentially complete disruption of 500 mg of dry weight of cells in 15 minutes has been reported. Still another unit is the Ribi cell fractionator which relies on pressure to disrupt the cells and which has good capacity and good temperature control during cell rupture (21).

2.1.2 Isolation of Cell Walls. The cells are disrupted by any of the methods outlined in the preceding section. When glass beads are used, the beads are separated from the disintegrated

bacteria by filtration on a coarse sintered glass filter. The beads are generally washed and then combined with the original filtrate and the extract is subjected to differential centrifugation. The typical layers formed on centrifuging the broken cells at 5 to 10,000 g are illustrated diagrammatically in Fig. 2. The layers consist of the intact cells, the granules, the cell walls, the membrane fragments, ribosomes, and the supernatant of soluble components.

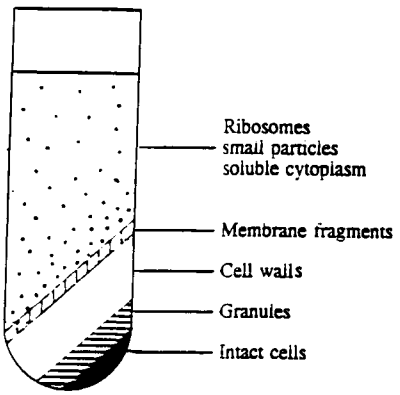


Fig. 2. Diagrammatic representation of the appearance of the pellet obtained by centrifugation at 5000 g of disrupted bacterial cell suspension (14).

The intact cells and granules sediment at about 5,000 g and are easily removed after centrifuging at this speed and discarded. The cell walls and soluble components remain in the supernatant. Recentrifuging the supernatant at about 10,000 g-20,000 g sediments cell walls which are recovered by decantation. The supernatant contains the solubles and fine particles and can be discarded. It may be necessary to re-centrifuge the cell wall preparation to remove any adhering material. The cell walls are washed thoroughly with buffer and used for isolating the antigenic polysaccharides.

In the early studies of preparation of cell walls enzymic treatments which might modify the cell wall polysaccharides were avoided. It was not known that wall components were not susceptible to enzyme attack. However, the removal of M-protein antigen by digestion with trypsin showed that proteolytic enzymes could be used in cell wall preparation without degrading cell wall polysaccharides (22). Enzyme treatment to remove proteins and

other impurities is now in use routinely in the preparation of cell walls (23,24).

2.1.3 Isolation of Microbial Cell Wall Polysaccharides. Trichloroacetic acid extraction at low temperature has been used often to isolate cell walls (25). To illustrate the isolation of cell walls from a *Streptococci* is described. *Streptococcus faecalis* was grown in 8 liters of Todd-Hewett broth supplemented with 0.1% glucose. Cell walls from 80 g of whole cells, wet weight, were prepared by utilizing a Mickle disintegrator. The carbohydrate components were extracted from the cell walls at 4°C for 24 hr with 10% trichloroacetic acid. Protein impurities in the trichloroacetic acid-soluble extract were precipitated by the addition of 2 N HCl-ethanol mixture (1:20). After removal of this precipitate by centrifugation, the trichloracetic acid-soluble extract was mixed with 5 volumes of acetone. The precipitate which formed was collected by centrifugation, redissolved in water and dialyzed against distilled water. Finally the solution was lyophilized to dryness.

A dilute KCl-HCl solution of pH 2 has been used to extract polysaccharides from the cell walls of *Streptococcus bovis* (18). The procedure is similar to one used originally by Lancefield (26) to isolate streptococcal carbohydrates. In the procedure the walls or washed cells were suspended in a KCl-HCl solution of pH 2.0 prepared by diluting 10.6 ml of 0.2 M HCl and 50 ml of 0.2 M KCl to a final volume of 200 ml. The resulting suspension was heated for short periods in a boiling water bath. After 10 minutes the residue was removed by centrifugation and the extract was saved for isolation of polysaccharides. The residue was resuspended in 200 ml of solvent and reheated for 60 minutes. The residue was removed and the extracts were used for isolation of polysaccharides. Both extracts were dialyzed separately against distilled water and concentrated by vacuum distillation. Contaminating protein in the concentrate was precipitated with chloroform and butyl alcohol mixture and removed by centrifugation. The solvent was evaporated by vacuum distillation and the residual solution containing the carbohydrates was lyophilized to dryness.

Formamide extraction has been used to isolate antigenic polysaccharides from microorganisms (27). Formamide dissolves the microbial cell completely and so gives a good yield of polysaccharides. Other solvents have been tested by Fuller (27)

and these were phenol, lactic acid, benzaldehyde, ethylene glycol, pyridine, glacial acetic acid, and saturated aqueous urea but none were as suitable as formamide. Since the polysaccharides were likely chemically bonded to other cellular components it is necessary to break such bonds before the desired compounds could be obtained. This was achieved with the formamide. In this procedure 5 ml of formamide is added to 100 mg of lyophilized cell walls, and extraction is carried out at 180°C with continuous stirring for 12 hrs. After extraction, the insoluble residue is removed by centrifugation and the carbohydrate polymer is obtained from the supernatant by the addition of 5 volumes of acetone. The acetone-precipitable carbohydrate obtained from the extraction is dissolved in water and dialyzed against distilled water. The sample is lyophilized to dryness.

Enzyme treatment of microbial cell walls followed by extraction of carbohydrate components has been used (28). The cell walls are suspended in M/30 phosphate buffer of pH 8 and mixed with the enzyme preparation from *Streptomyces albus*. The suspension is incubated for 16 hr at 37°C, during which time the suspension becomes an almost completely clear solution. The solution is then shaken with portions of chloroform and amyl alcohol until no further precipitate appears on centrifugation. The partially deproteinized solution is brought to 0.85 percent by the addition of solid sodium chloride and then mixed with two volumes of ethanol. A slight precipitate forms which is discarded. The carbohydrate is then precipitated from the supernatant by the addition of five volumes of acetone. The precipitate is collected by centrifugation, dissolved in water, dialyzed against distilled water, and finally dried from the frozen state.

2.1.4 Isolation of Plant Polysaccharides. There are many methods that have been devised for isolating plant polysaccharides and a number are outlined in reference (29). If the polysaccharide is present in plant seeds it will be necessary to grind the seed before extraction. Extraction of whole seeds may be done by soaking seeds in water for periods up to 22 hrs. (30). The thick liquid is squeezed away from the seed through fine linen, and the process then repeated until very little carbohydrate can be obtained from the extract. The extracts so obtained contain a small amount of flocculent matter which is allowed to settle. The carbohydrate in the supernatant is separated from the precipitate

by centrifugation. The solution is then treated with twice its volume with 90% alcohol and a white precipitate is formed. The precipitate is dehydrated with absolute alcohol, washed with ether and finally dried in vacuo.

In the preparation of polysaccharides from ground seeds the following protocol may be used (31). The ground material is stirred in water for several hours. To the solution ethanol is added with vigorous stirring to a concentration of 25%, and the precipitated material, which constitutes about 10% of the original sample is discarded. Thereafter, the ethanol concentration is increased slowly to 40%. On standing a white precipitate is formed constituting the polysaccharide. The pure polysaccharide is recovered by centrifugation. The precipitate is dried by lyophilization.

3. MICROORGANISMS

3.1 *Streptococci*

The *Streptococci* make up a relatively large group of coccus bacteria characterized by an arrangement of the cells in linear chains. The *Streptococci* are responsible for a variety of diseases of man and some diseases of animals. It is now well known that there are pathogenic forms as well as relatively harmless parasitic forms of *Streptococci*. The latter are constantly present in the human throat and in the intestinal tract and assume a pathogenic role only under circumstances in which normal resistance is markedly reduced. A few strains of *Streptococci* have been isolated from milk and milk products but these are generally non-pathogenic.

The early reports on *Streptococci* dealt chiefly with their morphology and pathogenic effects in humans and little attention was given to identification and classification of the organism. In extensive studies, Lancefield utilized the differences in cell wall antigenic carbohydrates for *Streptococci* classification (32). The antibodies induced by these antigens were used to develop a serological scheme. Although numerous biochemical and cultural methods of differentiation had been advanced previously a satisfactory classification scheme had not been developed. The scheme of Lancefield is based on the study of 106 strains of *Streptococci* which were isolated from a wide variety of sources. The results of this study have been valuable not only from the theoretical viewpoint of establishing an orderly grouping of

Streptococci, but also from an epidemiological aspect in providing a means of identifying a given strain from which treatment of a disease can be recommended. The degree of pathogenicity of various groups of *Streptococci* for different species of animals is recorded in Table 2 (32).

TABLE 2

Pathogenicity of Serological Groups of Streptococci

Animal source	Chief pathogenic groups	Occasionally pathogenic groups	Groups apparently non-pathogenic
Man	A	B, C, D, F, G, H	K, L
Cattle	B, C	A, G	D, E, H, L
Horse	A		
Monkey		A, G	C
Dog	G, L, M		C
Rabbit	C	A, B	
Guinea Pig	C		G
Goat	C		
Sheep	C		
Mouse		A, B, C	

3.1.1 *Streptococcus faecalis*. *Streptococcus faecalis* belongs in the group D Streptococcal class and there are many strains of this organism in nature. The organism was originally isolated from cheese but can be found in other dairy products and in humans. The organism is generally non-pathogenic but is occasionally pathogenic and can cause endocarditis and urinary infections in pregnancy.

As pointed out earlier, the polysaccharides are not only important structural elements of the bacterial cell wall but they confer immunological specificity to the cell. In the cell wall the polysaccharides are embedded in the matrix of glycoaminopeptide and teichoic acids. Structural analyses of many of the cell wall polysaccharides have been attempted, however in view of the fact that many of these polymers are often acid-labile or susceptible to other chemical degradation, the structural characterization of these compounds has been achieved only recently.

The preparation of a new type of antigenic polysaccharide from *S. faecalis*, strain N was reported a number of years ago (25). This strain was being used to study the thymidine sugar pathway for

the synthesis of deoxy sugars (33). Later the strain was used in the preparation of antibodies specific for cell wall carbohydrates. An immune serum was obtained by immunization of rabbits with non-viable cells of the organism and complete Freunds adjuvant (CFA). Agar diffusion tests were performed and these are shown in Fig. 3. The extract (Well E) contained two types of antigenic compounds, a di- and tetra-heteropolysaccharide since *S. faecalis* extract and the immune serum (well S) yielded two precipitin bands. Evidently two antigenic polysaccharides were present in the extract. Earlier studies of antigenic carbohydrates from *S. faecalis* were reported but the purification and structural characterization of the carbohydrates was not achieved (34). Later a report that the antigen of group D *Streptococci* was a type of teichoic acid has also appeared (35) but this has not been confirmed.

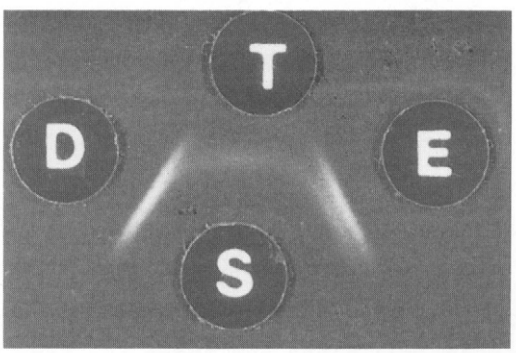


Fig. 3. Agar diffusion patterns for anti-S. faecalis serum (S) and the diheteropolysaccharide (D), the tetraheteropolysaccharide (T), extract of cells (E).

3.1.2 Antigenic Polysaccharides from *S. faecalis*. Two antigenic polysaccharides were isolated in pure form by methods described later. The polysaccharides induce the synthesis of two different sets of antibodies. Fig. 3 also shows agar diffusion tests with the purified samples (Wells D and T) and initial extract (Well E) with the immune serum (Well S). Each purified sample yielded single but different precipitin bands. Structural studies described in detail later have shown that one polysaccharide is a diheteropolysaccharide composed of glucose and galactose and the other is a tetraheteropolysaccharide composed of rhamnose, galactose, N-acetyl galactosamine and glucose and a small amount of phosphate. The monosaccharides were identified in acid

hydrolyzates of the polysaccharides by multiple ascent paper chromatography (36). Photographs of the paper chromatograms are shown in Fig. 4.

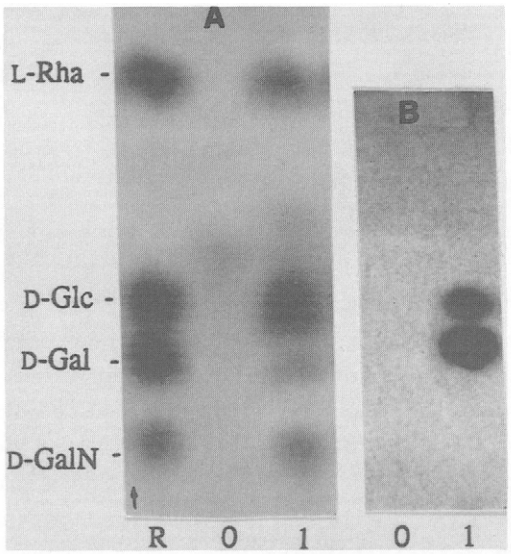
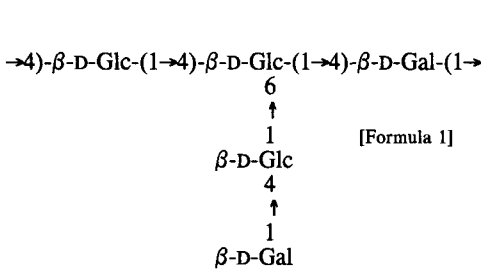
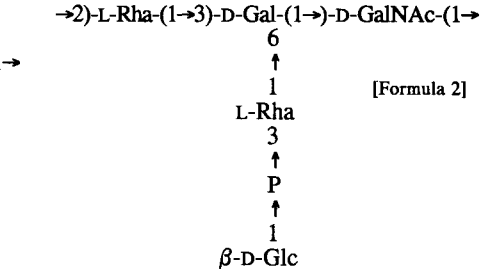


Fig. 4. Paper chromatogram of monosaccharides in hydrolyzates of tetrahetero-(A) and dihetero(B)-polysaccharides of *Streptococcus faecalis* stained by the silver nitrate method (37). O and 1 hr acid hydrolysis was used, B is reference monosaccharides.

The chromatogram strips were stained by the silver nitrate method (37). Comparisons of the Rf values of the products with Rf values of reference monosaccharides tentatively identified the monosaccharide constituents in the tetraheteropolysaccharide as rhamnose, glucose, galactose, and galactosamine which in the native polysaccharide is N-acetylated. On staining duplicate chromatograms with specific reagents positive tests were obtained for 6-deoxy hexose with aniline oxalate (38), for galactosamine by the amino sugar reaction (39), for galactose by galactose oxidase (40) and for glucose by glucose oxidase (41). Other tests showed that the amino sugar was N-acetyl-galactosamine. The diffuse spot in the 0 hr hydrolyzate of the tetra compound was not identified. The monosaccharide constituents of the diheteropolysaccharide were glucose and galactose (B of the figure). The structures proposed for these polysaccharides on this and subsequent evidence are shown by Formula 1 and 2.



[Formula 1]



[Formula 2]

Both of the polysaccharides possess some unusual structural features. The diheteropolysaccharide has many side chains of lactose units attached to the main chain. There are only a few compounds in nature which contain a lactose moiety in their molecular structure. The tetraheteropolysaccharide possesses side chains of β-glucose-1-phosphate. The phosphate which is also an unusual structural moiety of polysaccharides was determined by a colorimetric method (42). The lactose of the former and the β-glucose-1-phosphate of the latter are the immunodeterminant groups of the respective polysaccharides.

3.1.3 Immunization Procedure. Non-viable cells of *S. faecalis*, strain N were also prepared for use in the preparation of vaccine for immunization of rabbits. The bacteria were grown in 800 ml of Todd Hewett broth at 37°C for 16 hr. The cells were collected by centrifugation and suspended in 20 ml solution of 2% formaldehyde. The mixture was maintained at room temperature for 12 hr. Growth tests showed that the cells had become non-viable by this treatment. An aliquot of the suspension was mixed with an equal volume of complete Freunds adjuvant (CFA). Four rabbits were immunized intravenously with 1.0 ml of the non-viable cell CFA suspension by injections of the bacterial suspension in the ear vein. The injections were done weekly for 8 weeks. Blood samples were collected in the latter weeks and serum was obtained. Agar diffusion tests were performed with the serum and polysaccharide preparations. The results have been shown in Fig. 3. These results show that the synthesis of two populations of different antibodies had occurred and two different groups are present as immunodeterminants in the cell wall antigens.

3.1.4 Separation of *S. faecalis* Polysaccharides. The *S. faecalis* cell wall polysaccharides were prepared as described in Section 2.1.3. Since the TCA soluble extract contains two

antigenic carbohydrate polymers separation procedures were developed. Ethyl alcohol fractionation was found suitable for separating the polymers. A sample of 0.2 g of the extract was dissolved in 9 ml of 10% solution of trichloroacetic acid. After removal of a slight amount of insoluble material by centrifugation, one volume of ethyl alcohol was added to the clear supernatant and the mixture was maintained at 4°C for 18 hr. In this period a white precipitate formed and this precipitate was collected by centrifugation. The supernatant was saved for subsequent fractionation. The precipitate was washed with 3 ml portions of ethyl alcohol, finally with diethyl ether and collected by centrifugation. The sample was dried by lyophilization. The second polysaccharide from *S. faecalis* was obtained from the above supernatant by stirring with four additional volumes of ethyl alcohol at 4°C for 18 hr. Another precipitate was formed in this period. This precipitate was the tetraheteropolysaccharide and was collected by centrifugation, washed with ethyl alcohol and diethyl ether and dried by lyophilization.

The structure of both preparations has been determined by identification of acid hydrolytic products by paper chromatography, methylation analysis, specific enzyme hydrolysis, periodate oxidation, acetolysis, alkali degradation, hapten inhibition and ultracentrifugation in density gradients.

3.1.5 Methylation Analysis. Methylation analysis was performed following the protocols recommended by Hakomori (1) and Lindberg (43). Samples of 2 to 5 mg were dissolved in 0.5 ml of dry methyl sulphoxide in a 5 ml serum bottle sealed with a rubber cap (44). The bottle was flushed with nitrogen and 0.5 ml of 2 M methylsulphinyl sodium in methyl sulphoxide was added dropwise from a syringe. At conclusion of the addition the sample was agitated in an ultrasonic bath for 0.5 hr. The reaction was allowed to proceed for 8 hr at room temperature at which point 0.5 ml of methyl iodide was added and the reaction mixture stirred again in an ultrasonic bath for an additional 0.5 hr and the reaction mixture was poured in approximately 10 ml of water. The sample was dialyzed for 24 hr and then concentrated to dryness. The methylated polysaccharide was suspended in 1 ml of 90% formic acid and heated at 100°C for 2 hr in order to effect a dissolution of the methylated compound. Then the formic acid was removed by evaporation and the residue resuspended in 1 ml of 0.13 M sulfuric

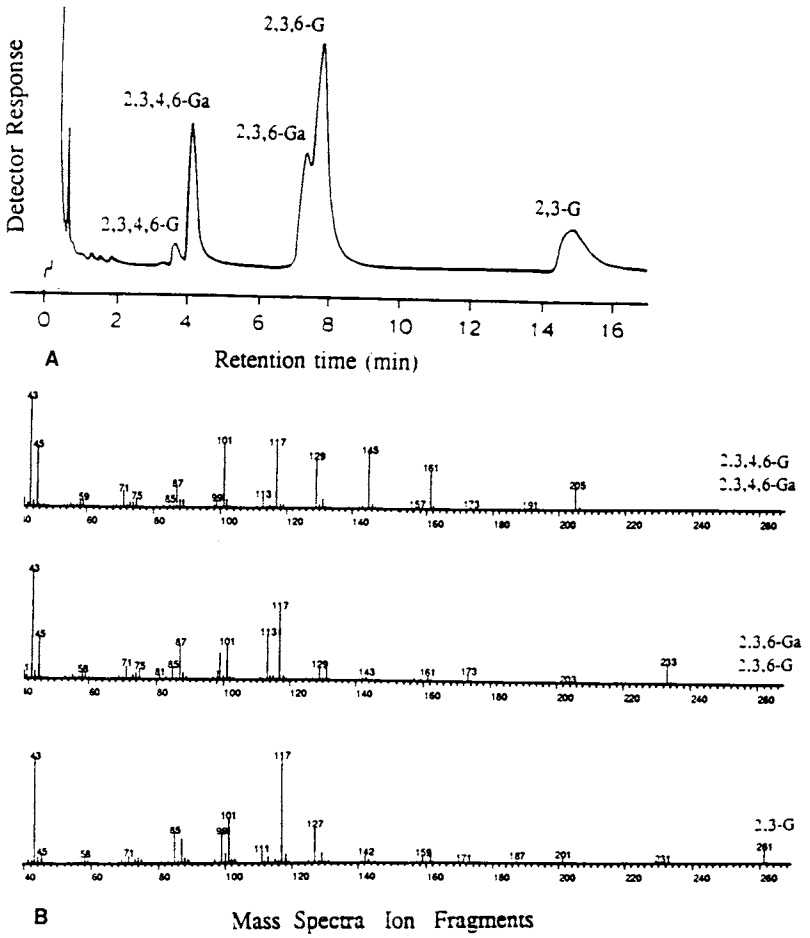


Fig. 5. A: Photograph of the GLC patterns for the methylated alditol acetates from the native diheteropolysaccharide (A). B: mass spectra of ion fragments of the glucose and galactose derivatives from the diheteropolysaccharide (44).

acid and heated at 100°C for 12 hr. The acid was neutralized and the hydrolytic products were reduced with disodium borohydride at 25°C for 12 hr. The solution was acidified and evaporated to dryness. The residue was dissolved in 0.5 ml of acetic anhydride and pyridine (1:1) and heated at 105°C for 2 hr. The sample was dissolved in chloroform and injected into a GLC-Ms unit for analysis. Fig. 5 shows GLC-Ms results for the S. faecalis diheteropolysaccharide. The GLC peaks were identified by retention times. The mass fragments were identical to those obtained from standard derivatives (45).

3.1.6 Enzymic Hydrolysis. Enzymic hydrolysis of the *S. faecalis* diheteropolysaccharide was performed with an almond preparation containing exo-β-galactosidase and exo-β-glucosidase (44). A sample of 60 mg of the diheteropolysaccharide was dissolved in 1.5 ml of water and mixed with 0.75 ml of 4% almond enzyme preparation in 0.1 M phosphate buffer of pH 6.8. The digest was incubated at room temperature for 44 hrs and checked periodically for the presence of reducing sugars by paper chromatography. The qualitative paper chromatograms of the digest revealed that D-galactose was liberated from the polysaccharide by the prolonged enzyme treatment. The enzymes were inactivated by heat and precipitated with an equal volume of 10% trichloroacetic acid. The supernatant was obtained and dialyzed for 48 hrs against distilled water. The sample was then lyophilized to dryness. Qualitative capillary precipitin tests showed that the enzyme modified polysaccharide yielded only a trace of precipitin complex with the immune serum. Two mg of enzyme modified polysaccharide was subjected to methylation analysis (3.1.5) and the products were identified by GLC and mass spectrometry. The quantitative data were obtained for the enzyme modified and the native polysaccharide by comparisons with known standards and integration of the area under the comparable peaks. The amounts of methyl products per mole of polysaccharide were calculated from methylation data and molecular weight determined by ultracentrifugation. These data are recorded in Table 3.

TABLE 3

Moles of methylated monosaccharides per mole of diheteropolysaccharide.

Compound	Native	Enzymically Modified
2,3,4,6-Tetramethylglucose	4.0	11.3
2,3,6-Trimethylglucose	35.0	29.4
2,3-Dimethylglucose	17.1	16.6
2,3,4,6-Tetramethylgalactose	17.5	9.4
2,3,6-Trimethylgalactose	17.6	17.2

3.1.7 Periodate Oxidation. A sample of 40 mg of the diheteropolysaccharide was oxidized in 50 ml of 0.02 M sodium periodate of pH 4.5 (44). The solution was maintained in the dark at 4°C for 18 hrs. At this point excess periodate was decomposed by addition of ethylene glycol. Low molecular weight materials were removed from the reaction mixture by dialysis against distilled water. The oxidized polysaccharide was recovered by lyophilization. A sample of 20 mg of the oxidized polysaccharide was reduced with sodium borohydride (5 mg) at room temperature for 24 hr. The reduced product was dialyzed and dried by lyophilization. Samples of 2 mg of the oxidized polysaccharide and 2 mg of native compound were dissolved in 0.1 ml of 0.1 N HCl and heated for 3 hr in a boiling water bath. Analysis of the hydrolysate for carbohydrates was performed by multiple ascent paper chromatography. In the hydrolyzate of the native polysaccharide, glucose, galactose and some oligosaccharides were identified and in the oxidized and reduced compound glucose, glycerol and D-threitol were present. D-Galactose was completely oxidized by this treatment.

3.1.8 Acetolysis. Acetolysis of 10 mg diheteropolysaccharide was effected in 1 ml of acetolysis mixture (10 parts of acetic anhydride, 10 parts of glacial acetic acid and 1 part of concentrated sulfuric acid). The mixture was heated in a stoppered reaction vessel at 40°C for 3 hours. The acetylated products in the mixture were converted to deacetylated products which were isolated following the directions of a published procedure (46). The following products were detected on the qualitative chromatograms D-glucose and D-galactose in low amounts and a high

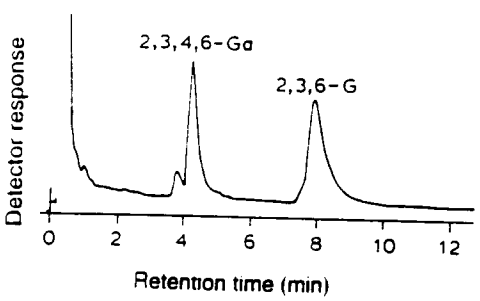


Fig. 6. The methylated products from the disaccharide from the acetolysis mixture of diheteropolysaccharide.

amount of a disaccharide, which moved on paper at the same Rf value as lactose. This disaccharide on methylation analysis yielded 2,3,4,6-tetramethyl-galactose and 2,3,6-trimethyl glucose identified as the alditol acetates. These are the products expected from lactose. The results are shown in Fig. 6.

3.1.9 Hapten Inhibition. The formation of the complex of antibody and antigen was observed qualitatively by agar diffusion. A 1% agarose solution was placed on a microscope slide in a thin layer. After the agarose solidified a punch was used to make an array of wells of uniform size. Immune serum (10 ul) or purified antibodies (10 ul) containing 10-20 ug protein were placed in the center well of the diffusion plate and the digest of the antibody and inhibitor was placed in another center well. The polysaccharide solution (10 ul) containing 10-50 ug compound was placed in the outer wells of the agar plate. The diffusion plates were placed in a petri dish on moist filter paper, covered, and maintained at room temperature for periods of 6-30 hr. The plates were checked periodically for precipitin formation and the number and intensities of the precipitin bands which formed were noted. The plates were also photographed for permanent record. The compounds which were inhibitors yielded weak or no precipitin bands at the low concentration of antigen.

The determination of quantitative precipitin formation utilized the Lowry protein test (47). The amounts of reagents used in the quantitative method were 0.1 ml immune serum and optimum concentration of polysaccharide (5 μg). The digest was incubated for 10 hrs. The precipitin formed is recovered by centrifugation and the amount is determined by the Lowry protein method. Hapten inhibition tests were performed with lactose, galactose and several derivatives. For the inhibition tests with the anti-Lac antibodies the concentration of inhibitors ranged from 0 to 5 mM. The results are plotted in Fig. 7. It is noted in the figure that lactose and derivative were strong (85%) inhibitors but galactose and derivatives inhibited less than 5%, thus the immunodeterminant group of the *S. faecalis* diheteropolysaccharide is the lactose moiety.

3.1.10 Molecular Weight. On sucrose density gradient ultracentrifugation (48), the two polysaccharides isolated from the cell wall of *S. faecalis* differed in sedimentation rates. From the density gradient sedimentation rate and an empirical formula (49)

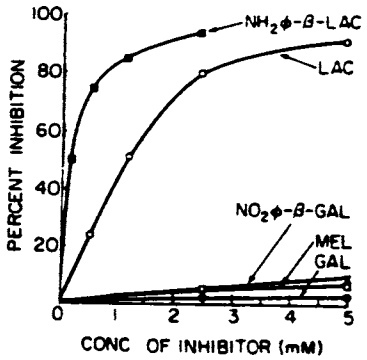


Fig. 7. Hapten inhibition of the anti-diheteropolysaccharide antibodies.

the molecular weight of the diheteropolysaccharide was calculated as 16,000. The molecular weight of the tetraheteropolysaccharide was calculated to be 6,000. In view of the foregoing data a diagrammatic representation of the dihetero polysaccharide of *S. faecalis* is shown in Fig. 8

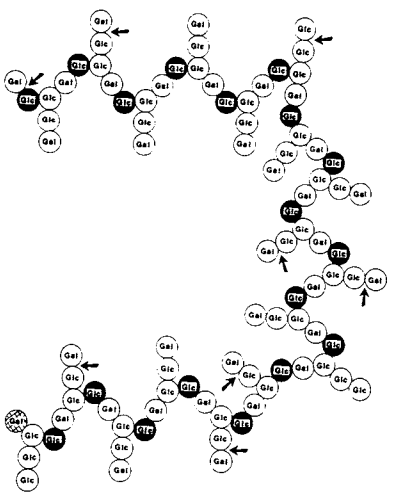


Fig. 8. Diagrammatic representation of the structure of a typical molecule of the diheteropolysaccharide from *S. faecalis*. Dark circles indicate residues linked to position 6 of adjacent residues; open circles indicate residues linked to position 4 of adjacent residues; the cross-hatched circle indicates the residue with the reducing group; circles adjacent to only one circle indicate terminal residues; the arrows indicate possible bonds hydrolyzed by the β-glycosidases.

3.1.11 Structure of Tetraheteropolysaccharide. The data on the structure of the tetraheteropolysaccharide are incomplete but a tentative structure shown in Formula 2 has been proposed on data obtained mainly on methylation analysis and alkali degradation. Two mg samples of the tetraheteropolysaccharide were used for methylation analysis and 50 mg for the degradation. The methylation analysis was performed by gas liquid chromatography and mass spectrometry as described in section 3.1.5. The rhamnose, glucose and galactose derivatives were observed by Glc analysis and the N-acetyl galactosamine derivative was not observed by GLC analysis but was identified as a constituent of the polysaccharide by color reaction and by Rf values for the amino sugar (39). On methylation analysis the following methyl alditol acetates were identified from the methylated tetraheteropolysaccharide: 1,2,5-tri-O-acetyl-3,4-di-O-methyl rhamnitol, 1,3,5-tri-O-acetyl-2,4-di-O-methyl rhamnitol, 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol, 1,2,3,5-tetra-O-acetyl-4-O-methyl rhamnitol, 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl galactitol and 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl galactitol.

The methylation data reveal two important structural features of the tetraheteropolysaccharide. First, the glucose units are terminal units and are present as side chains on a main chain. Second, the main chain is composed of rhamnose and galactose as deduced from the methylation data and N-acetyl-galactosamine as deduced from the galactose oxidase test (40). The latter showed that the N-acetyl-galactosamine unit was not terminal.

3.1.12 Evidence for β-linkage. To obtain evidence that the glucose units were linked to phosphate via a β linkage, a 50 mg sample of the polysaccharide was adjusted to pH 8.0 with 0.1 N NaOH and then reduced with 40 mg of sodium borohydride. Such a reduction of the reducing group of the polysaccharide was necessary to prevent alkaline degradation from the reducing end. The reaction mixture was allowed to stand at room temperature for 24 hr, then acidified to pH 4.5 with acetic acid and dialyzed against distilled water for 48 hr. The polysaccharide with the reduced end group was taken to dryness by lyophilization. The reduced tetraheteropolysaccharide was dissolved in 0.1 ml of 0.1 N NaOH in a glass vial. The vial was sealed and then heated at 115°C for 6 hr. It has been shown earlier (50) that under these conditions β-glucosides but not α-glucosides yield levoglucosan (α-1→6)

anhydroglucose. At the end of the heating period, the vial was opened and the solvent evaporated under vacuum. The reaction products were then acetylated in 0.2 ml of dry acetic anhydride and 0.2 ml of pyridine at room temperature for 6 hr. The reagents were removed by evaporation in a stream of nitrogen and the products were dissolved in a small volume of dry chloroform. This solution was subjected to gas liquid chromatography at a temperature of 190°C. Samples of β-D-glucose-1-phosphate, α-D-glucose-1-phosphate and levoglucosan were subjected to the same series of reactions. A sample of reduced tetraheteropolysaccharide was also subjected to acetylation and to GLC analysis. GLC patterns for some of the samples are shown in Fig. 9. It will be noted that the reduced tetraheteropolysaccharide when heated in dilute alkali yielded a product which on acetylation (B) possessed a retention time on GLC analysis identical to that of authentic 2,3,4-tri-O-acetyl levoglucosan (A). The reduced tetraheteropolysaccharide (C) which was not treated with alkali did not yield this product. Also β-D-glucose-1-phosphate when subjected to the above series of reactions yielded the levoglucosan derivative but α-D-glucose-1-phosphate did not.

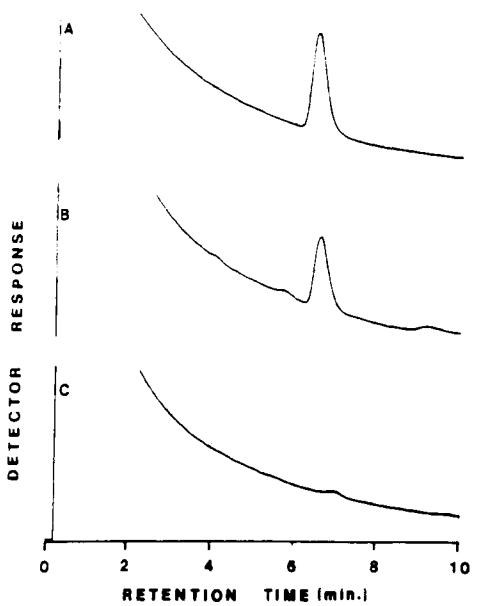


Fig. 9. Gas-liquid chromatography patterns for 2,3,4-tri-O-acetyl levoglucosan (A) and the products from the tetraheteropolysaccharide heated in alkali (B) and a control of tetraheteropolysaccharide (C).

3.1.13 Hapten Inhibition. Information on the structure and immunology of the tetraheteropolysaccharide of *S. faecalis* was obtained by hapten inhibition studies. These tests were performed with glucose, rhamnose, galactose, N-acetyl-galactosamine and several glucose derivatives by the quantitative hapten inhibition test. In these tests an optimal level of antigen (80 ug) a constant level of antiserum (0.1 ml) and varying amounts of inhibitor (0.04 to 0.2 mM) in a final volume of 1 ml were incubated overnight at 4°C. The precipitates which formed were collected by centrifugation and quantitatively determined by the Lowry protein method (47). Plots of some of the inhibition data are shown in Fig. 10. β-Glucose-1-phosphate but no other glucose derivative was an inhibitor. None of the other monosaccharides which are constituents of the polysaccharide gave inhibition. Thus β-glucose and phosphate are necessary for precipitin formation with the antibody.

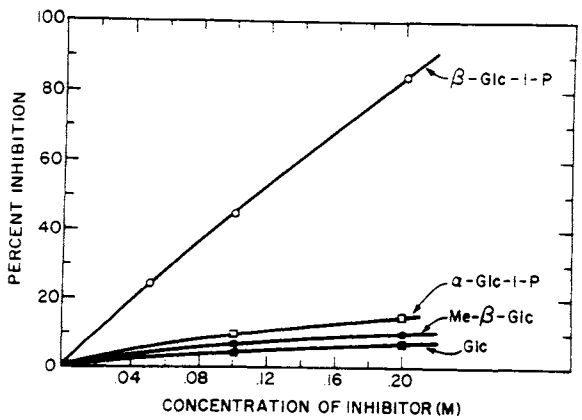


Fig. 10. Inhibition of the precipitin reaction between the tetraheteropolysaccharide and the anti-*S. faecalis* serum by glucose and derivatives.

3.1.14 Acid Lability of Phosphate Linkage. Tests were performed on the lability of the linkage of the polysaccharides to mild acid hydrolysis. Samples of 1 mg of the tetrahetero- and the diheteropolysaccharides were dissolved in 0.02 ml of 0.01 N HCl and heated in sealed tubes at 100°C for 1 hr. The hydrolyzates were neutralized with sodium bicarbonate and were then used in agar diffusion tests. The results are shown in Fig. 11.

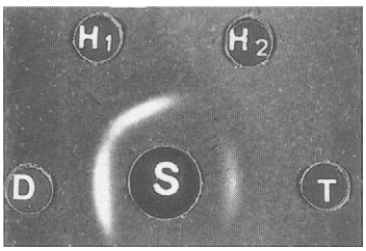


Fig. 11. Agar diffusion pattern of anti-*S. faecalis* serum (S) and *S. faecalis* cell wall diheteropolysaccharide (D), and tetraheteropolysaccharide (T) and 0.02 N acid hydrolyzates, H_1 for D and H_2 for T.

It will be noted in the figure that the dilute acid treatment had no effect on the antigenicity of the diheteropolysaccharide (D and H_1). However, heating the tetraheteropolysaccharide under the above conditions yielded a product which did not give a precipitin band in agar diffusion (T and H_2). In the hydrolysate of the tetraheteropolysaccharide glucose was identified by a test with glucose oxidase. In order to compare the hydrolytic rates for the glucose-1-phosphates and the polysaccharide, samples of the phosphates were subjected to acid hydrolysis. It was found that the same rate of glucose liberation occurs with all compounds. Evidence of the location of the phosphate linkage in the polysaccharide was obtained by an enzymic method utilizing alkaline phosphatase. These results showed that the linkage occurs as a phosphodiester between the glucose and rhamnose units of the polysaccharide.

3.1.15 Position of Phosphate Linkage. To establish the position of phosphate in the structure of the polysaccharide a sample of 1.5 mg of the tetraheteropolysaccharide was dissolved in 0.1 ml of 0.01 N HCl and heated in a boiling water bath for 30 minutes. A sample of 0.05 ml of the hydrolysate was analyzed for inorganic phosphate by a standard procedure (42). The hydrolysate was also tested in a precipitin reaction with immune serum. The remainder of the hydrolysate was adjusted to pH 9 with sodium bicarbonate and 50 ul of the sample was mixed with 50 ul of bacterial alkaline phosphatase (10 units). After incubation for 24 hrs at room temperature, the mixture was analyzed for inorganic phosphate. Suitable blanks of enzyme and the polysaccharide were analyzed for phosphate content. Data for this experiment are recorded in Table 4.

TABLE 4

Phosphate content in samples before and after enzymatic and acid hydrolysis of tetraheteropolysaccharide.

Sample	Total Phosphate μ Moles	Inorganic Phosphate μ Moles	Phosphate Liberated %
Tetraheteropolysaccharide (500 μg)	0.37	0.005	--
Acid hydrolyzed polysaccharide (a)	0.36	0.016	4.3
Phosphatase blank (50 μl)	--	0.005	--
Phosphatase hydrolyzate of (a)	0.36	0.260	72

Precipitin tests with anti *S. faecalis* serum and the modified polysaccharide samples prepared as above showed that neither the acid hydrolyzed- nor the acid hydrolyzed phosphatase treated-polysaccharide yielded positive reactions with immune serum. Over 70% of the phosphate was liberated by enzyme hydrolysis after the glucose was released by acid hydrolysis. The phosphate is present in the polysaccharide as a diester bond linking the glucose to the rhamnose of the polysaccharide (see Formula 2).

3.2 Streptococcus bovis

New dihetero and tetraheteropolysaccharides were extracted from the cell walls of *S. bovis*, a group D *streptococcus* (18,51). Agar diffusion tests were performed with the initial extract and the purified polysaccharides with immune serum prepared with non-viable *S. bovis* cells. The results are shown in Fig. 12.

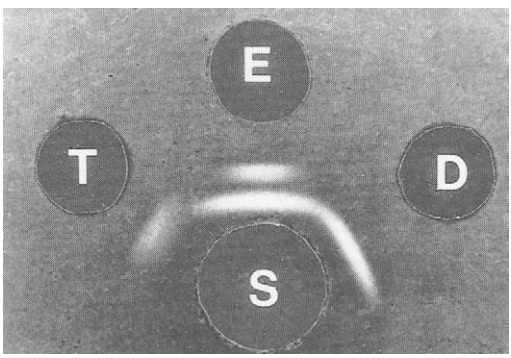
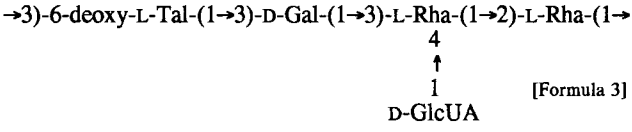


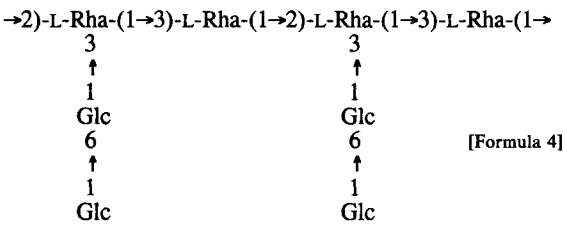
Fig. 12. A photograph of an agar diffusion plate: Well (T) tetraheteropolysaccharide of *S. bovis*; well (E), extract well (D), diheteropolysaccharide of *S. bovis*; well (S), immune serum against nonviable *S. bovis*.

It is noted in Fig. 12 that the extract (E) yields two bands with the serum, whereas the purified compounds from *Streptococcus bovis* tetraheteropolysaccharide (Well T) and diheteropolysaccharide (Well D) yield single but different bands.

The tetraheteropolysaccharide extract was obtained from *S. bovis* by very mild acid extraction of the cells with the solvent of KCl-HCl for a short period (10 minutes). The polysaccharide was purified by gel filtration to remove the diheteropolysaccharide. Acid hydrolysis converted the tetraheteropolysaccharide to galactose, glucuronic acid, rhamnose and a new deoxy sugar, 6-deoxy-L-talose identified by its specific rotation, nuclear magnetic resonance spectra, and x-ray diffraction of the osazone derivative. The second polysaccharide was obtained from the supernatant from a longer period of extraction (60 minutes) of *S. bovis* cells. This polysaccharide is made up of rhamnose and glucose and is therefore of the dihetero type. The tentative structures of the repeating units of the two polysaccharides have been determined and are shown in Formulas 3 and 4.



[Formula 3]



[Formula 4]

3.2.1 Preparation of *S. bovis* Polysaccharides. The polysaccharides were prepared from *Streptococcus bovis*, group D, from cells grown at 37°C for 24 hr in Todd-Hewitt media containing 0.1% D-glucose. The cells from 18 liters of culture were collected in a refrigerated Sharples centrifuge and extracted by the KCl-HCl method (Section 2.1.3).

In early studies (18,51) a separation of the two antigens was performed by Bio-gel filtration but the separation was not complete. An improved method was devised based on DEAE-cellulose

chromatography (52). In the new procedure, the column of DEAE cellulose was washed with 0.01 M potassium phosphate buffer of pH 7.5 in 0.01 M sodium chloride. A sample of 0.1 gm of the lyophilized product was dissolved in 5 ml of the buffer and placed on the column. Sodium chloride gradient from 0 to 0.5 M was used to elute the carbohydrates. Fractions of 8 ml of the eluates were collected and aliquots of 0.1 ml of these fractions were used for carbohydrate analysis by a phenol-sulfuric acid method (53). The biofiltration results are recorded in pattern A of Fig 13. The

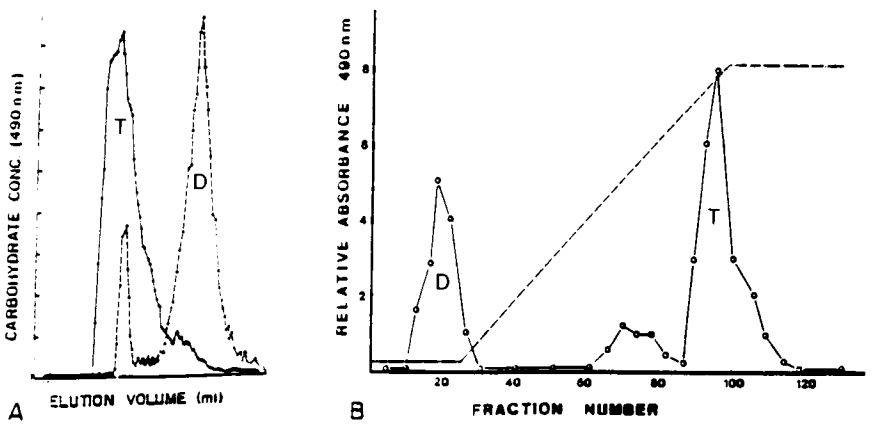


Fig. 13. A: Distribution of carbohydrates in eluates on gel-filtration of the 10 and 60 minute extracts (solid line and broken line 10 and 60 min extract, respectively) from cell walls of *S. bovis*; B: elution pattern of the polysaccharides of *S. bovis* from 60 min extract from a DEAE-cellulose column with a sodium chloride gradient. D=Diheteropolysaccharide and T=tetrahetero-polysaccharide.

DEAE results are shown in pattern B of the figure. The appropriate fractions corresponding to the various carbohydrate peaks were combined, dialyzed and taken to dryness by lyophilization. The lyophilized materials constitute the purified polysaccharides.

3.2.2 Identification of Monosaccharides. The monosaccharide constituents of the S. bovis polysaccharides were identified in acid hydrolysates of the polymers by paper chromatography (36). The chromatograms were developed in a solvent system of n-butyl alcohol, pyridine and water (6:4:3 by vol.) by the multiple ascent method. Three ascents of the solvent were used.

One chromatogram was stained with the silver nitrate reagent (37), another was sprayed with aniline oxalate (38) reagent and others with enzymes. The identity of the monosaccharides in the hydrolysates was established by comparison of the Rf values with values of standards. Identification of the monosaccharides was confirmed by colorimetric methods: the carbazole method for uronic acids (54), the cysteine-sulfuric acid method for deoxy sugars (55), and the copper sulfate method for neutral sugars (56). A sample of polysaccharide with reduced glucuronic acid was also hydrolyzed and analyzed chromatographically. A paper chromatogram is reproduced in Fig. 14.

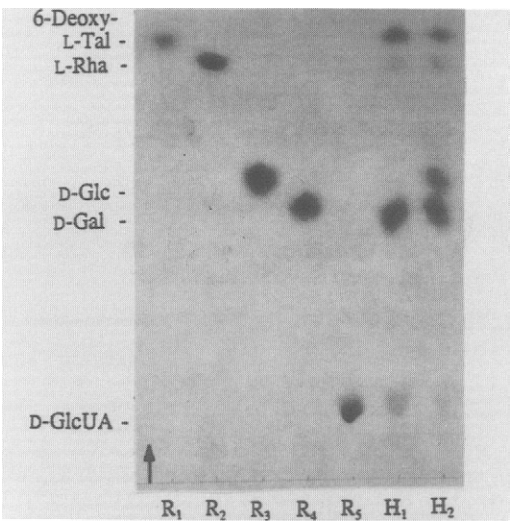


Fig. 14. Paper chromatogram showing the hydrolytic products from the native and reduced tetroheteropolysaccharide of *S. bovis* on hydrolysis of the native and reduced polysaccharide in N HCl at 100°C for 1 hr.

3.2.3 Esterification and Reduction. The reduction of the tetraheteropolysaccharide (60 mg) from *S. bovis* was effected by a two step reaction first by esterifaction with ethylene oxide at 0°C and reduction with sodium borohydride (57). The esterified product was recovered by dialysis and lyophilization. One-half of this product was used for reduction by 1.5% solution of cold sodium borohydride. After 48 hrs of reaction the mixture was acidified with 1 N HCl, dialyzed for 48 hrs and taken to dryness by lyophilization. In later experiments the reduction of uronic acids was effected by the carbodiimide-borohydride method (58,59).

Samples of 2 mg of native and of the reduced polysaccharide were heated in 0.2 ml of 0.1 N hydrochloric acid for two hrs at 100°C. The hydrolytic products were separated by multiple ascent paper chromatography. A photograph of a finished chromatogram of the hydrolysates stained by the silver nitrate method (37) and a paper strip is included in Fig. 14. A duplicate chromatogram was sprayed with glucose oxidase-peroxidase solution of pH 5.2 followed by O-tolidine. Only reference D-glucose and the new sugar in the hydrolysate of the reduced polysaccharide yielded a positive test with the glucose oxidase.

Samples of the esterified and the reduced polysaccharide were also used for the quantitative precipitin tests. The antigenicity of the native and reduced polysaccharide was tested at several concentrations of antigen and constant level of antibodies. The inhibition data are plotted in Fig. 15. Quite clearly, modification of the carboxyl group of the antigen markedly reduces the antigenicity of the polysaccharide and therefore glucuronic acid residues are necessary for precipitin formation.

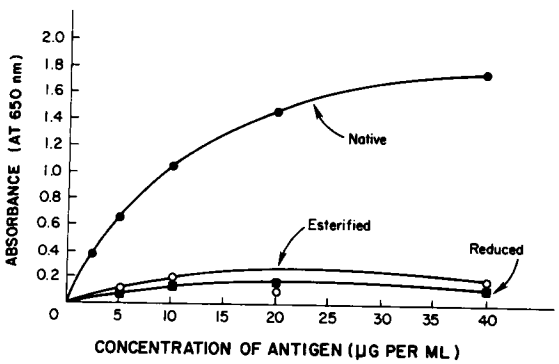


Fig. 15. Quantitative precipitin curves for the reaction of the native, the esterified, and the reduced tetraheteropolysaccharide and antiserum against *S. bovis*.

3.2.4 Quantitative Inhibition. Quantitative values for inhibition of precipitin reaction for the tetraheteropolysaccharide and its constituent monomeric sugars are presented in Table 5. Under the conditions of the test the optimum concentration of the polysaccharide for precipitin formation was 40 ug and this concentration was used for all tests except with the glucuronic acid and 6-deoxy-L-talose. Since the latter compound was available

in limited quantity, inhibitions with this compound were performed by a capillary precipitin method. Of the constituent sugars of the polysaccharide, only the D-glucuronic acid was a strong inhibitor of the precipitin reaction and over 80% inhibition was obtained with this compound. Galacturonic acid was also an inhibitor for the reaction. Evidently the orientation of the hydroxyl group at carbon 4 of the uronic acid is not essential for complex formation.

TABLE 5

Inhibition of precipitation and reaction of tetraheteropolysaccharide and immune serum.

Compound	Concentration mg/ml	Inhibition %
D-Glucuronic acid	0	0
	10	17
	20	56
	40	82
D-Galactose	40	8
L-Rhamnose	40	0
6-Deoxy-L-talose[a]		0
D-Glucurono-(6,3)-Lactone	40	3
D-Glucuronamide	40	6
D-Galacturonic Acid	40	70

[a]Measured by micro capillary method.

3.2.5 β-Elimination. β-Elimination of glucuronic acid of the tetraheteropolysaccharide (12 mg) was effected by a published method (60). The native polysaccharide was first methylated by the Hakomori procedure and analyzed by the Lindberg method (Section 3.1.5). Purification of the methylated polysaccharide was effected by pouring the entire reaction mixture into water and extracting the product with choloroform. The chloroform extract was taken to dryness and the product was remethylated by the Hakomori method. A sample of the remethylated polysaccharide was hydrolyzed and the products converted to their alditol acetates and analyzed by GLC and mass spectrometry.

The GLC patterns for several different samples of the polysaccharide are recorded in Fig. 16. The data for the native sample (A) show the composition of the polysaccharide to be 6-deoxy

talose, rhamnose, galactose and glucuronic acid with the uronic acid identified by the carbazole test (54). Data for the reduced polysaccharide (pattern B) show the glucuronic acid was converted quantitatively to glucose identified as 2,3,4,6-tetramethyl glucitol and is a terminal unit. The pattern (C) for the β-elimination product shows the glucuronic acid is attached to rhamnose which is converted to 2,4-dimethyl rhamnose on elimination of glucuronic acid.

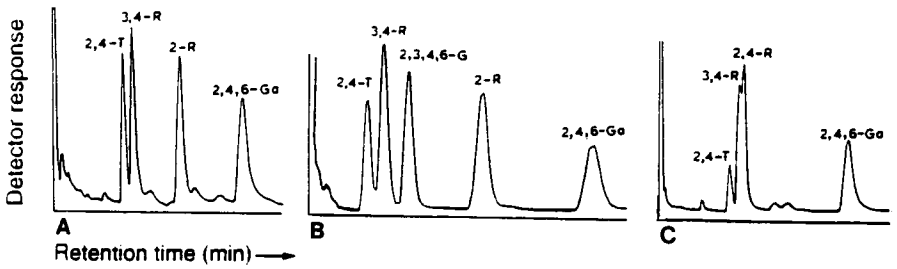


Fig. 16. A photograph of GLC patterns for the methylated alditol acetates from the native tetraheteropolysaccharide (A), the polysaccharide fragment after reduction of the D-glucuronic acid residues (B), and the polysaccharide fragment after β-elimination of glucuronic acid (C).

3.2.6 Diheteropolysaccharide of S. bovis. A second antigenic polysaccharide was isolated from *S. bovis* by the KCl-HCl extraction (60 min) of the cell walls of this organism (51). The derivatives obtained from the compound on methylation analysis (section 3.1.5) are listed in Table 6.

TABLE 6

Retention times and yields of methylated monosaccharides derived from the diheteropolysaccharide of *S. bovis*.

Derivative	Rt	Moles
2,3,4-trimethyl rhamnitol	0.47	1.0
3,4-dimethyl rhamnitol	0.87	25.2
2,4-dimethyl rhamnitol	0.92	8.0
2,3,4,6-tetramethyl glucitol	1.00	16.0
4-methyl rhamnitol	1.52	14.9
2,3,4-trimethyl glucitol	2.20	15.3

This antigen composed of glucose and rhamnose, has now been shown to consist of a main chain of rhamnose residues to which many isomaltosyl units are attached as side chains. The tentative formula suggested for the repeating unit of the polysaccharide is shown in Formula 4.

3.2.7 Chromium Trioxide Oxidation. Chromium trioxide oxidation is a relatively new technique which yields information on the configuration of glycosidic linkages in a polysaccharide (62). Oxidation by this procedure followed by methylation analysis of the native and oxidized samples revealed that the methyl alditol acetates from the oxidized polysaccharide were present in the same ratio as in the native polysaccharide. It has been established that α-linked residues are slowly oxidized and β-linked residues are oxidized rapidly by chromium trioxide (61). The results with the polysaccharide show the glycosidic linkages between residues of the *S. bovis* diheteropolysaccharide are alpha.

3.2.8 Hapten Inhibition. Glucose, maltose and isomaltose were tested as inhibitors of the quantitative precipitin reaction between the diheteropolysaccharide and anti-*S. bovis* serum. Figure 17 contains the results of the inhibition tests with these compounds. It is noted that isomaltose is the most effective inhibitor. Over 80% of inhibition was obtained with isomaltose as an inhibitor. Maltose and glucose effected some inhibition and only at concentrations of 20 times higher than isomaltose.

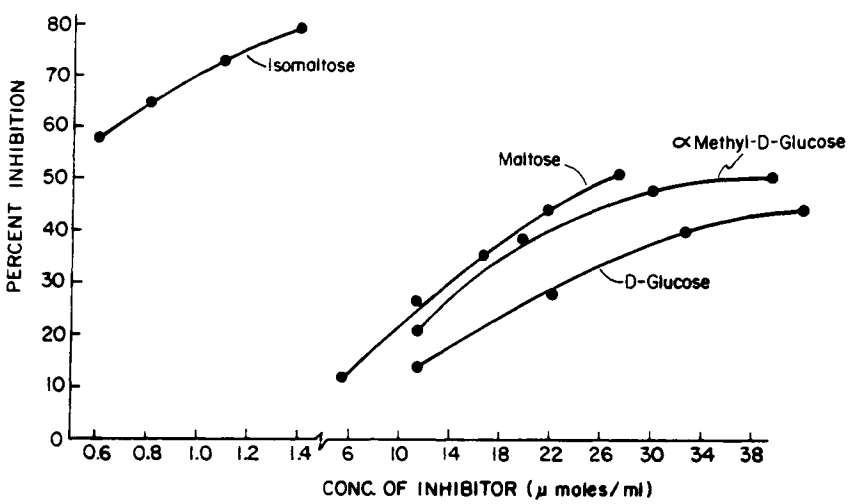


Fig. 17. Inhibition of the precipitin reaction between the purified diheteropolysaccharide from *S. bovis* and antiserum against *S. bovis* carbohydrates.

3.2.9 Molelcular Weight by Gel Filtration. To obtain data for molecular weight calculations, Bio-gel filtration of the *S. bovis* diheteropolysaccharide and of a reference diheteropolysaccharide of *S. faecalis* was employed. Samples of 11 mg of the diheteropolysaccharide from *S. bovis* and 10 mg of diheteropolysaccharide from *S. faecalis* were placed on a Bio-gel P-30 column (2 cm x 100 cm). The polysaccharides were eluted with a 0.1 M sodium phosphate buffer at pH 7 and fractions of 2 ml were collected. The total carbohydrate (solid circles) and the 6-deoxy hexose (open circles) content of each fraction were determined by colorimetric methods (53,55) and values are plotted in Fig. 18.

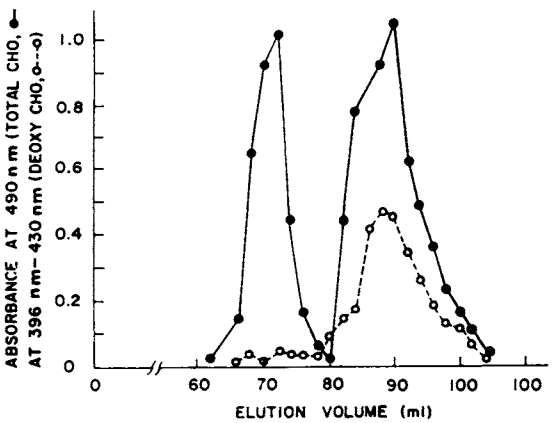


Fig. 18. The concentration of diheteropolysaccharides from *S. faecalis* and *S. bovis* in eluates from a column of Bio-gel P30.

From the chromatographic results and a molecular weight value of 16,000 for the *S. faecalis* diheteropolysaccharide (25) the molecular weight of the *S. bovis* diheteropolysaccharide was calculated to be 12,000. Molecular weight values were verified by density gradient centrifugation data.

3.2.10 Periodate Oxidation. The *S. bovis* polysaccharide was subjected to periodate oxidation following the method outlined in Section 3.1.7 to determine which residues were oxidizable. The oxidized material was dried by lyophilization and redissolved in 1 ml of water. The solution was analyzed for D-glucose and for L-rhamnose by standard colorimetric methods (53,55). The analysis showed that D-glucose was oxidized completely within 2 hours but only 50% of the L-rhamnose was oxidized on prolonged oxidation. The presence of a glycosidic bond at carbon 3 of rhamnose prevented the complete oxidation of this monosaccharide.

3.3 *Streptococci* Group L

Cell walls from a group L-*Streptococcus* for which complete taxonomic information is not yet available were prepared by the method in Section 2.1.3. For obtaining serologically active polysaccharides, it was necessary to extract the cell walls at low temperatures (63). Ten percent trichloroacetic acid was used as the extractant. Purification of the polysaccharide was achieved by fractional precipitation with ethyl alcohol and by filtration through a Biogel P-30 column. A 2 mg sample of the polysaccharide was hydrolyzed in 0.2 ml of 0.1 N hydrochloric acid for 2 hr. This hydrolysate was analyzed for reducing sugars by paper chromatography. The group L polysaccharide was found to be composed of rhamnose, glucosamine, galactosamine and galactose in the ratio of 2:1:1:2. The amino sugars were most likely in the N-acetyl form in the native polysaccharide. Phosphate analyses on different preparations of the polysaccharide yielded an average value of 2% phosphorus.

Rabbit antiserum against the group L non-viable cells of the organism was prepared in our laboratory by the method outlined in section 3.1.3. The serum contained antibodies with specificity for the group L carbohydrate and was used for immunological studies.

Since low temperatures were needed in the extraction process, the compound was checked for heat labile linkages (64). A 2 mg sample of the polysaccharide was dissolved in 0.2 ml of 0.01 N hydrochloric acid in a tightly stoppered tube and heated in a boiling water bath. Aliquots of the hydrolysates were examined by paper chromatography initially and after heating for 5, 20 and 60 minutes for reducing sugars which were detected by silver nitrate reagent. Only N-acetyl-glucosamine was released from the polysaccharide by heating in the dilute acid indicating a N-acetyl-glucosamine to phosphate linkage in the polysaccharide.

Group L carbohydrate was used as inducer for the enzyme system in an aerobic *Bacillus* strain 2aSm (64,65). It was anticipated that this organism would elaborate enzymes which would hydrolyze certain linkages of the polysaccharide and possibly destroy serological activity. An active enzyme preparation was obtained when the *Bacillus* cells were grown aerobically on media containing 0.1% of the L polysaccharide. The enzyme preparation liberated inorganic phosphate and N-acetyl-glucosamine from the group L polysaccharide. Agar diffusion data in Table 7 show that both the

enzyme treated and the mild acid hydrolyzed polysaccharide were no longer antigenic with anti-group L serum.

TABLE 7

Precipitin tests with native and modified L carbohydrate and various streptococcal antisera.

Sample	Antiserum	Reaction
L carbohydrate	group L	+4
Acid hydrolyzed	group L	0
Acid hydrolyzed	group G	0
Enzyme treated	group G	+2
Enzyme treated	group L	0

The enzyme treated sample no longer yielded a positive precipitin test with anti L serum but did yield a precipitin complex with anti group G Streptococcal serum (66). Since the

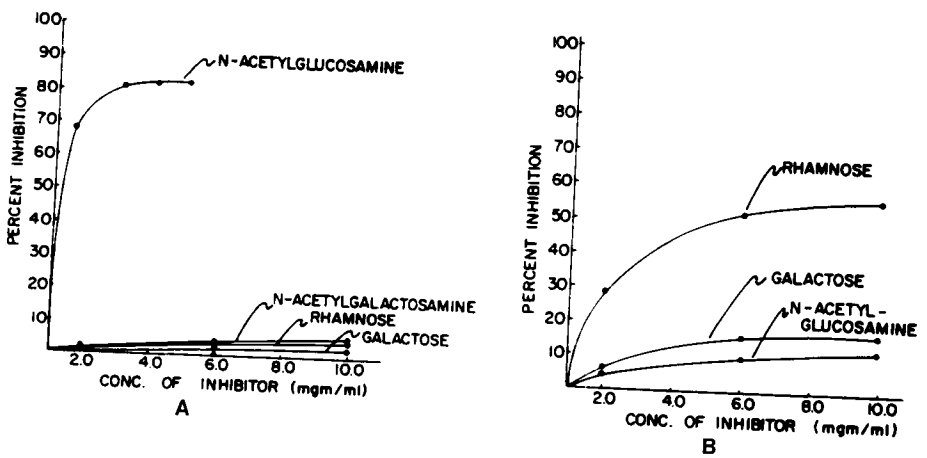


Fig. 19. A: Inhibition of quantitative precipitin reaction group L carbohydrate and serum with various monosaccharide inhibitors. B: Inhibition of precipitin reaction between enzyme-treated group L carbohydrate and group G antiserum with monosaccharides.

reactivity of the group G polysaccharide with its homologous antiserum is dependent upon terminal rhamnose units it was concluded that the N-acetyl-glucosaminyl-phosphoryl moieties are

linked to rhamnose units in the Group L polysaccharide. The immunodeterminant group of the L-carbohydrate is N-acetyl-glucosaminyl-phosphate. The inhibition data are presented in Fig. 19.

3.4 *Streptococcus mutans*.

Much research has been done on the agents involved in the formation of dental caries. *S. mutans* has been implicated as a causative agent. This organism produces an extra cellular polysaccharide (dextran) which adheres to the teeth. The deposit harbors other bacteria which may result in the development of plaques and dental caries (67). Antibodies against whole cells of *S. mutans* have been shown to inhibit the enzyme which produces the dextran (68,69). Methylation analysis studies in this laboratory have shown that the antigenic polysaccharide of the cell wall of *S. mutans* consists of L-rhamnose, D-glucose and D-galactose (70). The non viable cells of the organism stimulate the immune response to produce the specific antibodies.

3.4.1 Isolation of Cell Wall Polysaccharide. The cell wall polysaccharide of *S. mutans* was isolated from the cells by the KCl-HCl method described in Section 2.1.3. The carbohydrate polymers in the supernatants in the extract were precipitated by addition of 5 volumes of ethyl alcohol, collected by centrifugation and dried by lyophilization.

The monosaccharide constituents of the polysaccharide were determined by acid hydrolysis and paper chromatographic identification. The Rf values of the hydrolytic products from the polysaccharides and reference compounds were calculated and these were for the reference compounds: Rha (1.37), Glc (1.00), Gal (0.87), and for the *S. mutans* polysaccharide products Rha (1.38), Glc (0.98), Gal (0.86). Methylation analysis was also performed. GLC patterns for the methylated products are shown in Fig. 20.

It may be noted in this Figure that several rhamnose derivatives were produced from the polysaccharide, including a small amount of 2,3,4-tri-O-methylrhamnose derivative. Thus L-rhamnosyl residues of the polysaccharide are internal but a few are terminal residues. The D-galactosyl units are internal, but some of D-glucosyl units are internal and some are terminal. The polysaccharide activates the immune system to produce anti-Rha, anti-Glc and anti-Gal antibodies. These antibodies were isolated by affinity chromatography on Sepharose bearing the ligands of Rha,

Glc or Gal. The antibodies were eluted from the columns by rhamnose, glucose or galactose. The antibodies in the eluate were precipitated with ammonium sulfate collected by centrifugation and dried by lyophilization. The agar diffusion results with the polysaccharide are shown in Fig. 21.

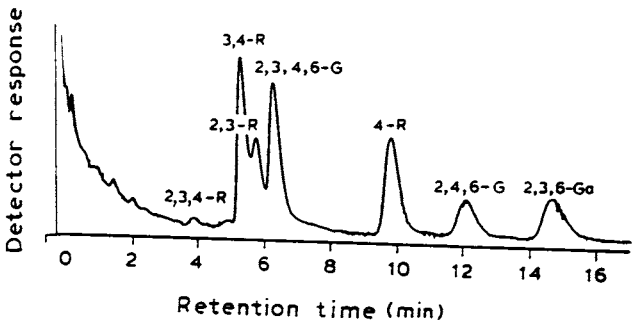


Fig. 20. GLC pattern for the methylated alditol acetates from the methylated polysaccharide preparation from *S. mutans*.

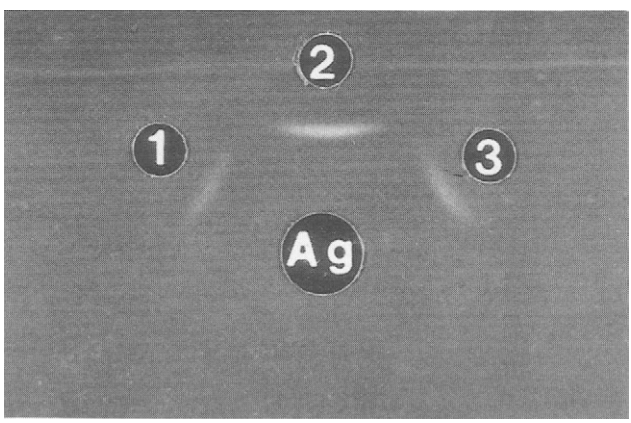


Fig. 21. Agar diffusion of polysaccharide preparation (Ag) from *S. mutans* and anti-rhamnose antibodies (1), anti-glucose antibodies (2), and anti-galactose antibodies (3).

3.5 *Streptococci* Group A

The group A Streptococcus is the chief pathogenic group in humans. Among the diseases for which this group may be a causative agent are: scarlet fever, endocarditis, erysipelas, puerperal fever, and rheumatic fever. *Streptococcus pyogenes* have been studied most extensively. Early work on this area was done by investigators at the Rockefeller Institute. The studies on virulent strains and intermediate strains of the organisms and the

relation to disease are excellent studies on group A and group C *Streptococci* (13).

3.5.1 Structure. The polysaccharides in the cell wall of group A strains were made up of rhamnose and N-acetyl glucosamine with the latter being the immunodeterminant group activating the immune system. The structure proposed for this polysaccharide is shown by Formula 5. More recent studies by periodate oxidation and methylation analysis have been published verifying the proposed structure of the polysaccharide (71,72). Table 8 contains methylation data and Table 9 periodate oxidation data. For comparative purposes it also contains data from the Group C polysaccharide.

TABLE 8

Relative amounts of the methylated monosaccharides determined as their alditol acetates obtained from streptococcal group-specific carbohydrates.

Compound	Group A	Group C
4-O-methylrhamnose	1.25	1.3
2,4-di-O-methylrhamnose	1.25	1.3
2,3,4-tri-O-methylrhamnose	Trace	Trace
3,4,6-tri-O-methyl-2-deoxy-2-N-methylacetamidoglucose	1	
3,4,6-tri-O-methyl-2-deoxy-2-N-methylacetamidogalactose		1.0
4,6-di-O-methyl-2-deoxy-2-N-methylacetamidogalactose		1.0

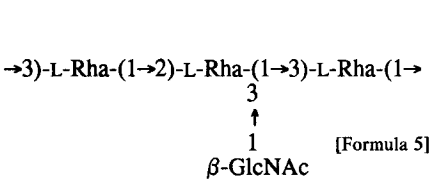
TABLE 9

Recovery of sugars after periodate oxidation of the streptococcal Group A and C carbohydrates.

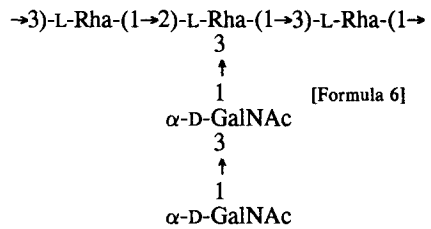
	Monosaccharide Present (moles)			
	Group A		Group C	
	Before $NaIO_4$	After $NaIO_4$	Before $NaIO_4$	After $NaIO_4$
N-acetylglucosamine	134	3.4	7.7	0
N-acetylgalactosamine			120	57.5
Rhamnose	365	390	292	288

The above periodate oxidation data and the methylation data confirm the structural repeating unit of Group A polysaccharide shown in Formula 5.

3.5.2 *Streptococci Group C*. The carbohydrate of Group C *streptococci* was prepared from cell walls utilizing digestion of an enzyme from *S. albus* (65) and a phage-associated lysin (73). The carbohydrate was isolated from both enzyme and phage digests of the cell walls by the same method and the samples were found to be identical. Chemical and serological analyses were performed on both samples. This information was used to propose the structure of Group C carbohydrate as shown in Formula 6 (72).



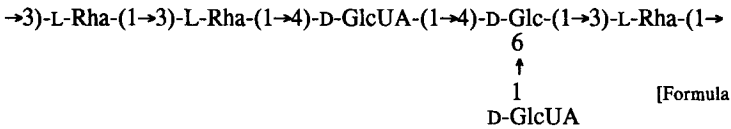
[Formula 5]



[Formula 6]

3.6 *Pneumococci*

The antigenic soluble substance of *Pneumococci* was the first substance of this type to be discovered being detected in filtrates in cultures of type II *Pneumococcus* (74). This substance was found to be present not only in the intact bacterial cell but also in the body fluids of the infected host. The substance yielded precipitins only with homologous immune sera. In subsequent and excellent work, Heidelberger and Avery (7) described the methods for the isolation as well as the chemical and immunological characterization of the specific substance from the organism. The soluble specific substance was shown to be a polysaccharide. This was the first demonstration that a polysaccharide functions as an antigen in the immune response. The early work is reviewed by Heidelberger (75). Structural studies have shown that the polysaccharide is composed of rhamnose, glucose and glucuronic acid (76). The proposed structure of the Type II polysaccharide of the group is shown in Formula 7).

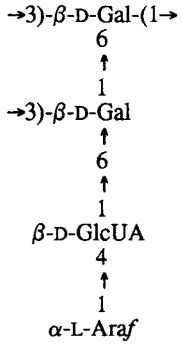


[Formula 7]

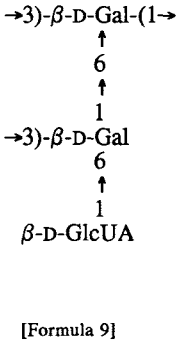
4. POLYSACCHARIDE GUMS

4.1 Gum Arabic

Gum arabic is isolated from the gum exudate from semi-tropical trees (*Acacia senegal*). It is a high molecular polysaccharide with unusual physical properties particularly high solubility, low viscosity and superior emulsification. Gum arabic consists of four monosaccharides, L-rhamnose, L-arabinose, D-galactose, and D-glucuronic acid, several types of glycosidic linkages and a unique arrangement of the sequence of carbohydrate residues (77). Since the fine structure of the gum molecule has not been completely elucidated (78) only the structure of the immunodeterminant groups are given in formulas 8 and 9. As a result when gum arabic is injected into animals, two sets of antibodies specific for the gum are produced. On the basis of methylation data, chemical degradations and hapten inhibitions of the precipitin reaction, structures for the two types of immunodeterminant groups have been deduced (5).



[Formula 8]



[Formula 9]

4.1.1 Preparation and Immunization. Gum arabic powder can be obtained from a number of commercial suppliers. The gum was purified by dissolving the powder in distilled water, separating the insoluble impurities by centrifugation and removing the soluble low-molecular weight impurities by dialysis. Following dialysis, the solutions of the gum were taken to dryness by lyophilization and the resulting residues were used for subsequent experiments.

To prepare a suspension for immunizing animals for producing antibody a concentrated solution of gum arabic was prepared by

dissolving 0.3 g of the gum in 2 ml of sterile phosphate buffer (0.02 M phosphate, pH 7.2) in saline. This solution was then mixed with an equal volume of Freund's complete adjuvant and used to immunize several rabbits. Samples of 0.4 ml of the gum-adjuvant suspension were injected intramuscularly in the hind leg of the rabbit and the injections were repeated weekly in alternate legs for 6 weeks. The animals were then allowed to rest for 2 weeks and the injection schedule was repeated. Several cycles of immunization were used to obtain serum of high titer. Blood samples were collected in the second and subsequent cycles and antisera were prepared from the samples by standard methods. An agar diffusion method was used for detecting precipitin formation and the results are shown in Figure 22. Two precipitin bands were obtained with the serum (Se) and the gum arabic (Ar) establishing that two different types of antibodies are synthesized in response to immunization by gum arabic. Reaction was not obtained with preimmune serum (P) and the gum (Ar).

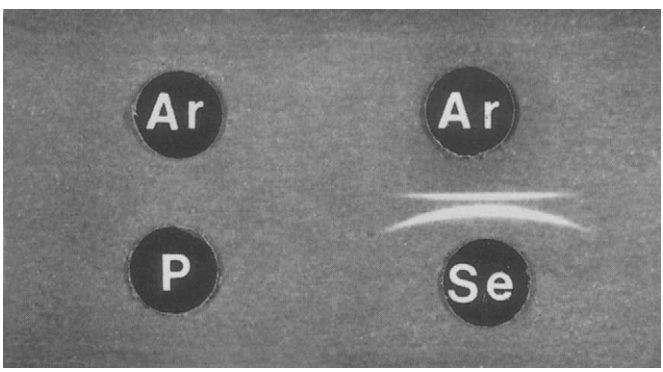


Fig. 22. Agar diffusion of anti gum arabic serum and gum arabic: P, pre-immune serum; S, immune serum; and Ar, gum arabic.

4.1.2 Evidence for Two Sets of Antibodies Specific for Gum Arabic. Evidence that the immune serum contained two types of antibodies specific for two different structural units of gum arabic is presented in Fig. 23. These results show that mild acid hydrolysis of gum arabic liberated arabinose and the resulting product did not form a precipitin band with one set of antibodies. Hydrolysis by concentrated acid liberated rhamnose, arabinose, galactose, and glucuronic acid as shown in Fig. 23. This treatment destroyed both types of precipitin forming abilities as seen in the diffusion plate of the figure.

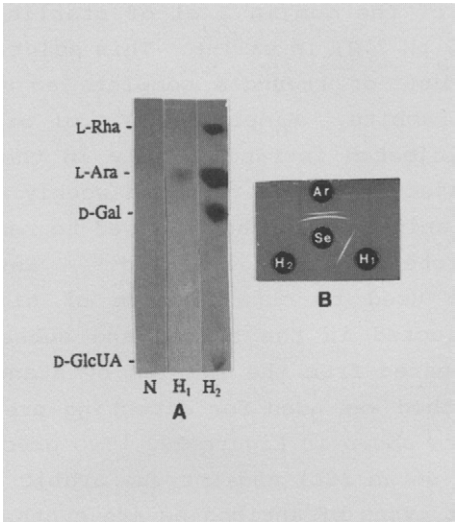


Fig. 23. Paper chromatograms of native (N) and hydrolysates of .01 N (H_1) and 1 N. (H_2) hydrolyzates of gum arabic (A) and the agar diffusion of these samples (B). Se, anti-gum arabic serum and Ar, gum arabic.

Terminal arabinose units in the native gum are in the furanose ring form (78) and these units are readily removed by dilute acid hydrolysis. Since one set of antibodies is no longer reactive with the gum, the arabinofuranosyl units are an essential part of the determinant group for antibodies of this set. In order to destroy completely the precipitin forming ability of gum arabic hydrolysis in concentrated acid was required.

Support data for the structures given for the immunodeterminant groups of gum arabic have been obtained by reduction and methylation analysis. Portions of the GLC methylation analysis charts for native and reduced gum arabic are reproduced in Fig. 24. In this analysis the major types of the methylated alditol acetates obtained from the reduced gum arabic (patternB) were: 1,3,4-triacetyl-2,5-dimethyl-arabinitol; 1,5-diacetyl-2,3,4,6-tetramethyl-glucitol; 1,5-diacetyl-2,3,4,6-tetramethylgalactitol; and 1,4,5-triacetyl-2,3,6-trimethyl-glucitol. The two glucosyl derivatives were not present in the patterns of the native gum arabic (pattern A). The trimethyl glucose derivative comes from the internal and reduced glucuronic acid residues of the gum and the tetramethyl glucose derivative

comes from the reduced terminal glucuronic acid residues. Thus the gum has glucuronic acid residues in terminal position and these residues participate in the immunodeterminant group of the gum.

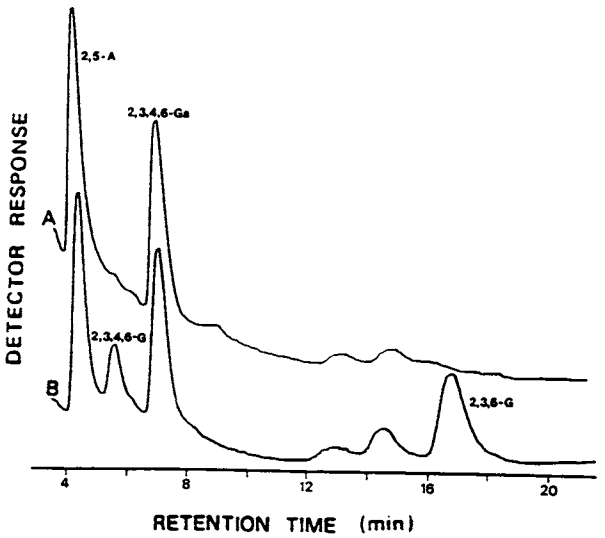


Fig. 24. Comparable portion of the GLC patterns for partially methylated and acetylated derivatives from native (A) and reduced (B) gum arabic. 2,5-A=1,3,4-triacetyl-2,5-dimethyl-arabinitol; 2,3,4,6-G=1,5-diacetyl-2,3,4,6-tetramethyl-glucitol; 2,3,4,6-Ga=1,5-diacetyl-2,3,4,6-tetramethyl-galactitol; 2,3,6-G=1,4,5-triacetyl-2,3,6-trimethyl-glucitol.

4.2 Gum Mesquite

Gum mesquite is composed of L-arabinose, D-galactose and 4-methyl D-glucuronic acid in the ratio of 5:3:2 but having a highly complex structure. The presence of the 4-methyl group in the glucuronic acid moiety of the native gum imparts unusual properties to the gum. There are considerable uncertainties about the complete structure of the gum (79). It has been proposed that the main chain consists of (1→6) linked galactose units and the side chains are composed of a disaccharide moiety of 4-methyl-glucuronic acid and galactose and a tetrasaccharide moiety of L arabinofuranose units. The gum was used to immunize rabbits and immune serum was obtained (5). Gum arabic and gum mesquite were found to have a common feature in reactivity with anti-arabic and anti-mesquite serum. The results are shown in Fig. 25. Gum mesquite yields only one precipitin band with homologous antiserum

and one precipitin band with anti-gum arabic serum. Therefore the two gums possess common immunodeterminant groups. This group is β-glucuronyl (1-6) galactose as evidently the methyl group of gum mesquite does not affect the immune response and the precipitin forming ability.

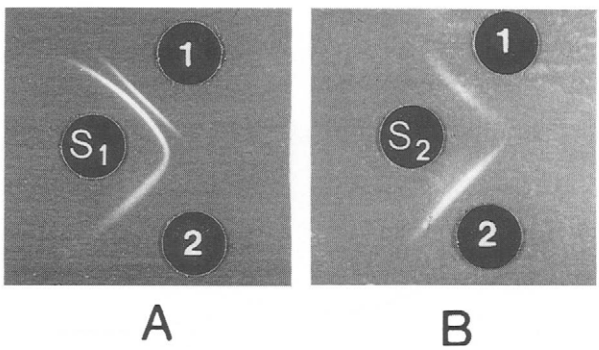


Fig. 25. Agar diffusion patterns for the anti-gum arabic serum (S_1) and anti-gum mesquite serum (S_2) against gum arabic (1) and gum mesquite (2).

4.2.1 Hapten Inhibition. The inhibition of reaction of gum mesquite with its antibodies was determined by the use of a micro inhibition method (80) and the oligosaccharide, β-D-glucuronic acid (1→6)-D-galactose. This oligosaccharide was isolated from an acid hydrolyzate of gum arabic. The disaccharide was a strong inhibitor of gum mesquite antibody reaction. The results of the test are shown in Fig. 26. The number of precipitin bands in the control is 5 and in digests with the oligosaccharide only 1 at the highest concentration of antigen. The oligosaccharide is a very effective inhibitor of anti-gum mesquite antibodies.

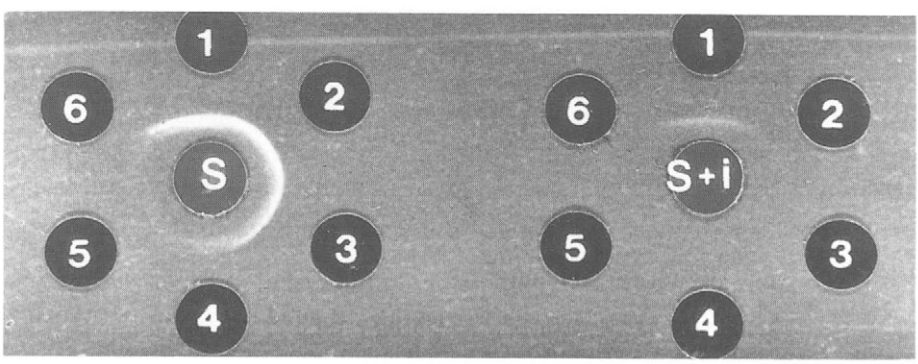
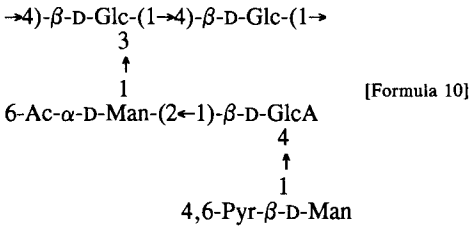


Fig. 26. Inhibition of precipitin formation between gum mesquite immune serum by disaccharide of glucuronic acid (1→6) galactose.

4.3 Xanthan

Xanthan is an exocellular polysaccharide produced by *Xanthomonas campestris* (81). The properties of this microbial polysaccharide are comparable to properties of plant gums. In structure, the polysaccharide is composed of a pentasaccharide repeating unit with a main chain of (1→4)-β-DGlc units and a trisaccharide side chain of mannose and glucuronic acid 4,6-pyruvate mannose units linked to the 3 position of alternating glucose residues of the main chain. The structure of the repeating unit of xanthan is shown in Formula 10 (82).



[Formula 10]

The polysaccharide is a large polymer with a molecular weight of several millions. As a result of the unique structure and the high molecular weight, xanthan has some remarkable rheological properties and can function as a thickening, stabilizing, and emulsifying agent in aqueous solutions. Based on these properties many uses for xanthan have been developed for food items, adhesives, pharmaceuticals, and personal care products (83).

4.3.1 Preparation of Xanthan. Xanthan prepared from *Xanthomonas campestris* by an aerobic submerged fermentation process involves the use of a multistep inoculum fermentation process, heat treatment of the final broth to kill the organism and recovery of the xanthan from the broth by alcohol precipitation. The alcohol is removed and the precipitate is collected and dried. The dried product is used for the characterization studies being reported.

4.3.2 Anti-Xanthan Antibodies. In order to test xanthan for antigenicity, rabbits were injected for possible antibody production by the method described in Section 3.1.3. Agar diffusion showed antibodies were produced. These antibodies were isolated by affinity chromatography (84). An affinity adsorbent of AH-Sepharose 4B with ligands of xanthan was also prepared (85). The adsorbent was transferred to glass columns (1x20 cm) and

equilibrated with 200 ml saline and 0.02% phosphate buffer of pH 7.2 and used for affinity chromatography.

The sample of the serum was applied to the adsorbent in the column and, after the adsorption of the sample, the column was washed with 0.02 M phosphate buffer pH 7.2 containing saline until the unadsorbed proteins were removed. The antibodies were then eluted with 1 M solution of ammonium thiocyanate and the eluate was monitored with a UV analyzer. The antibody was eluted as a single peak and was collected. This fraction was mixed with an equal volume of saturated ammonium sulfate. The precipitate which formed on refrigeration overnight was collected by centrifugation and dissolved in a small volume of phosphate buffer. Agar diffusion tests to detect antibodies were performed on this solution and are shown in Fig. 27. Also shown in the figure is the elution pattern obtained in the purification of the anti-xanthan antibodies.

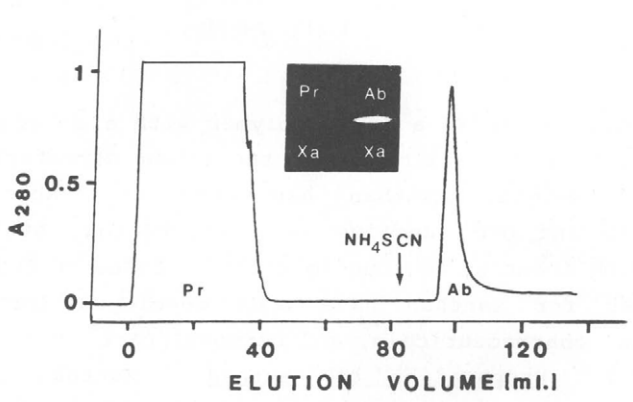


Fig. 27. An affinity chromatography pattern and agar diffusion of the fractions of the immune serum: Pr = protein, Ab = antibody, and Xa = xanthan.

4.3.3 Reduction of the Carboxyl Group of Glucuronic Acid. The glucuronic acid residues of xanthan gum were subjected to reduction of the carboxyl groups by using water-soluble carbodiimide and sodium borohydride according to a published procedure (58). One g of xanthan was reacted with 0.8 g of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate (CMC) for two hr at room temperature. At the end of the

reaction period, 0.8 g of sodium borohydride was added to reduce the product. The pH of this reduction mixture was allowed to rise to 7 and after the pH of the reaction mixture stabilized, the mixture was cooled to a temperature of 4°C and allowed to react for 16 hr. The excess borohydride was removed by dialysis. The reduced gum was recovered by lyophilization.

4.3.4 Periodate Oxidation. A sample of 50 mg of native xanthan was subjected to periodate oxidation (86). This sample was dissolved in 10 ml of 0.02 M sodium meta-periodate of pH 5. The oxidation was allowed to proceed at room temperature and in the dark for 24 hr. At this point the excess periodate was destroyed with ethylene glycol and the oxidation mixture was dialyzed for 24 h. The oxidized xanthan was recovered from the dialysis tubing and taken to dryness by lyophilization. The native and oxidized xanthan were tested for reactivity with the antibodies by agar diffusion. The results are shown in the next figure.

4.3.5 Deacetylation. A sample of 5 g of xanthan was deacetylated using the protocol of a published procedure (87). A 5% solution of polymer in 0.1% KCl was treated with 0.2 N KOH for 1 hr at 60°C with constant mixing. The solution was cooled to room temperature, neutralized with 0.05 N hydrochloric acid, and the product was recovered by precipitation with isopropanol. The recovered product was redissolved in 0.1% KCl, dialyzed for 24 hr, passed through ion exchange resins, and reprecipitated with isopropanol. The dried product was used for investigation.

4.3.6 Enzyme Treatment. α-Mannosidase was prepared from jack bean meal by extraction with phosphate buffer of pH 7.5 and recovered by alcohol precipitation. The enzyme solution hydrolyzed methyl α-D-mannoside at a high rate. Although the enzyme is an exo-enzyme it seemed possible that it was capable of hydrolyzing the α-glycosidic linkage joining the side chains to the main chain of xanthan. However, treatment of a solution of xanthan with the α-mannosidase preparation had no effect on the xanthan as no hydrolytic products were produced and no loss of antigenicity had occurred.

4.3.7 Depyruvylation. Depyruvylation of the xanthan polymer was performed by mixing the xanthan gum with 5 mM trifluoroacetic acid at 100°C for 1.5 hr (88). The solution was then dialyzed against water and passed through ion-exchange resins. The xanthan was precipitated with isopropanol and recovered by centrifugation.

The recovered product was dissolved in 0.1% KCl and reprecipitated with isopropanol. Several samples with different pyruvate content were prepared by varying the time of trifluroacetic acid treatment. The samples were hydrolyzed in 1N HCl and analyzed for pyruvate content (89). The analyses showed that the samples contained 6.0, 4.0, 2.8, 0.5, and 0.2% pyruvate. Solutions (0.5%) of these samples and standard xanthan were subjected to agar diffusion with anti-xanthan antibodies.

4.3.8 Micro Inhibition and Diffusion. A micro method combining inhibition and agar diffusion has been developed in this laboratory for detecting potential inhibitors of the antibody-antigen reactions (80). The method was used to determine whether glucuronic acid and mannose were inhibitors of the precipitin reaction of xanthan and antibodies. In these tests 0.1-5 mg of the potential inhibitor were dissolved in 20 μl of the appropriate antibody solution and incubated for 4 hr. At the end of this time, a 10-μl sample of the incubation mixture was placed in the center well of a diffusion plate. Antigen solutions (10 μl) containing decreasing amounts of the antigen (100 μg, 50 μg, 25 μg, 12 μg, 6 μg, and 3 μg) were placed in the outer wells of the agar plate. The plates were placed in a moist chamber and allowed to develop for 24 to 48 hr. The extent of inhibition was determined by comparing the intensity of the precipitin bands at the different concentrations of antigen on the inhibition plates with the intensity of bands on a plate with the antibody solution and antigen but no inhibitor. Some results are shown in Fig. 28.

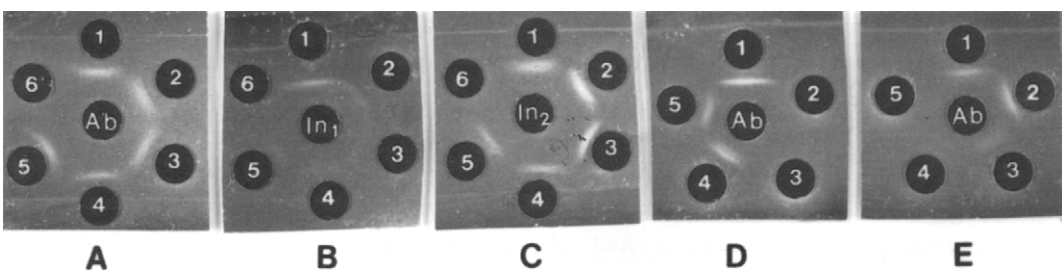


Fig. 28. Agar diffusion patterns of antibodies and of xanthan and chemical modified xanthan. Inhibition plate A, B and C: Wells 1 to 6 contain 100 μg, 50 μg, 25 μg, 12 μg, 6 μg, and 3 μg solutions of xanthan; In_1, antibody + glucuronic acid; In_2, antibody + mannose. Plate D: 1, xanthan, 2, xanthan reduced with borohydride, 3, xanthan oxidized with periodate, 4, deacetylated xanthan, 5, enzymically modified xanthan. Plate E: 1, xanthan with 4% pyruvate, 2, xanthan with 6% pyruvate, 3, xanthan with 2.8% pyruvate, 4, xanthan with 0.5% pyruvate, and 5, xanthan with 0.2% pyruvate.

Frame A of Fig. 28 shows the result with no inhibitor. Plate B shows the result with glucuronic acid and plate C with mannose. Only glucuronic acid was an inhibitor of the precipitin reaction. In frame D of Fig. 28 is shown the effects of chemical modification of xanthan on reactivity with Ab. Reduction of glucuronic acid and periodate oxidation of xanthan destroys reactivity but deacylation or enzyme treatment does not. In frame E it is noted that the pyruvate group is essential for precipitin formation. Depruvylation decreases the amount of precipitin formed and none is formed when pyruvate is less than 0.2%.

4.3.9 Analytical Applications. The utility of anti-xanthan antibodies as an analytical reagent is illustrated by the results in Fig. 29.

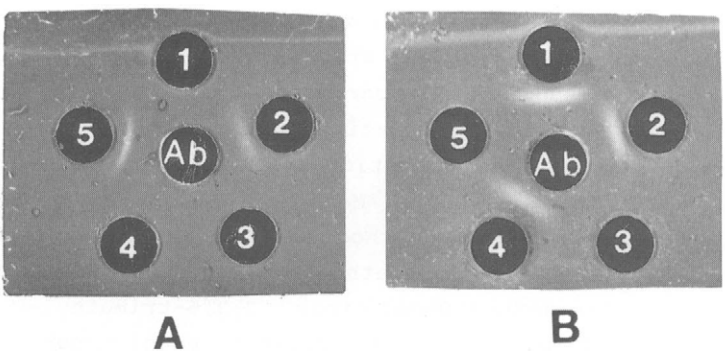
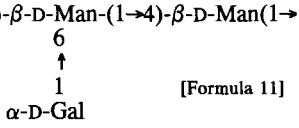


Fig. 29. Detection of xanthan in gum preparations and in food items by anti-xanthan antibodies and agar diffusion. Plate A: 1, blank, 2, xanthan, 3, sample A, 4, sample B, and 5, sample C. Plate B: 1, xanthan, 2, salad dressing, 3, salad dressing with other gum, 4, ice cream, 5, ice cream with other gums.

Plate A shows agar diffusion results with three commercial preparations of gum blends. The gum solutions were placed in wells 3, 4 and 5, standard xanthan solution in well 2 and a blank (H_2O) in well 1. It is apparent the sample in well 5 contains xanthan but samples in well 3 and well 4 do not. Plate B shows results of agar diffusion of antibodies with extracts of food items. These items include salad dressing containing xanthan (well 2), salad dressing with other gums (well 3), ice cream containing xanthan (well 4), ice cream with other gums (well 5) and (well 1) reference xanthan. The items containing xanthan are readily detected by the precipitin bands while the items containing other gums did not yield precipitin bands with the anti-xanthan antibodies. Some additional applications of the gum are listed (90).

4.4 Guaran

The structure determined for guaran is shown diagrammatically in Formula 11 (9).



[Formula 11]

4.4.1 Purification. Guaran was isolated and purified from guar flour which is available commercially. The flour is suspended in water and centrifuged at 13,000 g to remove insoluble material. Low molecular compounds are removed by dialysis against distilled water for 24 hr. The solution was then filtered and taken to dryness by lyophilization. The dried product was readily soluble in water and was used in the experiments.

4.4.2 Structure. Information concerning the structure of this gum was obtained by methylation analyses, periodate oxidation and x-ray diffraction (91,92). Methylation was performed by the disulfate sodium hydroxide method and by the micro method as outlined in section 3.2. The methylated products from the guaran were 2,3,4,6-tetra-methyl-D-galactose, 2,3,6-trimethyl-D-mannose and 2,3-dimethyl-D-mannose in approximately equal amounts. A GLC pattern is reproduced in Fig. 30.

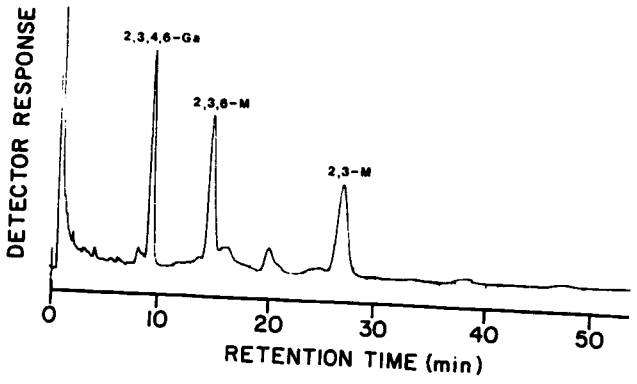


Fig. 30. Photograph of methylated alditol acetates from native guaran.

The molecule of guaran consists of a main chain of D-mannose units of which on an average every other one bears a single D-galactose unit as a side chain. From the x-ray data it was deduced that the main chain units are joined by β-(1→4) linkages. From the methylation data the position of the linkages was deduced.

4.4.3 Immunization. Antibodies against guaran were obtained by immunization of rabbits with a suspension of the gum and CFA. The enzyme galactose oxidase has specificity for terminal galactose units. Oxidation of guaran by this enzyme destroys the precipitin reaction of guaran and antibodies. These results are shown in Fig. 31. Thus side chains of terminal galactose units are the immunodeterminant groups of guaran.

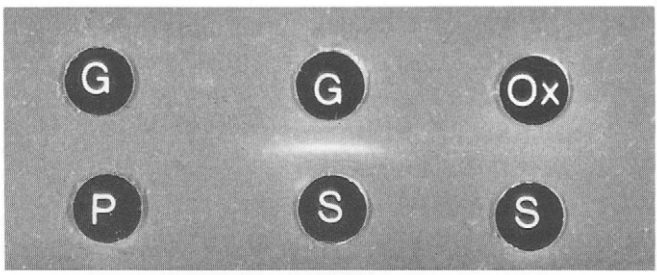


Fig. 31. Agar diffusion of guaran and anti-guaran serum, G, guaran, Ox, guaran oxidized with galactose oxidase, P, pre-immune serum, S, immune serum.

4.4.4 Periodate Oxidation. Periodate oxidation of guaran was conducted by dissolving 50 mg in water, then stirring and sonicating if necessary. The solution was pre-cooled to 4°C and mixed with an equal volume of precooled 0.04 M sodium metaperiodate. The pH of the mixture was adjusted to 4.5 and the reaction was allowed to proceed in the dark for 50 hr at 4°C. The excess of periodate was decomposed by adding ethylene glycol. The solutions were then neutralized, dialyzed against distilled water for 48 hr, and lyophilized.

A sample of the periodate oxidized guaran was subjected to methylation analysis (Section 3.1.5) and another sample of oxidized gum was subjected to reduction (Section 3.1.7) and then methylated (86). The GLC patterns of the products are reproduced in A and B of Fig. 32.

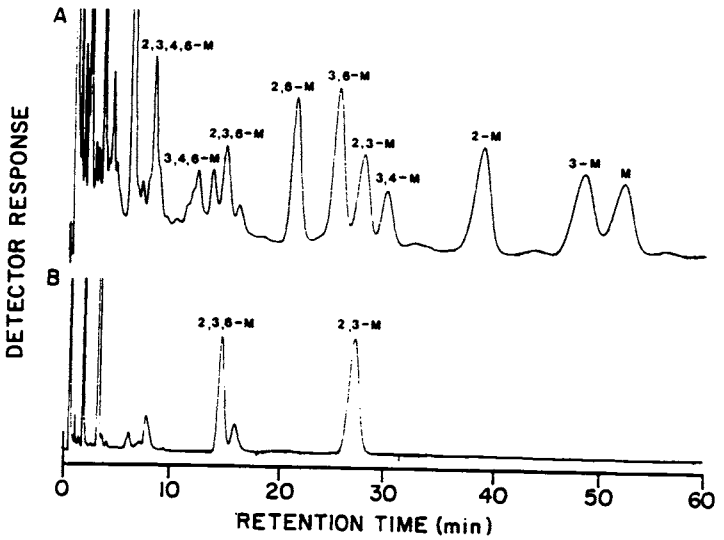


Fig. 32. Photograph of GLC patterns of the methylated alditol acetates from periodate oxidized (A) and oxidized borohydride reduced (B) guaran.

The identity of the methylated products was verified by mass spectrometry. Quantitative values for the products were obtained by integration of the area under the peaks. These values in molar ratios are shown and retention times are recorded in Table 10.

TABLE 10

The monosaccharide derivatives in acid hydrolyzates of methylated native and oxidized guaran: A = Native; B = periodate-oxidized

Hexoses and location of methyl groups	Rt	Mole percentage	
		A	B
2,3,4,6-Man	0.99		6
2,3,4,6-Gal	1.14	32	
2,3,6-Man	1.88	34	7
2,6-Man	2.72		14
3,6-Man	3.19		17
2,3-Man	3.54	34	9
3,4-Man	3.87		5
2-Man	5.01		15
3-Man	6.23		14
Man	6.67		13

The products from native guaran were 2,3,4,6-tetramethyl galactose, 2,3-dimethyl and 2,3,6-trimethyl mannose identified as the alditol acetates. Of significance is the finding that the periodate oxidized guaran yielded numerous GLC peaks on methylation analysis. The five major peaks were identified as the alditol acetates of 2,6-dimethyl-Man, 3,6-dimethyl-Man, 2-methyl-Man, 3-methyl-Man, and mannitol, and the minor peaks were identified as derivatives of 2,3,4,6-tetramethyl-Man, 2,3,6-trimethyl-Man, 2,3-dimethyl-Man, and 3,4-dimethyl-Man. The new derivatives arise from residues protected by hemiacetal bonds. Such bonds are formed during the oxidation of methyl lactoside (93). In guaran it can be calculated that about 80% of the unoxidized mannose residues were protected against oxidation by formation of hemiacetal bonds. The remaining 20% of the mannose derivatives were composed of residues that survived the oxidation even though not protected. The protection of oxidation of mannose was by hemi acetal bonds from the aldehydic group generated by the oxidation of mannose residues to free hydroxyl groups of the adjacent mannose residues.

4.4.5 Hemiacetal Bond Formation. A typical segment of a guaran molecule showing the oxidized residues and the resulting hemiacetal bonding types is drawn in Fig. 33. In constructing such

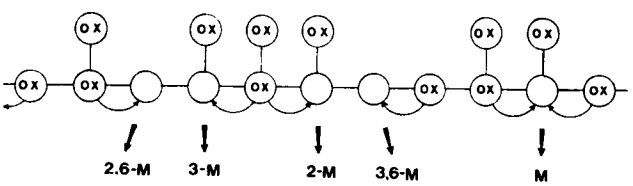


Fig. 33. Diagram of the structure of a segment of a guaran molecule: top row of circles, galactosyl residues, bottom row of circles, mannosyl residues, OX, oxidized residues, open circles non-oxidized mannose residues, vertical lines, (1→6) linkages, horizontal lines, (1→4) linkages, arrows, hemiacetal bonds.

a model sequence, the participation of oxidized galactose residues in inter-residue hemiacetal formation is considered unlikely for stearic reasons. Also, previous kinetic studies of periodate uptake in guaran have shown that the galactose side groups are oxidized rapidly and independently from the mannose chain. In the case of the oxidized mannose residues, hemiacetal bonds can be

formed with the aldehyde groups and the available hydroxyl groups on adjacent unoxidized residues to yield the 6 membered structures which are stable. It is also assumed that oxidized unbranched residues tend to participate in only one inter-residue hemiacetal, due to the possible formation of the more stable intra-molecular hemiacetals.

4.5 Gum Linseed (mucilage)

Gum linseed consists of several types of polysaccharides. The monomeric residues of these polysaccharides have been identified as L-rhamnose, L-galactose, L-fucose, D-xylose, and D-galacturonic acid (94). Extraction of seeds with cold water is a satisfactory method for obtaining the gums (30). Linseeds are suspended in water and soaked for 24 hr. The liquid is squeezed from the seed through fine linen, and the process then repeated until very little gum could be obtained from the extract. The extracts so obtained contained a small amount of flocculent matter which was allowed to settle and the gum in the supernatent was decanted therefrom. The supernatant was then treated with phosphotungstic acid solution and sulphuric acid to precipitate any impurities. The gum solution was then treated with twice its volume of 90 percent alcohol and the gum was obtained as a white fibrous mass, which was dehydrated with absolute alcohol, washed with ether and finally dried in vacuo.

Linseed polysaccharide has been separated into neutral and acidic polymers by cetyltrimethyl ammonium bromide (95,96). The acidic fraction was further separated into two fractions by the use of cupric acetate solution. Methylation of the neutral fraction followed by hydrolysis yielded 2,3,4-tri-O-methyl-D-xylose, 2,3-di-O-methyl-D-xylose, 2,4-di-O-methyl-D-xylose, and 4-O-methyl-D-xylose. The earlier study had shown that the neutral fraction is branched. One fraction of acidic polysaccharides contained L-rhamnose, L-galactose, and D-galacturonic acid. The second acidic fraction consisted of L-rhamnose, L-fucose, L-galactose, and D-galacturonic acid. The methylation analysis of this reduced fraction gave on hydrolysis high amounts of 2,3,4,6-tetra-O-methyl-D-galactose, arising from the reduced galacturonic acid. Periodate oxidation indicated a highly branched structure for the acidic fraction.

The mixture of polysaccharides was used in a vaccine for immunizing rabbits for antibody production by the method outlined in section 3.1.3. The serum was tested for precipitin formation

with the mixture of polysaccharides. The results are shown on a plate A in Figure 34. The agar diffusion pattern shows that the amount of antibody increased with the number of immunizations. The figure also shows that two sets of antibodies are produced. These sets can be isolated by affinity chromatography. An affinity absorbent of linseed polysaccharide-Sepharose was prepared and used to isolate the two types of antibodies. Anti-xylose antibodies and anti-galacturonic acid antibodies were obtained from the immune serum by selective elution with xylose and then with galacturonic acid (97). The affinity technique described in Section 4.3.2 was used. The agar diffusion figure shows that both of these antibodies were obtained in purified form. This represents the first isolation of anti-xylose and anti-galacturonic acid antibodies.

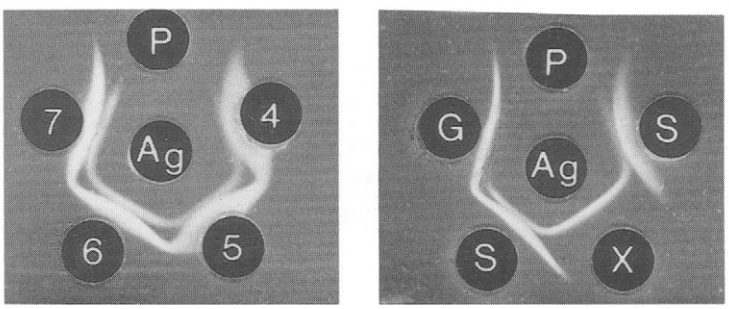


Fig. 34. The agar diffusion results in Frame A of the figure show an increase in antibody from the 4th to the 7th week of immunization. The results in Frame B of the purified anti-xylose antibodies (X) and the purified anti-galacturonic antibodies (G).

5. SUMMARY

Novel polysaccharides composed of two, three or four types of monosaccharide residues (D-glucose, D-galactose, D-mannose, L-rhamnose, 6-deoxy-L-talose, D-glucuronic acid, D-galacturonic acid, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, D-xylose or L-arabinose) have been isolated and characterized from microorganisms and plants. The isolation was performed by extraction of microbial cell walls or plant seeds with 10% trichloroacetic acid, dilute KCl-HCl solution of pH 2, formamide, or distilled water at various temperatures. The purification was by fractional precipitation with acetone and ethyl alcohol and by gel filtration.

The characterization procedures for the polysaccharides were paper chromatography, methylation, gas liquid chromatography, mass spectrometry, enzymatic hydrolysis, periodate oxidation, chromium trioxide oxidation, acetolysis, β-elimination, and hapten inhibition. The antigenicity of the polysaccharides was determined by immunization of laboratory animals and isolating antibodies with specificity for carbohydrate residues of the polysaccharides by affinity chromatography methods.

Comments on some interesting polysaccharides that were obtained follow. Two of the polysaccharides contain phosphate as integral units of the molecular structure. The phosphate occurs as β-D-glucose-1-phosphate or N-acetyl-β-D-glucosamine -1-phosphate as side chains of the polysaccharides. These units function as the immunodeterminants in the immunological response. Another polysaccharide of D-glucose and D-galactose possesses many lactose units as side-chains on a main chain of glucose and galactose and the lactose units are the immunodeterminants. On immunization of animals with this polysaccharide anti-lactose antibodies are produced. A tetraheteropolysaccharide of D-galactose, D-glucuronic acid, L-rhamnose and a rare sugar 6-deoxy-L-talose was purified. The biosynthesis of the latter sugar has been shown to proceed by the thymidine sugar pathway (33).

The antigenic polysaccharides from gums have useful properties such as high viscosity, gel forming ability and stabilizing of suspensions. Included in this class are gum arabic, gum mesquite, gum xanthan, gum guaran and gum linseed. Of interest is the finding that gum arabic possesses two immunodeterminant groups of arabinosyl-glucuronic acid and glucuronsyl-galactose structures. Two sets of antibodies are synthesized on immunization of animals with this gum. Gum mesquite has only the glucuronsyl-galactose as an immunodeterminant but the glucuronic acid bears a methyl group on position 4. The gum induces the synthesis of one set of antibodies which also react with gum arabic. Gum xanthan possesses a unique immunodeterminant group which is a mannosyl-glucuronic moiety but the mannose bears a pyruvate moiety. The pyruvate is linked by a ketal linkage at positions 4 and 6 to the mannose. Gum guaran has many galactose units as side chains attached to the main chain of mannose residues. The galactose units activate the immune system to produce anti-galactose antibodies. Gum linseed consists of several interesting polysaccharides and has been used recently

to immunize rabbits. One polysaccharide has many terminal xylose units and another has many terminal galacturonic acid units. Two types of antibodies, anti-xylose and anti-galacturonic acid, were produced when the gums were used to immunize the animals. The antibodies have been purified by affinity chromatography. The anti-xylose antibodies and anti-galacturonic acid antibodies have been prepared for the first time.

The polysaccharides have been used to prepare many anti-carbohydrate antibodies and these have specificity for glucose, galactose, glucuronic acid, rhamnose, glucose-1-phosphate, N-acetyl-glucosamine-1-phosphate, and oligosaccharides of glucose, galactose, glucuronic acid 4,6-pyruvyl-mannose, arabinose, N-acetyl-glucosamine and N-acetyl-galactosamine. Such anti-carbohydrate antibodies will likely have value for the detection and identification of glycoproteins and glycolipids in normal and diseased tissues. A program in diagnostic testing with the purified anti-carbohydrate antibodies should be initiated to develop immunological methods for the early detection of diseases.

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The Dereplication of Plant-Derived Natural Products

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INTRODUCTION

It has been conservatively estimated that the number of flowering plants on the planet Earth is 250,000 (1). From the small percentage of these species that has been evaluated for their medicinal and biological potential, the number of therapeutically useful compounds isolated from plants and currently used is about 120 (2). Even though medicinal chemists can modify established structures in order to increase efficacy and bioavailability and/or reduce toxicity, we still look to the chemical diversity of nature to provide us with novel, active structural types (lead compounds) to provide pharmacophores for optimization.

There have been many approaches described in order to facilitate the discovery of biological and medicinal agents from natural sources. The approaches to the discovery of bioactive plant secondary metabolites that have been and are still being used include the ethnomedical, the chemotaxonomical/taxonomical, the "random" collection, the information based approach, and serendipity (3-5).

The ethnomedical approach to plant collection is where information on the global indigenous uses, mode of preparation, and on the type of disease treated is acquired, analyzed and prioritized. Those plants of high priority are collected and then evaluated in a number of assays related to the disease state. Since it has been estimated that nearly 65% of the world's population relies on traditional medicinal practices for their primary health care needs, the extensive experience on a day-to-day basis of this large group of people should provide valuable insights into the primary evaluation of plants for natural product drug discovery (2).

The chemotaxonomical/taxonomical approach to plant collection relies on the premise that related taxa have inherited the genetic ability to produce similar secondary plant metabolites. The chemotaxonomic/taxonomic approach is therefore of varying utility depending on programatic need. If the purpose of the natural product drug discovery process is to obtain novel biologically active compounds, this process will probably afford compounds that are very similar in structure, albeit new, to known, active natural products. Thus, the chemical diversity in this instance may provide important information concerning structure-activity relationships regarding efficacy and toxicity. The alkaloids of *Rauvolfia* are an example of this search strategy. Reserpine was discovered from *Rauvolfia serpentina* (Apocynaceae) and was introduced as a therapeutic agent for hypertension and as a tranquilizer. With a successful drug discovered, pharmaceutical companies focused their efforts looking for analogs of reserpine in this and related genera in the Apocynaceae. The results of this process were many novel indole alkaloids, and two, rescinnamine and deserpidine, were actually marketed for similar indications to those already being treated with reserpine (6).

The "random" collection approach is where all plants within a geographic area are collected and are evaluated for a broad spectrum of biological activities. The random collection approach is most often used when financial resources are not a primary limiting factor and the biological screens have high throughput. An example of this approach is the National Cancer Institute human cancer cell-line panel of sixty cell lines and HIV screen, where extracts of all samples are analyzed for cytotoxicity and anti-HIV activity (7, 8). As discussed elsewhere (4, 9), many pharmaceutical companies are using this approach to create genetic banks of natural chemical diversity for now and for the future.

The information-based approach to plant collection is predicated on the acquisition of a prioritized list of plants from lists of plants that i) have ethnomedical information related to a specific disease profile, ii) have been reported active *in vivo, in vitro,* or to possess other biological activity relevant to the selected disease, iii) contain known natural products which show activity in one or more of the "relevant" assays. Plants that have a recorded ethnomedical use, activity *in vivo, in vitro,* or in other biologically relevant studies, and no previously isolated compounds responsible for the biological activity are acquired for subsequent analysis (5, 10).

The last approach, serendipity, is not a systematic approach and relies on the keen observations of an individual scientist or group of scientists. The discovery of vinblastine as an antineoplastic agent on the laboratory investigation on a plant reputedly used as an antihyperglycemic agent is an example (11).

Following collection and identification of the organism, the starting point of most natural product drug discovery programs is the screening of organic or aqueous extracts using an array of bioassays. After such primary screening, those extracts which show a strong and reproducible activity may be subjected to bioassay-guided fractionation. Bioassay-guided fractionation is the procedure whereby the extract is chromatographically fractionated and refractionated until a pure, biologically active compound is isolated. Each fraction that is produced during the fractionation process is evaluated in a bioassay system and only those fractions that are active are refractionated. At this point, there are three possibilities. The first possibility is that the compound is known and has been previously found to be active. The second is that the compound is known, but has not been found to be active in the particular bioassay being used. The third possibility is that the active compound is novel. In the majority of cases, the first two possibilities prevail. However, it is the latter instance which is usually preferred from a drug discovery and development perspective.

The "hit-rate", the number of active samples generated as a result of biological evaluation, is usually in excess of the research facility's capacity to handle them expeditiously. With the present technology, the rate-limiting step is not the selection of plant material nor is it the collection, extraction, and biological evaluation of the plant material, but rather it is the bioassay-guided fractionation stage (5). Depending on the definition of what is active, there may be many extracts that are subsequently chosen for the time-consuming process of bioassay-guided fractionation and structure elucidation. However, only a few extracts can be studied at one time by a given bench investigator. If the bioassay-guided fractionation and subsequent structure elucidation results in the re-isolation of known biologically active compounds, then time spent is wasted, since novelty of structure is widely regarded as a prerequisite to establishing a strong patent position (9).

In an effort to enhance efficiency, many natural product drug discovery groups have developed strategies to decrease the number of extracts entering the fractionation stage or to decrease the probability of re-isolating known, biologically active natural products. In some cases, these strategies involve submitting biologically active extracts to a secondary screening process in which additional biological information on the extract is obtained in order to increase or decrease the level of interest in the extract (12). If an information-based approach is used, the details of any ethnomedical, *in vivo* or *in vitro* data, or the chemical information on the organism or its genus can be used to increase or decrease the level of interest at this point. In theory, previous, related, biological or ethnomedical observations may strengthen interest, while previously published chemical isolation data may detract from its importance by suggesting that the activity is due to a known compound.

Although important, the information obtained from the previously described dereplication schemes typically were too general in nature (substance classes or functional group information) and could not be used to compare with a set of known compounds. For the biologically based dereplication schemes, the information obtained can not be used reliably to identify specific known compounds in a natural product extract. With these serious limitations to these basic approaches, we describe here a strategy which combines aspects of several available technologies to develop a more accurate and meaningful approach to natural product dereplication.

A simple method to obtain minimal, significant, mass spectral information has been developed in the last two decades with the advent of the electrospray ionization (ESI) interface, among others (19). The first application of electrospraying solutions into a mass spectrometer date back to 1968 when Dole and coworkers investigated dissolved macromolecules at atmospheric pressure and obtained intact, high molecular weight ions (20). Electrospraying solutions into a vacuum was then investigated by Evans and coworkers and was called electrohydrodynamic ionization (EHI) (21). Iribarne and Thomson investigated the process of the emission of ions from liquid droplets, and this greatly contributed to the understanding of the mechanism of gas-phase ion formation using a pneumatically nebulized electrospray interface (22). Recognizing the early experiments of Dole and Iribarne and Thomson, Yamashita and Fenn refined the electrospray interface design (23). The drawback of the older designs was that only low flow rates could be utilized. In order for higher flow rates to be used, the electrospray interface was combined with pneumatic nebulization to afford the newer interface called ionspray (24). The present commercially available electrospray interfaces have a sheath gas, which acts similarly to the pneumatic nebulization of the ionspray, in order for higher flow rates to be used.

Several mass spectrometric techniques have been used to analyze natural products. One important technique that has been used more recently with many compound classes, with much success, has been thermospray ionization mass spectrometry (TSP-MS). Some of the classes which have been analyzed include flavonoids, alkaloids, and terpenes, and several reviews have appeared on the importance of MS, and especially TSP-MS, to natural product chemistry (25-27). There are many parameters that need to be addressed in optimizing TSP-MS, including the mobile phase composition, the temperature of the vaporizer and ion source, and the position and potential of the ion source. The temperature of the vaporizer and the ion source particularly affect the appearance of the TSP spectrum.

Electrospray ionization has some substantial advantages over other ionizing methods. For example, there are no liquid matrices used, such as glycerol in continuous-flow fast atom

bombardment (CF-FAB) and matrix-assisted laser desorption ionization (MALDI), consequently no extraneous ions are seen in the detector. The difference between TSP and ESI is in the design of the interface. Using the TSP interface, the source and vaporizer temperatures greatly affect the spectrum, and both temperatures are compound dependent. Increasing the source temperature, increases the amount of fragment ions seen in the spectrum (26,28). The vaporizer temperature also has similar effects on the spectra of compounds (29). One of two disadvantages with the use of TSP as a general tool in the analysis of extracts is that there are many types of compounds in an extract and that due to compound temperature dependence, broad screening of a mixture of structural types can be difficult (26). The second disadvantage is in the pseudomolecular ion formation. Using the TSP interface, the ions observed are sometimes in the protonated $[M+H]^+$ or deprotonated $[M-H]^-$ forms, but can also appear as $[M+NH_4]^+$ or $[M+NH_4-H_2O]^+$ adducts, and all or some of these ions can be seen in the mass spectrum of a compound simultaneously (26). ESI and TSP, are both considered soft-ionization techniques, where the ions observed are usually protonated or deprotonated with little fragmentation, unlike electron-impact ionization. Thus, the primary information that can be obtained using an electrospray mass spectrometer is molecular weight information, and it is this information which can be used for comparison with a database of natural products. Using a combined liquid chromatography/mass spectrometry system with an electrospray ionization source (ESI LC/MS), one could therefore crudely separate an extract into its constituents and obtain minimal, structural information (UV and mass) and then use that information to correlate with a database of known natural products.

The second element to successfully dereplicate natural products is a comparison of the structural information obtained from the analysis of an extract with information in the natural product literature, which is potentially vast and very widely distributed. The literature holds chemical information and pharmacological information pertaining to compounds and extracts, and chemotaxonomic information. In order to quickly and efficiently compare information obtained from the analysis of natural product extracts, the use of databases which comprise abstracted information from the literature can be used. Available databases include Natural Products Alert (NAPRALERT) (30), among others (14), all accessed through the Science and Technology Network (STN) created by the American Chemical Society through its Chemical Abstracts Service. A general method for the dereplication of moderately lipophilic extracts using an ESI LC/MS system and the NAPRALERT database that is very efficient has been published (31). Before discussing this procedure in more detail, the history of the development of the chemically and biologically based dereplication strategies will be described.

Chemically Based Dereplication Strategies

There have been many types of chemically based dereplication strategies published over the decades. The acceptance of these "chemical screening" strategies was heightened when simple and stable chromatographic techniques were developed. The major chromatographic technique used was thin-layer chromatography (TLC). A second development in chemically based strategies came in the early 1970s when computers were introduced that could handle large amounts of searchable data. Originally, researchers desired a method to obtain information on the chemical classes of compounds in a plant extract. One way of obtaining chemical class information was to use chemical reaction tests.

The earliest type of screening procedure was the approach initiated by researchers in the latter part of the 19th century and the early part of this century called phytochemical screening. Phytochemical screening of plants is the process where information on the structural classes of natural products present in a plant extract is obtained, usually through specific color reactions with selected chemical reagents. Reviews on the types of chemical reagents used for different classes of compounds have been published previously (15,16,32). Some of the chemical classes that may be screened for include alkaloids, polyphenols, cardiac glycosides, saponins, and flavonoids. The drawback of many of the chemical reactions that were used in large-scale phytochemical screening programs were that they were non-specific for the compound class, thereby yielding numerous false-positives, or in some cases, false-negatives, or that they were not sensitive enough to detect small amounts of particular compound classes in plant material (15).

The advent of paper, and more notably of thin-layer, chromatographic techniques (17,33) provided researchers with rapid visualization techniques for the separation and identification of chemical classes of natural products. Many different chromogenic reagents have been developed for the analysis of the presence of different classes of plant secondary metabolites and many reviews on the specificity and sensitivity of these reagents have been published (16,17,33,34). The identification of the chemical class of a natural product in a plant extract before subsequent isolation and identification is of particular use if the goal is to find compounds of a certain chemical class. However, it is of limited use if the goal is to find novel, biologically active compounds independent of chemical class.

Many different TLC systems and extraction schemes have been employed for phytochemical screening. One system that has been published is the system described in detail by Marini-Bettòlo and collaborators in 1981 (16). The extraction system consists of extracting the plant material with solvents of increasing polarity to afford five extracts from each plant. The extracts are then

submitted to two to four TLC solvent systems using silica gel or cellulose plates (Table 1). The plates are then dried and sprayed with a variety of spray reagents (Table 2).

Table 1. Extraction Scheme and Solvent Systems Used in Chemical Screening[1]

Extraction Scheme		
Not Used	light pet. ether extract (50°C)	
Extract A	chloroform/acetic acid (99:1) (50°C)	
Extract B	methanol/chloroform/acetic acid (49.5:49.5:1) (50°C)	
Extract C	methanol/water (1:1) (50°C)	
Alkaloid extraction: Dilute (1%) HCl (40°C), check with Dragendorff's reagent, if positive, then adjust to pH 9 and extract with chloroform. Chloroform phase is extracted with 1% HCl. The chloroform phase is extract D and the aqueous phase is E.		
Solvent System	**Solid Phase**	**Extract Used**
Toluene/chloroform (9:11)	Silica	A
Toluene/acetone/chloroform (40:25:35)	Silica	A
n-Butanol/acetic acid/water (4:1:5-upper phase)	Silica	B,C
n-Butanol/acetic acid/water (4:1:5-upper phase)	Cellulose	C,E
2-Butanone/toluene/methanol/acetic acid/water (80:10:5:2:6)	Silica	B
Ethyl acetate/2-butanone/formic acid/water (5:3:1:1)	Silica	B
Chloroform/acetic acid/water (50:45:5)	Silica	B
Ethyl acetate/pyridine/water (5:1:4)	Cellulose	C,E
Cyclohexane/diethylamine (9:1)	Silica	D
t-Butanol/chloroform/diethylamine (2:7:1)	Silica	D

[1]See Ref. 16.

Table 2. TLC Spray Reagents Used in Chemical Screening[1]

Spray Reagent	**Chemical Class**	**Color Reaction**
Antimony (III) chloride	Flavonoids/steroids	Fluorescence,UV
Diphenylboric acid-β-aminoethyl ester	Flavonoids	Fluorescence,UV
Fast Blue B Salt and NaOH	Phenols, tannins, flavonoids	Various colors
$K_3Fe(CN)_6$ - $FeCl_3$	Phenols, tannins, flavonoids	Blue color
Acetic anhydride/H_2SO_4 (Liebermann-Burchard)	Steroids, saponins	Dark spots/fluorescence
Trifluoroacetic acid and heat	Steroids, saponins	Dark spots
Dragendorff's Reagent	Alkaloids	Reddish brown color
3,5-Dinitrobenzoic acid-KOH (Kedde Reagent)	Cardenolides	Blue-violet color
KOH in methanol	Coumarins, anthranoids, phenols	Fluorescence
Magnesium acetate and heat	Anthraquinoids	Orange-violet color

[1]See Ref. 16.

A number of TLC systems and chromogenic spray reagents have been used in the field of microbial research since the early 1960s, and these techniques have been extensively used in the analysis of new microbial compounds (35).

Three groups have been primarily responsible for developing dereplication methods using TLC and spray reagents to find novel microbial compounds. These groups were lead by Umezawa in Japan (36-38), Aszalos in the United States (35,40), and Wink and Zeeck in Germany (18, 40-48). Tables 3 and 4 summarize the TLC solvent systems, and the spray reagents used by the research groups in Japan and Germany. Each group has used different spray reagents and solvent systems, but the premise is the same. Namely, to use TLC and a variety of spray reagents in order to search for newly appearing metabolites that are not constituents of the media and are not frequently formed microbial products. The goal is to find metabolites which are unique to the screened microbial isolate and which would therefore merit further attention (18). This type of screening can be conducted either independently or in consideration of biological activity.

Table 3. TLC Solvent Systems Used in Antimicrobial Compound Screening[1]

Umezawa

Peptide antibiotics	*n*-Butanol/acetic acid/water (3:1:1)
Basic, water soluble antibiotics	Chloroform/methanol/17% ammonia (2:1:1) Propanol/pyridine/acetic acid/water (15:10:3:10)
Polyene antibiotics	Ethanol/ammonia/water (8:1:1)
Macrolide antibiotics	Ethanol/water (4:1) *n*-Butanol/acetic acid/water (3:1:1) Ethanol/ammonia/water (8:1:1)
Nucleoside antibiotics	Ethyl acetate/methanol (100:15)

See Ref. 36-38.

Wink and Zeeck

Hydrophobic compounds	Chloroform/methanol (9:1) Ethyl acetate/*n*-hexane (3:1)
Hydrophilic compounds	Ethyl acetate/methanol/water (6:2:1) Butanol/acetic acid/water (4:1:5/ upper phase) Ethanol/ammonia/water (8:1:1) Methanol/water (4:1) on RP-8 silica plates

See Refs. 18, and 40-48.

[1] On Silica gel plates unless noted.

Table 4. TLC Spray Reagents Used in Antimicrobial Screening Programs[1]

Ehrlich's Reagent	Vanillin/H_2SO_4
Barrollier Reagent	Molybdato-phosphoric acid
Tetrazolium Blue Reagent	Wood Reagent
Phenothiazine perbromide	Diacetyl Reagent
Anisaldehyde/H_2SO_4	Ninhydrin
Orcinol Reagent	

[1] See Refs. 18, and 36-38.

TLC was used by Aszalos to find novel microbial compounds in fermentation broths, and also to create a system for the classification of microbial compounds based on the mobility of the compound in selected solvent systems. The classification system consisted of four major groups and 15 subgroups (35,39).

Table 5 lists the solvent systems and the classification scheme used. The extract would be first subjected to the primary solvent systems. It would then be given a group number, and be subjected to the specific TLC solvent systems for that group. The identification methods used in the above scheme were UV and bioautography using a variety of microbes. The limitations to this method are that when used alone, it will not identify a particular antibiotic in a crude mixture, although it will narrow the choice to a limited number of known possibilities in a compound class.

The principal drawback of the use of TLC and spray reagents in natural product drug discovery is that the outcome of the visualization process only yields structural class approximations, and thus can not give a clear indication that the metabolite, though it may be unique to the screened species, is indeed novel.

Another screening method that has recently been published uses cartridge packs filled with different solid phases to obtain an elution profile of an extract (48). The strategy was developed for the prioritization of a large number of HIV-inhibitory extracts derived from marine invertebrates and plants, cyanobacteria, lichens, and terrestrial plants.

Table 5. Aszalos' Antibiotic Classification System

Primary Solvent Systems	
α: methanol	
β: chloroform/methanol (90:10)	
γ: chloroform	
Compound Group	**Mobility**
I	no mobility in α, β, γ
II	mobility in α only
III	mobility in α and β only
IV	mobility in all
Secondary Solvent Systems	
Ia:	pyridine/water (1:1)
Ib:	pyridine/water/ethanol (1:1:1)
Ic:	pyridine/water/ethanol (1:1:3)
IIa:	butanol/methanol (1:1)
IIb:	chloroform/methanol (1:1)
IIc:	ethanol
IIIa:	methanol/benzene (12:88)
IIIb:	methanol/benzene (6:94)
IIIc:	methanol/benzene (4:96)
IVa:	methanol/benzene (1:99)
IVb:	methanol/benzene/chloroform (1:49:50)
Compound Subgroup	**Mobility**
I-1	no mobility in Ia, Ib, Ic
I-2	mobility in Ia only
I-3	mobility in Ia and Ib
I-4	mobility in Ia, Ib and Ic
II-1	no mobility in IIa, IIb, IIc
II-2	mobility in IIa or IIb
II-3	mobility in IIa and IIb
II-4	mobility in IIa, IIb and IIc
III-1	no mobility in IIIa, IIIb and IIIc
III-2	mobility in IIIa only
III-3	mobility in IIIa and IIIb
III-4	mobility in IIIa, IIIb and IIIc
IV-1	no mobility in IVa or IVb
IV-2	mobility in IVa
IV-3	mobility in IVa and IVb

Early in the screening phase, it was found that the aqueous extracts of many marine and plant organisms showed activity in the HIV whole cell assay. On analysis many of these active extracts were determined to contain sulfated polysaccharides and tannins in marine invertebrates and terrestrial plants, respectively, which were responsible for the unusually high occurrence of viral inhibition (8,49). In order to remove both sulfated polysaccharides and tannins, the screening procedures were refined to include a method to precipitate the sulfated polysaccharides from marine sources (49) and a step to remove tannins from plant extracts, by irreversible binding to a polyamide containing column (50). Only after a processed extract was still active in the HIV assay, following the removal of sulfated polysaccharides or tannins, was the extract subjected to a chemical screen.

The chemical screen consisted of three different cartridge packs. The cartridge packs used were C_4 and C_{18} cartridges to obtain information on the relative polarity of the compounds, and a Sephadex G-25 cartridge to obtain information on the relative molecular size and weight of the compound. The eluents of the cartridges were then submitted, along with the original extract, to the HIV assay. The elution profile of the extract consisted of a 3 x 4 matrix. Each cartridge afforded four fractions. The C_4 and C_{18} cartridges gave a water, a MeOH/water (1:2), a MeOH/water (2:1) and a 100% MeOH fraction. The Sephadex G-25 cartridge yielded fractions derived by elution with varying amounts of water (2 mL, 0.75 mL, 0.75 mL, and 5 mL).

This procedure quickly eliminated known compounds (in this case sulfated polysaccharides and tannins) and an elution profile was obtained. This elution profile could be used to discern recurring patterns related to particular classes of compounds. In the case of sulfated sterols from marine sponges, a novel, consistent, pattern was determined (51). However, as with the other chemically based screening systems, the information obtained from the elution profile gave only limited structural information.

For any further advances to accrue, the information obtained from the above described processes must be combined with chemotaxonomical information. This additional step is dependent on an ability to correlate large datasets quickly and efficiently with either available or specifically constructed computerized databases.

Computerized Databases

With the advent of computerized database systems, the information on natural products, which originally was in a variety of different journals, conference abstracts and books, can now be found in a small number of databases. The databases which deal with natural products are: the Scientific and Technical Network (STN) files (Chemical Abstracts (CA), MEDLINE, EMBASE,

BIOSIS, BIELSTEIN, SPECINFO, JICST-E, Chemical Abstracts Registry (Registry), and NAPRALERT), the Bioactive Natural Products Database (the Bérdy Antibiotic Database), Chapman and Hall's *Dictionary of Natural Products*, DEREP, MARINLIT, the Marine Natural Products Database, and NAPRALERT at the University of Illinois at Chicago. The similarities and differences of each of these databases are outlined in the following paragraphs.

STN Database Files

The STN is a network of databases (referred to as files) which are searchable. The STN files can be separated into two different types. Information can be stored in a bibliographic style (an abstract of an article) or in a structural/numerical style. The files that store bibliographic information include BIOSIS, CA, EMBASE, JICST-E, and MEDLINE. These files contain the abstracts of journal articles and academic proceedings and the keywords from the title, abstract, and paper are separately indexed (e.g. compound, taxonomy, biological activity). The second type of information stored in the files is the structural/numerical type, and examples include BIELSTEIN, SPECINFO, and the Registry file. The Registry file can be viewed as the chemical substance index. There are over 12 million compounds in the Registry file, and approximately 70,000 compounds are derived from natural products. Table 6 briefly describes the information in these files and the types of searches that can be performed over dial-up lines or over the Internet (14,52).

Table 6. Selected STN Files

File	Reference	Updated	File Structure	RN[1]	SSS[2]
BIELSTEIN (1779-1990)	organic chemistry	periodically	structure/numeric[3]	Yes	Yes
BIOSIS (1969-)	biosciences (life sciences)	weekly	bibliographic	Yes	No
CA (1967-)	chemistry and chemical engineering	biweekly	bibliographic	Yes	No
EMBASE (1980-)	biomedicine and pharmaceutics	weekly	bibliographic	Yes	No
JICST-E (1985-)	Japanese journals (Multidisciplinary)	biweekly	bibliographic	No	No
MEDLINE (1972-)	medicine	biweekly	bibliographic	Yes	No
NAPRALERT (1650-)[4]	natural products	monthly	bibliographic with factual data	Yes	Yes[5]
SPECINFO	infrared and nmr spectra	~ quarterly	structure/numeric (spectral data)	Yes	Yes
REGISTRY (1957-)	chemical substance index	weekly	structure/numeric	--	Yes

[1] Links to the REGISTRY file.
[2] Sub-structure searchable.
[3] Has UV/VIS information.
[4] See separate section.
[5] All structures are coded by chemical class and functional group and can be searched by these codes.

Bioactive Natural Product Database (Bérdy Database)

The Bioactive Natural Product database, or the Bérdy Antibiotic Database, contains information on many of the microbial metabolites that have been published. It was created from the card index of Dr. J. Bérdy of the Research Institute for Pharmaceutical Chemistry in Budapest, Hungary. With the collaboration of Drs. M. Bostian, K. McNitt, and A. Aszalos of the National Cancer Institute, the card index was transferred to a MS-DOS based system (53). The characteristics of the database consists of the antibiotic name and synonyms, the chemical type and antibiotic code number (according to the Bérdy classification system, (54)), chemical formula, elemental analysis, producing organism, molecular weight, compound color/crystal structure, optical rotation, UV spectrum, solubility, chemical reactions, chromatography, stability, bioactivity, toxicity, and isolation methods, with a reference file on the structure and IR spectrum. It has entries on over 23,000 metabolites with updates being made available half-yearly (53,55).

The Bérdy chemical classification system separates the antibiotics into Family, Sub-family, Group, Type, and Sub-type with each having a single digit. The families are carbohydrate antibiotics, macrocyclic lactone (lactams) antibiotics, quinone and similar antibiotics, amino acid and peptide antibiotics, *N*-containing heterocyclic antibiotics, *O*-containing heterocyclic antibiotics, alicyclic antibiotics, aromatic antibiotics, aliphatic antibiotics, and miscellaneous antibiotics (54). Each compound has a unique code number which represents the chemical classification (5 digits) and the sequence number (56). The classification of antibiotics is based on the structure of the compound and has sometimes been arbitrary because compounds may have properties of more than one family. One particular attraction of this database is the ability to search using UV/VIS information.

MS-DOS/MacIntosh Based Databases

Chapman and Hall's *Dictionary of Natural Products* is a CD-ROM based database of natural products for the Windows environment. It contains 90,000 compounds, of which approximately 65,000 are natural compounds with the rest being semisynthetic derivatives. The structure of the database consists of the names and synonyms, molecular formula, molecular weight, bioactivity, taxonomy and structures, all of which are searchable, including sub-structure searching, with UV/VIS information being added for future upgrades. The database has approximately 95% of all of the natural compounds published.

The MS-DOS based database DEREP indexes 7,000 compounds using names and synonyms, molecular formula, formula weight, UV/VIS information, bioactivity, and taxonomy (14).

MARINLIT is a specialty database indexing marine natural products on the MacIntosh platform. MARINLIT indexes compound name and synonyms, the molecular formula, formula weight, UV/VIS, bioactivity, taxonomy, and structural information. One of the advantages of using MARINLIT is the ability to do sub-structure searching (14). Another MacIntosh platform specialty database is the Marine Natural Product Database (MNP Database) which is a private database of marine natural products constructed and used by Dr. D. J. Faulkner at the University of California, San Diego, for writing reviews in *Natural Products Reports* published by the Royal Society of Chemistry. It indexes name, molecular formula, formula weight, and taxonomy.

NAPRALERT Database

The last database to be discussed is the Natural Products Alert Database (NAPRALERT), which references over 80,000 compounds isolated from natural sources. The database consists of numerical and textual classification codes and is not merely a bibliographic database. The structure of the database consists of relational tables. These tables hold the bibliographic information on the articles and authors, the information on the organisms found in the articles, the compound information consisting of the compound name and synonyms, the sources of the natural product (taxonomy), and the pharmacological information relating to the biological activities noted in the articles. The NAPRALERT database is a relational database and stores information in defined fields. A group of related fields is then a part of an individual record.

The NAPRALERT database can provide information on natural products and their sources (class, family, genus, species, subspecies, and variety, plant part used and geographic origin), their pharmacological activities, the extraction and purification procedures used, the yield, and the ethnomedical uses of plants (part and type of extract and type of condition treated) (30). The molecular formula, UV/VIS, and formula weight information of natural products are presently being indexed in a separate file for future use.

Biologically Based Strategies

The second fundamental type of dereplication strategy is based on the biological activity characteristics of a given compound. A biologically based dereplication scheme uses biological activity data to correlate the activity profile of an unknown sample with that of known compounds with the anticipation of obtaining information regarding the mechanism of action.

The National Cancer Institute (NCI) screening program is an example of a biologically based dereplication strategy. The premise has been stated that known active antitumor and cytotoxic

agents possess a biological activity profile over the 60 human cancer cell-lines that comprise the NCI's screening program, and that this characteristic can be used to predict the general mechanism of action of a new compound or extract. Pattern recognition software is used to compare the biological fingerprint of the new compound or extract with that of the known active compounds in the NCI database (12).

The first step in obtaining information from the 60 cell-line panel is to develop a meaningful representation of the differential growth inhibition of a given sample. The first graphical representation was that of the Mean Graph of the IC_{50} values and the delta values (12,57). The mean graph is a vertical bar graph constructed from the delta values obtained from the test sample. It is the difference between the log IC_{50} value of the sample to a cell-line and the mean log IC_{50} value of the sample from all of the cell-lines which is referred to as the delta value. Thus, the delta values indicate if a given cell-line is significantly more or less sensitive than the other cell-lines to the test sample. In order to obtain one value in searching and sorting samples with differential growth patterns, a best delta (Delta) value was chosen. Two definitions of the Delta value could be considered. The first definition is the simplest and is the largest delta value of all the delta values for a given sample. However, a better definition of the Delta value takes into account inherent sensitivities of some of the cell-lines. The definition for the Delta value chosen was the delta value of a sample in a cell-line that has the highest number of standard deviations from the mean delta value which is obtained from the delta values of all samples tested in the same cell-line. Sorting by Delta values therefore offers an efficient way of identifying samples that exhibit differential growth inhibition.

The second step was to create an algorithm to compare the mean graph pattern of a given sample against all of the graph patterns of active compounds in the database. This algorithm was embodied in the COMPARE program (57). More specifically, the COMPARE program compares the test sample delta values for each cell line with the delta values of all of the active compounds in the database on a cell-line by cell-line basis. The program generates two values. The first value (Av) is the average difference between delta values, which is the mean of the absolute values obtained by subtracting the delta value of the compounds in the database from the sample's delta value for each cell-line. The second value is the maximum difference (Max), which is the largest absolute value obtained by subtracting the delta value of the compounds in the database from the sample's delta value for each cell-line. The COMPARE program then sorts the Av values first in increasing order and then the Max value in increasing order. The Av and Max of the test sample is 0. The

disadvantage of the program is that it gives a best match even if there is none. It also yields no direct chemical information.

An ongoing approach to analyzing the data from the 60 cell-line screen and thereby predicting the general mechanism of action of compounds is to use neural networks (58,59). Neural nets "learn" from an example set instead of memorizing the set. The neural net used at NCI had 60 input processing elements (PE), one for each cell line, seven hidden PE, and six output PE which were: alkylating agents, topoisomerase I inhibitors, topoisomerase II inhibitors, RNA/DNA antimetabolites, DNA antimetabolites, and antimitotic agents. Each input vector is connected to each of the seven hidden layers and each of the seven hidden layers is connected to each of the outputs (60). The connections have different weights associated with them. When the network is trained on a set of test samples, incorrect answers are fed back into the computer to update the weights. The ability to train the computer and update the weights of the connections is achieved by using the "back propagation" algorithm. The neural net "learned" on 90% of a set of 141 dose-range input vectors (delta values), the neural network was then used to predict the other 10% of the set. This learning and testing was repeated 10 times and the percentage of correct predictions was 91.5% (60). Both the COMPARE algorithm and the neural network are powerful tools when one wants to obtain information about the mechanism of action of new compounds.

Another use of the COMPARE program is to dereplicate extracts based on their differential activity profile. Using the program and information from a literature survey of taxa, compounds that show differential cytotoxicity could be used to compare with the differential cytotoxicity data of extracts. An example of this type of use has been in the study of the cucurbitacins. Some cucurbitacins have a characteristic, differential cytotoxicity pattern, and this pattern, along with a literature survey of taxa known to contain cucurbitacins, was used to isolate more cucurbitacins (61). Cucurbitacins E and I were isolated from *Iberis amara* L. (Cruciferae) quickly and efficiently. Using the profile for the compounds and extracts of *I. amara*, a similar differential cytotoxicity pattern was seen for the extracts of *Begonia plebeja* Liebm. (Begoniaceae), a genus known to contain cucurbitacins, and *Gonystylus keithii* Airy Shaw (Thymelaeaceae), a genus not known to contain cucurbitacins. Utilizing the same isolation and separation technique applied to *I. amara*, cucurbitacins B and D were isolated from *B. plebeja* and *G. keithii*, respectively (61). The use of the COMPARE program to dereplicate an extract on a structural basis using the differential cytotoxicity profile is based on a fundamental assumption that the individual profile is linked to one and only one structural class of active metabolites. In addition, for the system to be used effectively on a large scale, it is also assumed that every class of compound should have a unique profile. However, it was

noted that further confirmation from LC/MS or HPLC using a photodiode array detector could be used if one required more specific identification of the bioactive compound.

The principle advantage of using the COMPARE and neural network programs is in the ability to find compounds or extracts with a novel mechanism of action or novel compounds with a known mechanism of action. The information obtained can then be employed to evaluate prospective agents for the expensive *in vivo* testing (12,57,60-62).

The dereplication schemes that have been developed to date have significant limitations. Chemical screening of extracts does not take biological activity information into account and provides only functional group or structural class information on the constituents of a sample (15,16,18,36). Some dereplication strategies, while taking biological activity into account, yield only structural approximations (35), while other strategies only afforded elution profiles or biological activity profiles that could be used, rather crudely, to obtain chemical information, if and only if, similar elution or biological activity profiles were known (8,57).

With the tremendous strategic pressure on the decision-making required for the fractionation of active leads resulting from high-throughput screening, it has become clear that there is an urgent need to more effectively dereplicate biologically active, plant extracts. In order to achieve this goal, one needs to combine, in an interactive manner, physical, chemical, and biological activity data. It is crucial to obtain key information on the physical and chemical characteristics of the constituents of an extract, and to obtain concurrent biological activity information of those components. From the physical and chemical information, it should be possible to use databases in order to obtain information on compounds with the same characteristics. Mass information is a key parameter that can be used to derive such a list of specific metabolites, although because of extensive duplication, mass information cannot be used as the sole criteria for the elucidation of those constituents. In order to effectively obtain biological activity data, there is a profound need to quickly and efficiently obtain such information on subfractions of an extract. The ability to compare and interrelate biological activity and mass information directly, and compare that with database information, is a key step in the process of effectively dereplicating biologically active natural product extracts. Such a procedure will now be described.

With the advent of EIS LC/MS instrumentation, mass information could be obtained on partially separated constituents of a sample, and, with a 96-well plate fraction collector, the eluent could be collected for subsequent biological evaluation. Since the EIS LC/MS system is linked in time to the fraction collector, the biological activity profile can be directly compared to the information obtained from the mass spectrometer, for both are accumulating data on the same time

axis. With the mass information available that corresponds to the biological activity profile, these masses could be used to obtain a list of known compounds that have previously been found in the genus or species under investigation and a list of metabolites which possess activity in similar bioassays. The NAPRALERT database was chosen to be used in the dereplication scheme since it indexes taxonomic, pharmacological, and chemical information.

DEREPLICATION METHODOLOGY

The LC/MS system used for the dereplication of plant extracts consists of a Hewlett-Packard Electrospray System (Wilmington, NC) containing a 1090 Series II L HPLC, a 59987A Electrospray, and a 5989B Single Quadrupole Mass Spectrometer. The system is controlled by the HP ChemStation Software. The splitter is a 1/50 : 1/125 splitter made by LC Packings (San Francisco, CA). The fraction collector is a FC 204 Series made by Gilson Inc. (Middleton, WI) fitted with a narrow bore needle. The post-column addition pump is an ABI MicroGradient System. The column used was a Kromasil C_{18} column purchased from Technikrom (Wilmette, IL) with the dimensions 0.32 x 25 cm and using 5 µm packing material.

The present dereplication process can be divided into two separate phases. The first phase consists of the separation and data collection phase and the second phase is the data analysis phase. Variables of the first phase include the chromatographic parameters, sample handling, and biological testing. The second phase involves strategies for the analysis of the spectroscopic and biological activity data.

Chromatographic Parameters and Sample Handling Methodology

The mobile phase system consists of water (A) and acetonitrile (B) and is a gradient set up as: 0 to 1 minute 100% A, a linear gradient from 1 to 8 minutes to 50% A/ 50% B, a linear gradient from 8 to 20 minutes to 100% B and held at 100% B for a final 10 minutes. After the end of the run (30 minutes), the mobile phase is then returned to starting conditions (100% A). There is a 10 minute equilibration before the next injection. Experimentally, it was found, using a C_{18} column, that this gradient system worked well for defatted alcoholic or chloroform extracts.

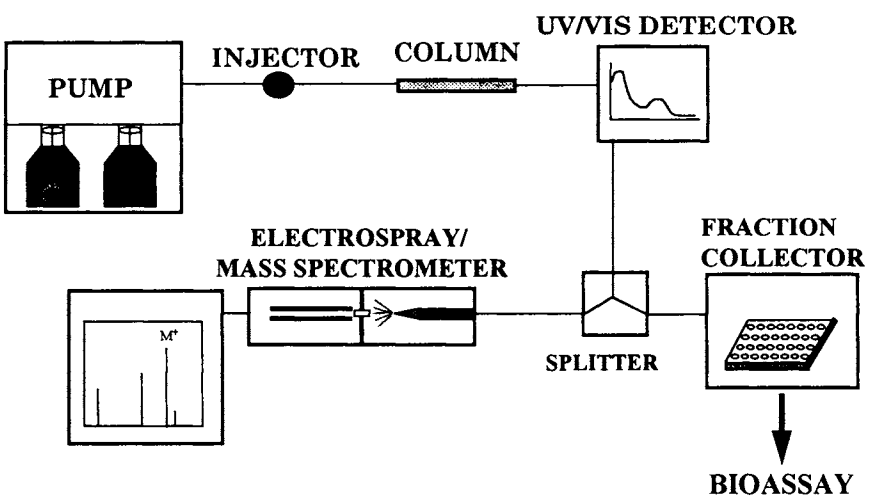
For more polar extracts, the gradient system used is: 0 to 10 minutes at 80% A / 20% B, a linear gradient from 10 to 18 minutes to 60% A / 40% B, a linear gradient from 18 to 28 minutes to 25% A / 75% B, a linear gradient from 28 to 30 minutes to 100% B and held at 100% B for 7

minutes. After the end of the run, the mobile phase is returned to starting conditions (80% A / 20% B), and there is a 10 minute equilibration before the next injection.

Each extract is injected three times in the EIS LC/MS instrument generating three, 96-well microtiter plates (the last row of each plate is left blank for bioassay controls) and three sets of spectroscopic data. The injection volumes are relative to the ED_{50} value of the extract. Thus, the first two separations are based on 25 µL injections of a 4.0 mg/mL sample, which is at least five times the calculated effective dose (ED_{50}) obtained from the preliminary screen. They differ, however, in that the eluent from the first separation is post-column treated with 0.2% acetic acid in 20% aqueous methanol and the mass spectrometer adjusted to observe positive ions, while the second separation is post-column treated with 0.2% triethylamine (TEA) in 20% aqueous methanol and the mass spectrometer adjusted to observe negative ions. The third injection is a 5 µL injection of the 4.0 mg/mL sample which is one equivalent of the effective dose. It serves to detect the most active compound(s) present in the extract. Only the biological data from this run is truly relevant since the activity seen in the 5 µL injection matches the dose that was used in the primary screen.

The present configuration of the LC/MS system is shown in Figure 1. The extract is injected onto the column and is partially separated. The UV/VIS absorbance spectra of the compounds in the eluant are obtained by the photodiode array detector (PDA). After the PDA, the eluent is split unequally. Two percent of the eluent goes into the mass spectrometer to determine the masses of the

Figure 1. LC/MS Outline



metabolites in the eluent at that point in time, while the remainder of the eluent is deposited into 96-well plates for biological activity testing. The ESI LC/MS system can furnish mass information on almost all classes of compounds, if they have ionizable groups. The PDA, the MS and the biological assay can be viewed simply as detectors for the HPLC instrument.

Analysis Methodology

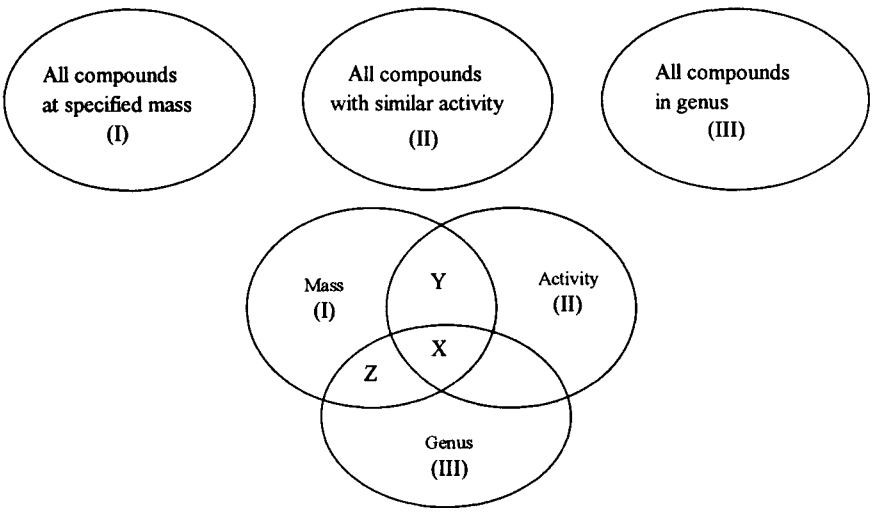
Following sample separation and distribution, three sets of data are obtained. The first set of data is the mass information in both the negative and positive ion modes, the second set of data is the UV/VIS information from the photodiode array detector and the last set of data is the biological activity profile. It is of critical importance that all of these data sets have the same time axis. The activity profile from the 5 μL injection is analyzed for the time range of activity. If no activity is seen in the separated fractions from the 5 μL injection sample, then the data from the 25 μL injection is analyzed. Since each well corresponds to a specific time period, by finding the active wells, the time range for the elution of the active metabolites can be determined. This time range information is subsequently used to select and analyze the mass spectral data. Those masses which predominate during the active time range and that closely follow the activity profile are used for subsequent correlation with the NAPRALERT, and if warranted, other databases.

The dereplication process is based on a comparative analysis of datasets already developed with libraries of chemotaxonomic, pharmacological, and molecular weight information. Specifically, after the bioassay is completed, masses associated with the active time range(s) are determined from both the positive and negative Total Ion Chromatogram (TIC). The time profile of the masses that appear in the area of activity are also retrieved from the TIC profiles as Extracted Ion Chromatograms (EIC). If the time profile of a mass is not similar to that of the activity profile, for example, if activity has been found in only 1 well (a 0.34 minute time range) and the ion profile time range for a mass is 1 minute, then that mass can be ignored.

The NAPRALERT database is used to correlate the masses found in the TIC profiles with all known natural products that have the specified masses (Figure 2, Top I). In addition, the database also produces a second list of metabolites which have been reported to possess activity in a similar bioassay (Figure 2, Top II). The list that is used in the comparison with compounds found in the genus is the metabolite list obtained from the overlap of the lists I and II (Figure 2, bottom: overlap of I and II, areas X and Y). A third list is also retrieved from the database. This list contains all of the compounds that have been previously been isolated from the genus in question (Figure 2, Top III). If there is a correlation between the genus and the mass/bioactive lists, that is, a compound

having a mass correlating with the observed area of activity has been reported active and has been previously found within the genus (Figure 2, bottom: overlap of I, II, and III, area X only), then it will be noted as having a high probability that it will be subsequently isolated.

Figure 2. Analysis Methodology: Database Correlation



The other potential outcomes are that the mass relating to the peak of activity may be a known compound which has been found in the genus, and which has not been previously described to be active in a similar assay (Figure 2, bottom, overlap of I and III, area Z), or that the mass(es) that correspond to the area of activity do not relate to any compounds previously isolated from the genus. If there has been no correlation observed using the NAPRALERT database, then STN files are used. The genus is searched in the CA file and registry numbers are retrieved from the abstracts. The Registry is used to obtain a list of compounds that match the registry numbers retrieved from the abstract search. The list retrieved from the Registry file may also contain compounds obtained by synthesis or semi-synthesis and thus care is needed in evaluating the information received from the Registry. Compound names that are matched by mass information and genus information, and masses with no correlation, are provided to the phytochemists who will be fractionating and isolating the biologically active compounds.

Dereplication Studies

The following examples illustrate important aspects of the present dereplication method. Over 60 plant extracts previously shown to have a strong and reproducible cytotoxic activity from our anticancer drug discovery program (10,63) were subjected to the dereplication process. Some of the samples that were subsequently chosen for bioactivity-guided fractionation, and compound isolation and structure elucidation were *Mesua ferrea* L., *Ratibida columnifera* (Nutt.) Woot. & Standl., *Allamanda blanchetii* A. DC., and *Rubia cordifolia* L.

Mesua ferrea

Mesua ferrea L. (Guttiferae) leaf samples were collected in Thailand and evaluated in a panel of human cancer cell cytotoxicity assays at the University of Illinois at Chicago (UIC) (10,63). The chloroform extract was active in the human cancer cell lines Mel2 (Human melanoma) and ZR-75-1 (hormone-dependent breast cancer), with IC_{50} values of 4.5 and 7.0 μg/mL, respectively, and was studied by dereplication. Figure 3 is the negative ion TIC (Figure 3A), the LC chromatogram at 280 nm (Figure 3B), the positive ion TIC (Figure 3C), and the biological activity profile from the ZR-75-1 bioassay of the sample (Figure 3D).

From the activity profile, three distinct areas of biological activity may be noted. The most active area is the well that corresponds to the time range of 20.78 to 21.12 minutes, and consequently this time period was used to analyze the electrospray mass spectra. Figure 4A is the TIC of the time frame of maximum activity (± 0.5 min) and Figure 4B is the dominant ion in the area of this activity. Figure 5 is the EIC of the ion observed in the spectrum. The positive ion mode did not show any particular ions in that time range.

The only ion found in the mass spectrum of the most active region was at *m/z* 405.2 (Figure 4B). The difference between the TIC and the EIC at *m/z* 405.2 suggests that a different ion (*m/z* 391.2, data not shown) was responsible for the peak between 20.3 to 20.6 minutes. Since the ion at *m/z* 391.2 does not follow the activity profile, it was rejected.

Therefore, assuming unity for charge, the molecular mass of the ion at *m/z* 405.2 would be 406.2 amu. Consequently, this molecular mass was used to correlate with the information obtained from the NAPRALERT database. In the case of *Mesua ferrea*, the search identified that the prenylated 4-phenylcoumarin mammeisin correlates well in all respects. It has a MW of 406 amu, it has been found in the genus *Mesua*, and it has been previously reported to possess activity in a similar biological assay.

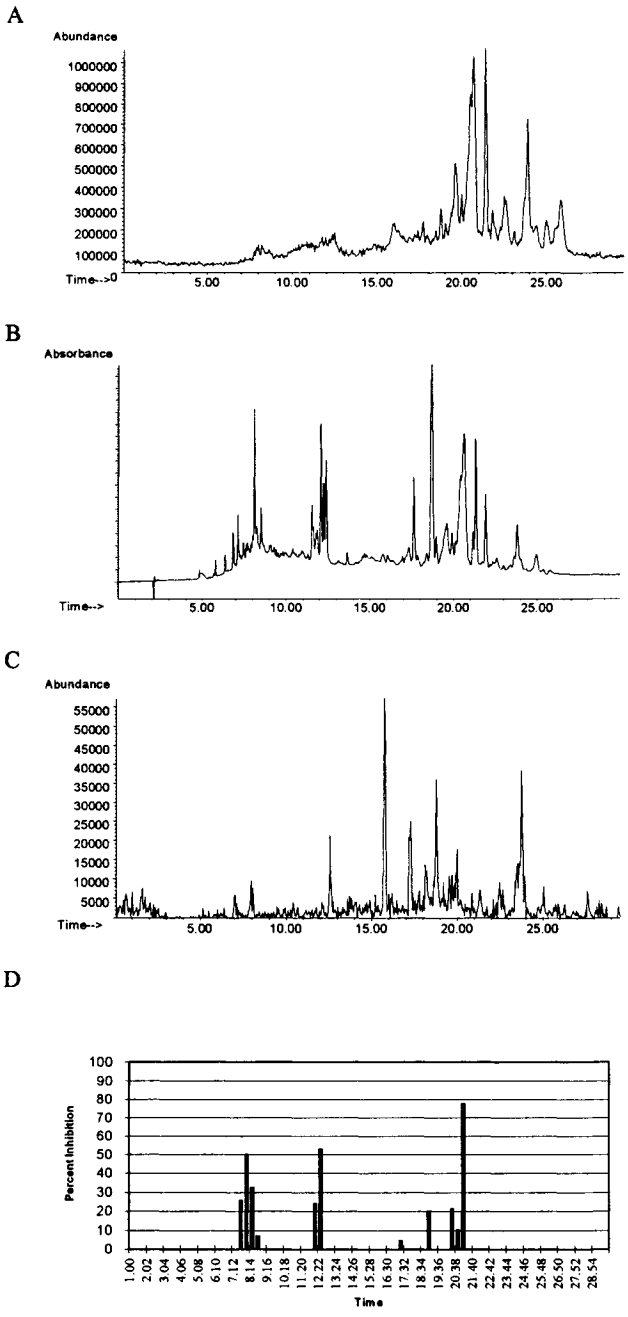


Figure 3. Negative Ion TIC (A), LC Chromatogram (B), Positive Ion TIC (C), and Biological Activity Profile (D) of *Mesua ferrea*

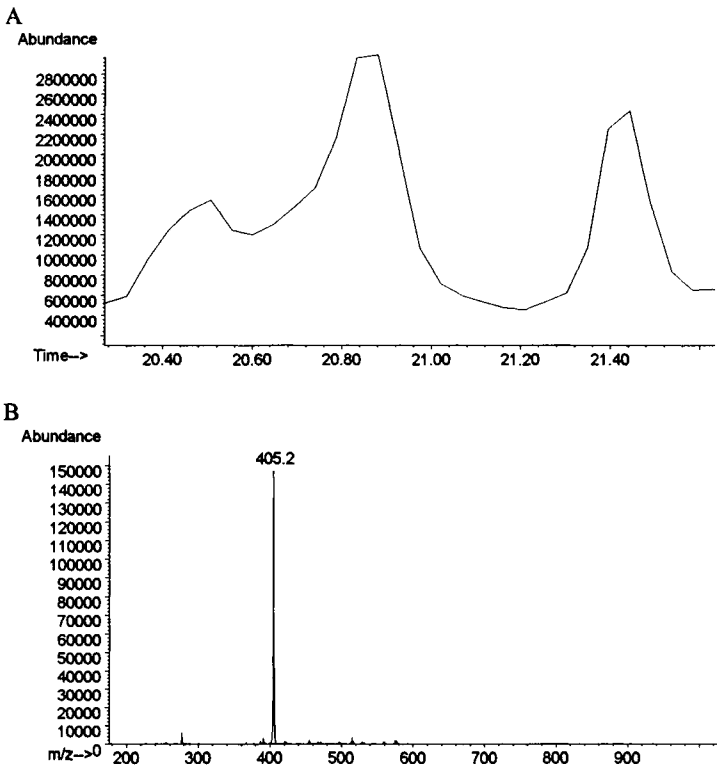


Figure 4. Negative Ion TIC (A) and mass spectrum (B) correlating to the biologically active area of *Mesua ferrea*

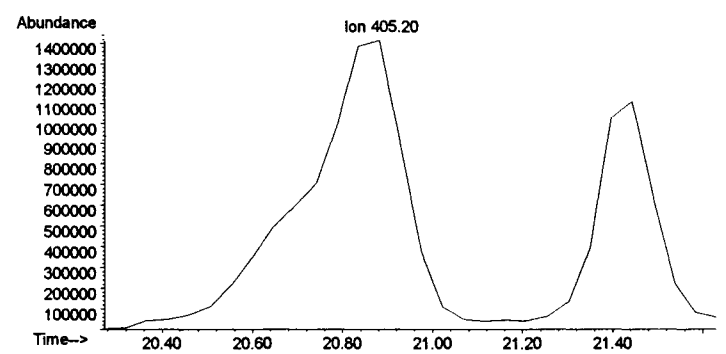
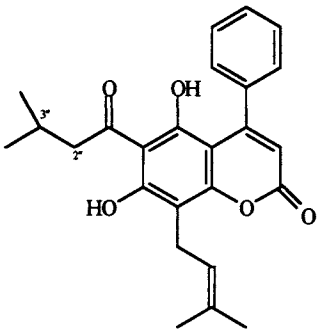
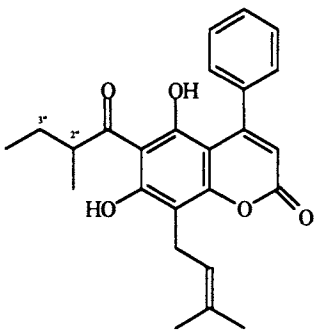


Figure 5. Extracted Ion Chromatogram of the ion observed in the mass spectrum of *Mesua ferrea*

In order to establish the predictive nature of this strategy, the active principle was isolated from the sample. Subsequent structure determination revealed that the metabolite was an isomer of mammeisin called mammea A/AB.



Mammeisin



Mammea A/AB

The structural difference between these two isomers is that the methyl group located at the C-3'' position in mammeisin is shifted to the C-2'' position in mammea A/AB. In the proton NMR spectrum of mammea A/AB, the signals for the H-3''a and b appeared as multiplets at 1.73 and 1.38 ppm, respectively, and the signal for H-2'' was at 3.57 ppm. In mammeisin, H-3'' is a multiplet at 2.20 ppm and H_2-2'' is a doublet at 2.82 ppm (64). This compound had not been isolated previously from this genus and had not been reported to possess this type of biological activity. Therefore, it would not have shown up in our database searching strategy.

Ratibida columnifera

Above ground parts of *Ratibida columnifera* (Nutt.) Woot. & Standl. (Compositae) were collected in the United States and were found to be cytotoxic in a select group of human cancer cell lines. The IC_{50} for the extract in the LNCaP (hormone-dependednt human prostrate cancer) cell line was 0.82 μg/mL, whereupon the extract was submitted for dereplication.

Figure 6 is the negative ion TIC (Figure 6A), LC chromatogram (Figure 6B), positive ion TIC (Figure 6C), and the activity profile (Figure 6D) (using the LNCaP cell-line) of the extract. Areas of strong activity were found between 10.86 to 12.22 minutes and between 13.24 and 13.58 minutes. Using these time ranges, both the negative (Figure 7A) and positive (Figure 9A) ion mode

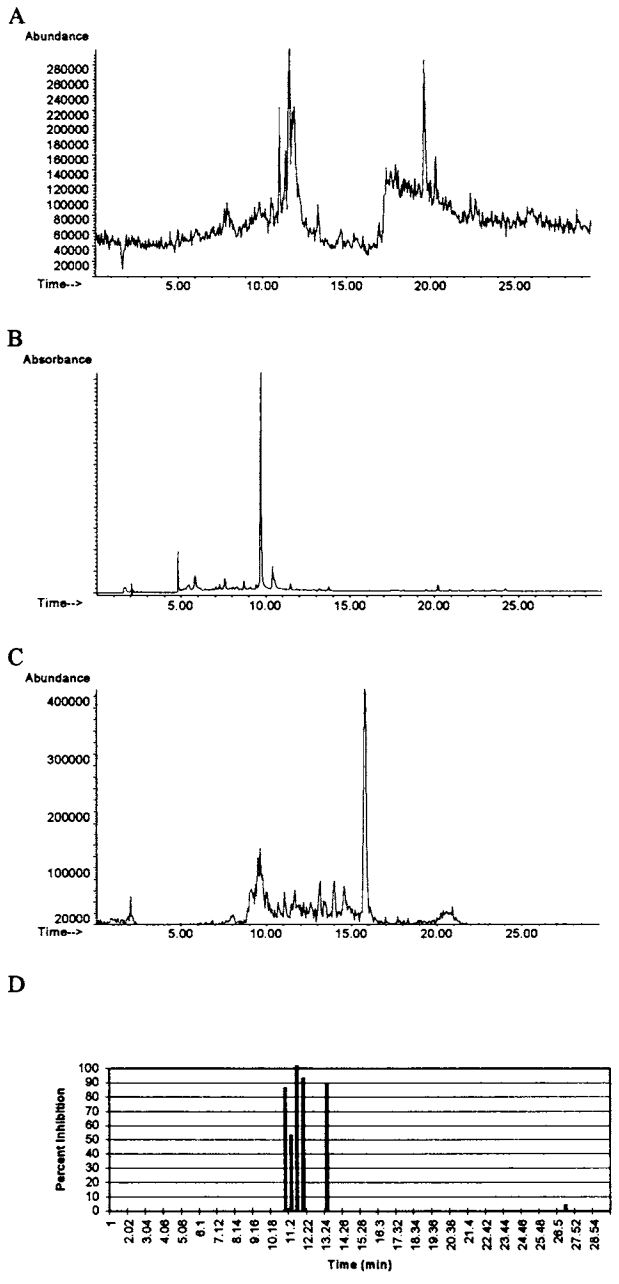
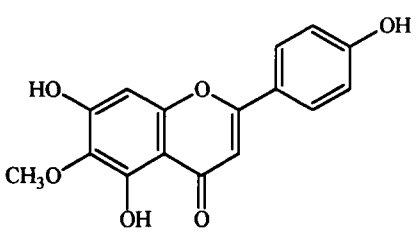


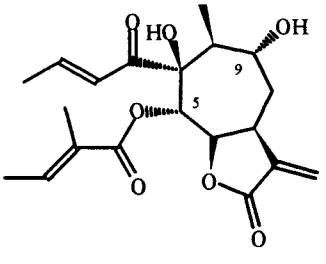
Figure 6. Negative Ion TIC (A), LC Chromatogram (B), Positive Ion TIC (C) and Biological Activity Profile (D) of *Ratibida columnifera*

TICs were analyzed. The ions found in the negative ion mode that closely followed the first area of activity were at *m/z* 365.0, 377.2, 379.2, 413.0, 414.9, and 422.9 (Figure 7B and Figure 8A). As can be seen from Figure 8A, these ions group under two separate peak areas, since the area of activity includes both peaks, all ions should be used for subsequent correlation with the database. For the second area of activity, only the ion at *m/z* 374.8 followed the biological activity profile (Figure 7C). The ion at *m/z* 411.7 did not closely follow the activity (Figure 8B). The ions found in the positive ion mode for the first area of activity were at *m/z* 366.9, 378.9, 381.1, 391.0, 396.1, and 416.8 (Figure 9B), however, only the ions at *m/z* 366.9, 378.9. 381.1, and 416.8 closely followed the activity profile (Figure 9C). The ion at *m/z* 396.1 was observed between 9 and 10.5 and the ion at *m/z* 391.0 was background noise. No particular ions appeared in the second area of activity in the positive ion mode. The molecular masses observed in these time ranges would then be 366, 376, 378, 380, 414, 416, and 424 amu. These masses were used to correlate with the information in the NAPRALERT and STN databases. There have been many seco-ratiferolides and xanthones previously reported from the genus *Ratibida*, but the NAPRALERT database compound subfile is not complete in this regard, consequently, the STN database was also used. The MWs that matched with compounds in the STN database were those at 366, 376, 378, 380, and 424 amu. The structures corresponding to these registry numbers, all appeared to have the basic skeleton of a seco-ratiferolide.

After subsequent isolation and structure elucidation, hispidulin and 9α-hydroxy-*seco*-ratiferolide-5α-*O*-angelate (378 amu) (shown below) (65) were obtained. A compound of mass 376 amu was also isolated and identified as a novel metabolite. It was not 9-oxo-*seco*-ratiferolide-5α-angelate. The mass of hispidulin at 300.2 amu was not observed in the 5 μL injection activity profile, but it was observed in the biological activity profile from the 25 μL injection (data not shown).



Hispidulin



9α-Hydroxy-*seco*-ratiferolide-5α-*O*-angelate

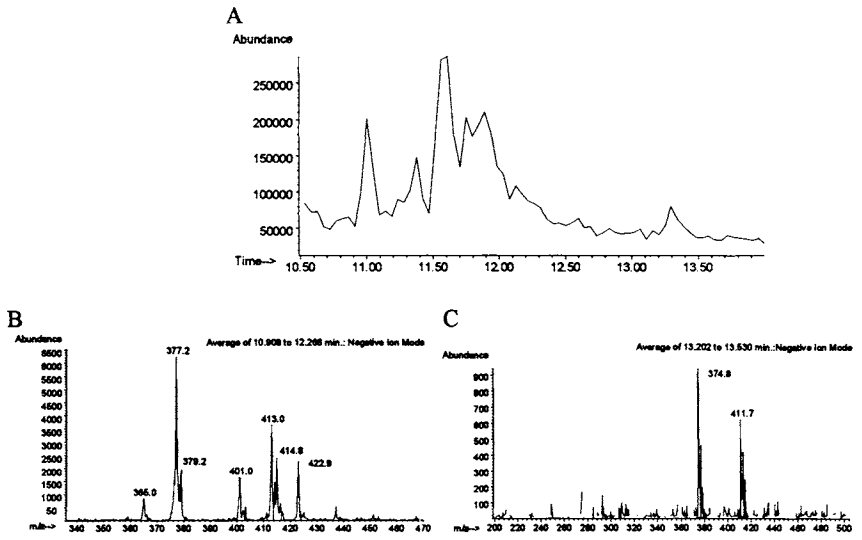


Figure 7. Negative Ion TIC (A) and mass spectra correlating to the biologically active areas (B and C) of *Ratibida columnifera*

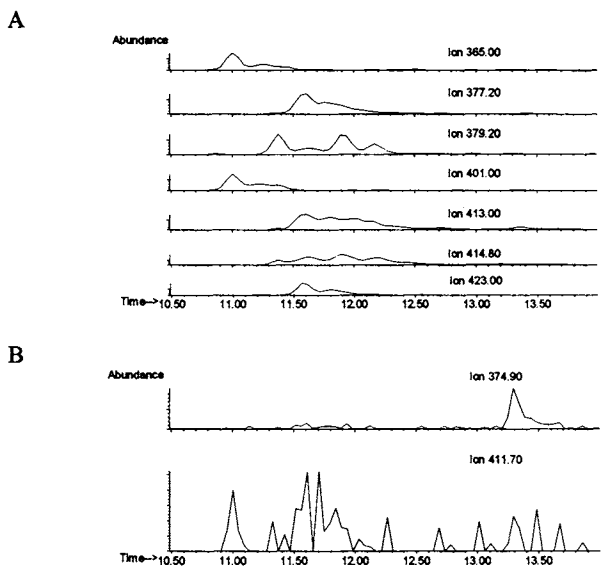


Figure 8. EICs of selected ions observed in the mass spectra of *Ratibida columnifera*

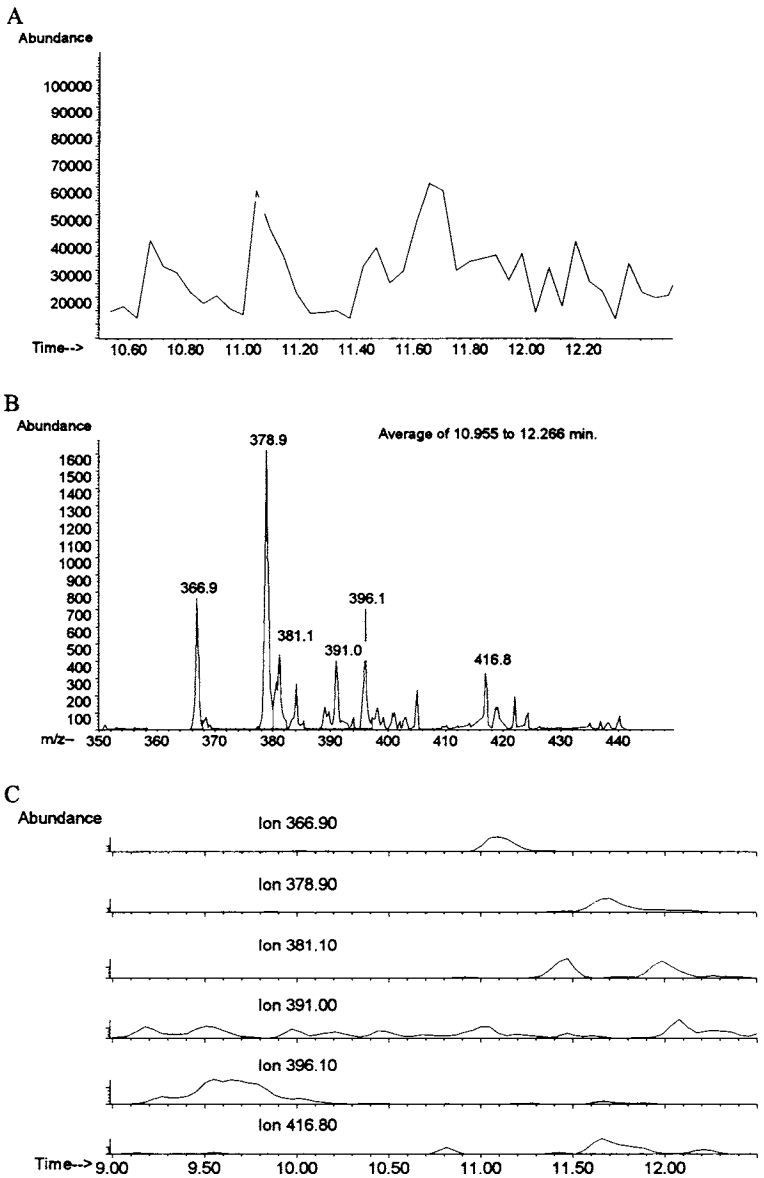
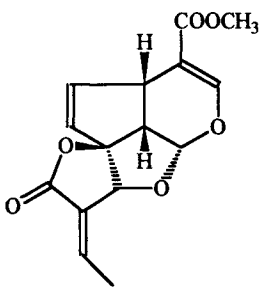


Figure 9. Positive Ion TIC (A), Mass spectrum correlating to the area of biological activity (B), and EICs of selected ions of *Ratibida columnifera*

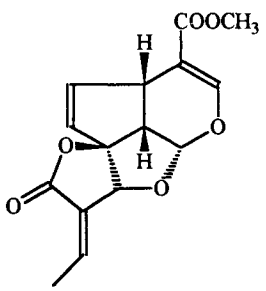
Allamanda blanchetii

Roots of the plant *Allamanda blanchetii* A. DC. (Apocynaceae) were collected in Brazil. After extraction and primary screening, this plant was found to display cytotoxic activity in a number of human cancer cell lines. The IC_{50} values for the extract using the human cancer cell lines LNCaP, ZR-75-1, and KB-V (drug-resistant human epidermoid carcinoma) were 1.3, 1.3, and 1.2 μg/mL, respectively. At this point, the extract was submitted for dereplication.

Using the larger 25 μL injection for the analysis of activity, two distinct ranges could be seen. The first range was from 7.8 to 9.9 minutes and the second range was from 10.8 to 12.2 minutes. Using these time ranges, the negative and positive TICs were analyzed. The ions found in the negative ion mode spectra that closely followed the area of activity in the first range were at *m/z* 615 and 645, corresponding to molecular masses of 616 and 646 amu. No particular ions were found for the first range in the positive ion mode. The ions found in the second range in the negative ion mode that closely followed the area of activity were at *m/z* 213, 245, 265, 289, and 321, and the ions found in the second range in the positive ion mode were at *m/z* 267, 291, 308, 353, 383, and 603. However, only the ions at *m/z* 267, 291, 308, and 603 closely followed the biological activity profile. The masses used for the correlation with the databases were therefore 214, 246, 266, 290, 307, 322, 602, 616, and 646 amu. NAPRALERT data correlated the mass at 290 amu with the isomeric compounds isoplumericin or plumericin, and the mass at 616 amu with the compound plumeride-*p*-coumarate. After subsequent isolation and fractionation of the extract, isoplumericin and plumericin were identified as the active principles (shown below) (66,67).



Plumericin



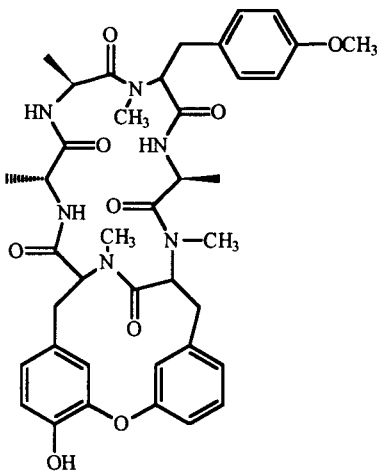
Isoplumericin

Rubia cordifolia

The stembark of *Rubia cordifolia* L. (Rubiaceae) was collected in India and the extract was discovered to be broadly cytotoxic in the human cancer cell cytotoxicity panel at UIC, whereupon the extract was submitted for dereplication. The ions seen in the negative ion mode that closely

followed the biological activity profile were at *m/z* 315.0 and 755.1, and therefore the masses used to correlate with the databases were 316 and 756.1 amu. No matches were obtained with the NAPRALERT database, however, the STN files did correlate the mass at 756.1 amu with the cyclic polypeptides RA2 or RA5. Cyclic peptides RA2 and RA5 are known to be cytotoxic (68-70), and were the constituents proposed to be responsible for the activity of the extract.

Isolation and structure elucidation of the active compounds was performed and the metabolite isolated was one of the proposed cyclic hexapeptides, RA5 (shown below).



Cyclic Peptide RA5

Flavonoid Dereplication

For specific types of natural products, where the nature of the metabolite is known, the sample manipulation strategy may be varied. Thus, for flavonoid dereplication, the HPLC method for more polar extracts is used and different electrospray parameter sets, or tune files, are used in negative ion mode. The first tune file used is similar to that used for standard dereplication, i.e. the electrospray parameters are set so that only the pseudomolecular $[M-H]^-$ ions are observed. A second tune file is used where the electrospray parameters are specifically adjusted to induce collisions of the flavonoid glycosides within the MS so that the sugars are removed. Consequently, the ions that are now detected are those of the aglycones. The major differences between the two tune files are that, for the tune file adjusted to induce collisions in the MS, the entrance lens voltage is decreased to filter out high molecular weight molecules, the capillary exit voltage is decreased and

the first skimmer lens voltage is slightly increased thereby increasing the voltage difference between the capillary exit voltage and the first skimmer voltage. This increase in the voltage difference can cause collision induced dissociation of the molecules.

Flavonoid Standards

Frequently, biological activity may reside in an area of the chromatographic separation where the resolution is rather poor. This is particularly true where the activity is found within the first 10 minutes of the standard gradient.

It became apparent that some assays were sensitive to compounds in this area. Therefore, a second method was developed to establish what information on the active principle could be obtained. The metabolites that elute within the first 10 minutes are very polar, including flavonoids and flavonoid glycosides and saponins, among others. Therefore, an alternative gradient system was proposed for more polar extracts and the technique applied. The technique induces collisions, called collision-induced dissociation (CID), between the ions and other molecules within the electrospray source which induces fragmentation of ions. Using the new gradient system and collision-induced dissociation, it was possible to analyze these polar extracts and determine the molecular weights of both the glycosylated derivative and the aglycone (71).

Figure 10 is a negative ion TIC and LC chromatogram of a number of flavonoids and flavonoid glycosides. The flavonoid glycosides elute within the first 15 minutes while the non-glycosylated flavonoids elute subsequently. Table 7 is the list of the flavonoids used experimentally and their retention times in the chromatographic system used. Figure 11 comprises the spectra of rutin and naringin where the ESI-MS is in the normal (top) and CID mode (bottom). In the normal mode, rutin and naringin show ions at *m/z* 609 $[M-H]^-$ and *m/z* 579 $[M-H]^-$. However, the CID mode shows rutin with ions at *m/z* 300 and 609 for the $[quercetin-2H]^-$, where the extra proton lost was proposed to be due to the conversion of the B-ring to a quinone and for the $[M-H]^-$, respectively. The aglycone quercetin, using the tune file for cleavage, also shows the ion at *m/z* 300. The spectra for naringin, in the CID mode, show ions at *m/z* 271 and 579 for $[naringenin-H]^-$ and $[M-H]^-$, respectively. A plant extract was submitted for dereplication using the new hplc method and tune files in order to evaluate the effectiveness of the method.

Analysis of *Eugenia jambos*

The aqueous extract of *Eugenia jambos* L. (Myrtaceae) was used to establish if the previously described LC/MS protocol could be utilized to analyze substantially more polar extracts

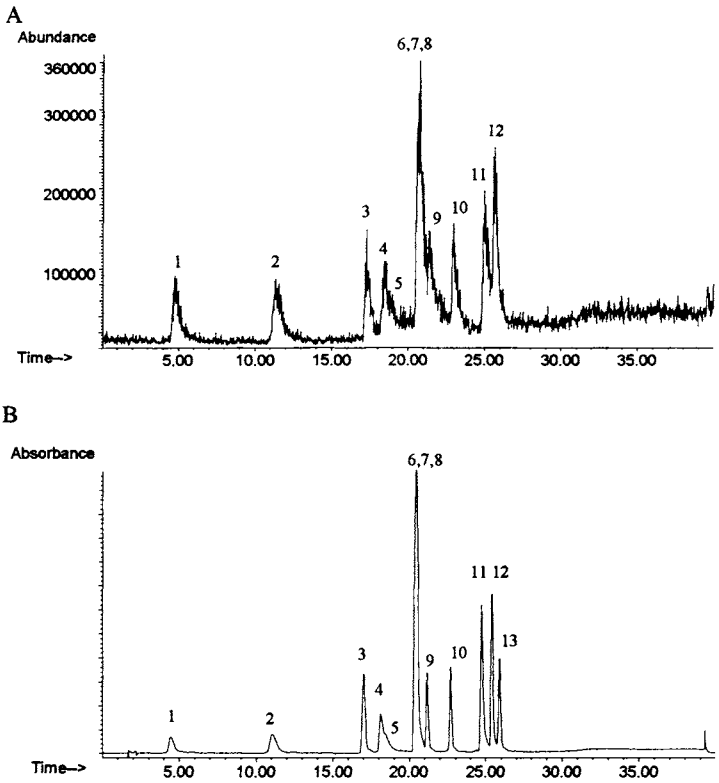


Figure 10. Negative Ion TIC (A) and LC chromatogram (B) of flavonoid standards

Table 7. Retention Time Table of Flavonoids

Number	Flavonoid	R_t (LC)
1	Rutin	4.44
2	Naringin	11.05
3	Daidzein	17.02
4	Luteolin	18.15
5	Quercetin	18.48
6,7,8	Apigenin/Genistein/Naringenin	20.45
9	Hesperetin	21.18
10	Formononetin	22.70
11	Chrysin	24.75
12	Biochanin A	25.40
13	Tangeretin	25.90

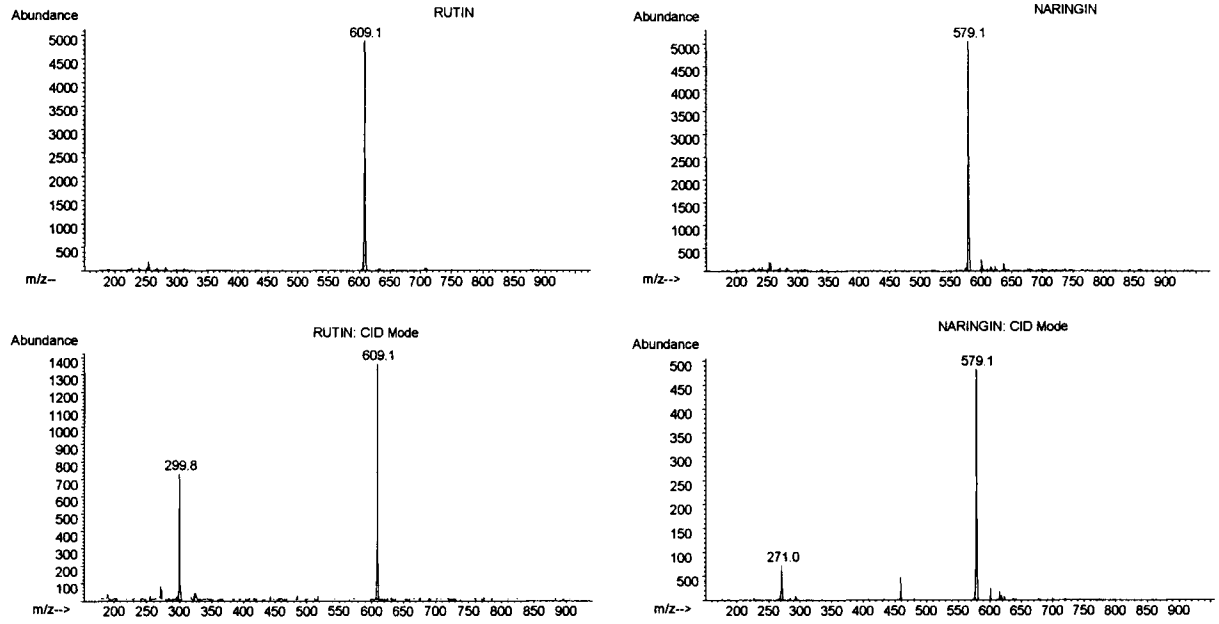


Figure 11. Mass spectra of rutin (left) and naringin (right) in the normal (top spectra) and CID mode (bottom spectra)

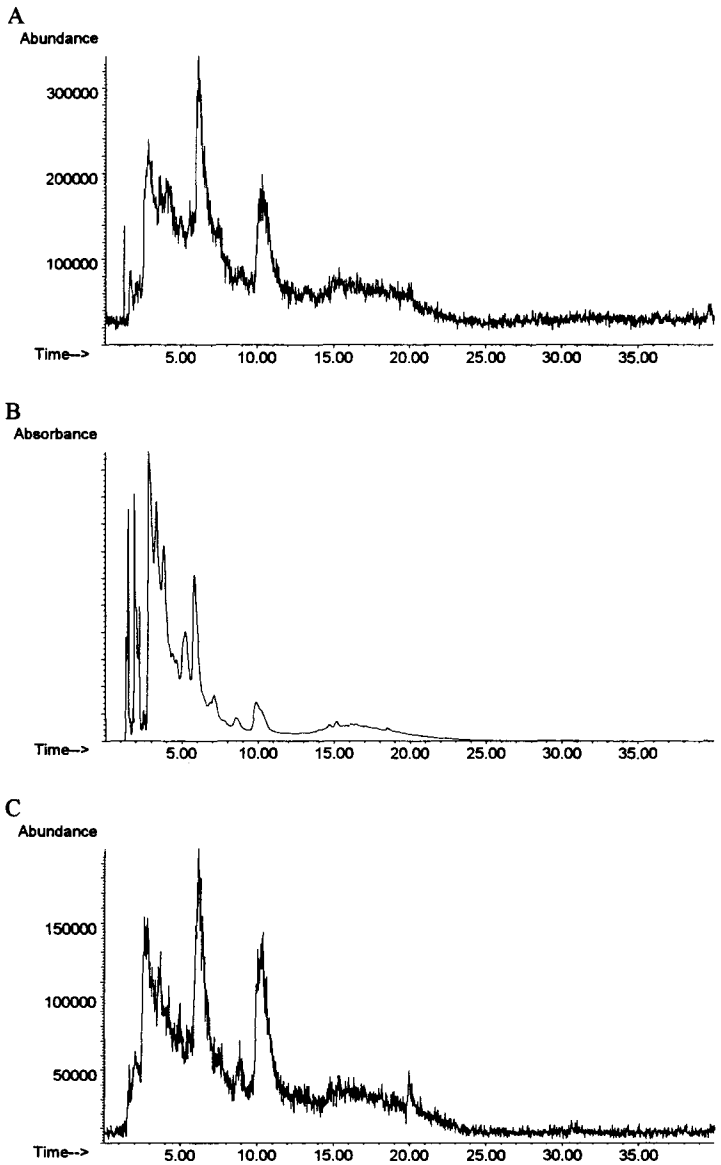


Figure 12. Negative Ion TIC (A) LC Chromatogram (B) and Negative Ion TIC using CID mode (C) of *Eugenia jambos*

from plants. Figure 12 shows the negative ion TIC and LC chromatogram of the aqueous extract of *E. jambos* using the normal tune file, whereas the bottom figure is the negative ion TIC adjusting conditions for the cleavage of sugars. Three known compounds were isolated from *Eugenia jambos* namely myricetin-3-*O*-xylose-rhamnose, quercetin-3-*O*-xylose-rhamnose and quercetrin, which were active in the cyclooxygenase assay (72). The retention times for these three compounds in the TIC are 6.2, 10.2, and 10.7 minutes for myricetin-3-*O*-xylose-rhamnose (mw 596 amu), quercetin-3-*O*-xylose-rhamnose (580 amu) and quercetrin (448 amu), respectively (see top figures in Figure 13). Analyzing the same areas in the TIC obtained using the tune file for cleaving sugars, both ions at *m/z* 316 and 595 were observed in the area of 6.2 minutes corresponding to the aglycone and the glycoside, respectively. In the area of 10 to 11 minutes both of the quercetin-based glycosides yielded an ion at *m/z* 300, in addition to their parent ions at *m/z* 579 and 447 (see the bottom figures of Figure 13). While these results do not define the chemical identity of the compound any more than the previous techniques, they do confirm the glycosidic nature of the isolate, the number of sugars attached to it, and the nature (i.e. mass) of the aglycone.

DISCUSSION

The dereplication of natural products is becoming of even greater importance in optimizing the process of natural product drug discovery as the pace of evaluating natural product extracts for their biological potential has increased. Over the years, as new technologies have been introduced into analytical chemistry, they have been applied to detect known compounds before isolation and structure elucidation was initiated. However, it was not until concurrent advances in mass spectrometry and computerized natural product databases were made that dereplication at the level reported here could be achieved. Initially, chromogenic reagents were used to obtain information on the chemical classes of compounds found in natural product extracts (15,16,32). Though not very powerful in predictive nature as to the identity of compounds present, chemical class information was used in order to prioritize natural product extracts for subsequent isolation, for example, extracts that contained alkaloids were sometimes regarded as being of more importance than extracts that did not contain alkaloids (15).

The power of partially separating extracts with paper and thin-layer chromatographic techniques before subsequently spraying with chromogenic reagents, increased the ability of researchers to distinguish different classes of compounds from one another (16,18,34) and TLC

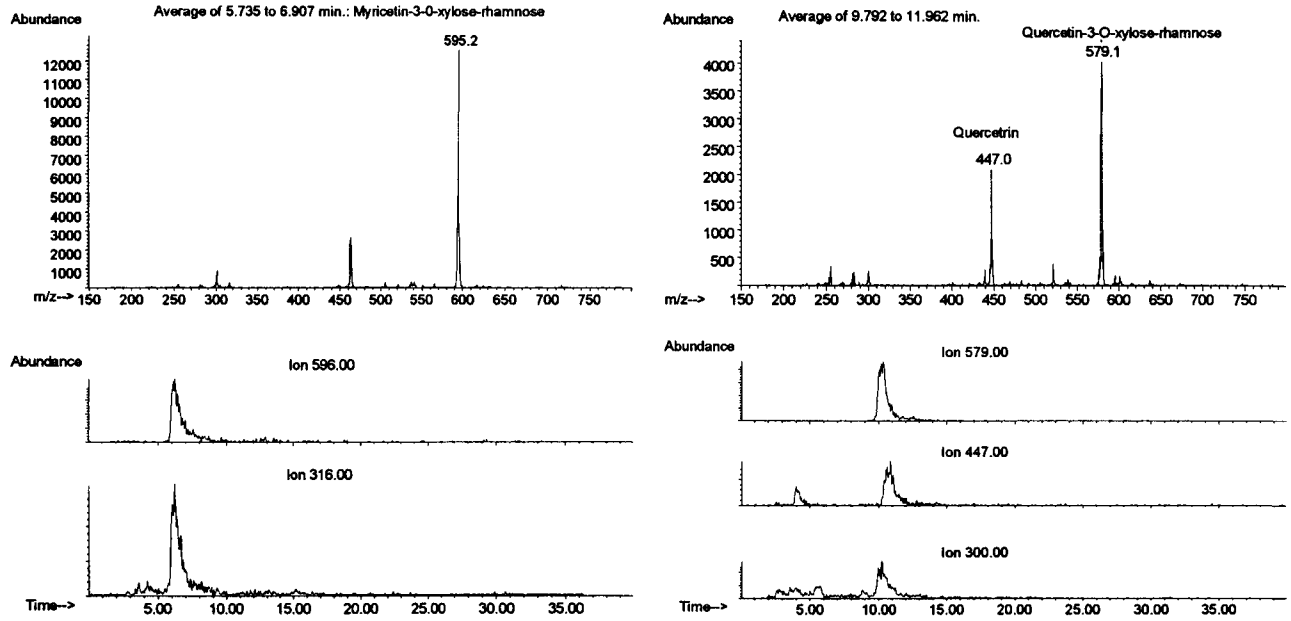


Figure 13. Mass Spectra in the normal mode of the flavonoids (top) and EIC of selected ions in CID mode (bottom) of *Eugenia jambos*.

techniques were of major importance to many natural product drug discovery groups and are still widely used today (18,35,36).

One of the most critical technologies to become available in the past decade and to advance the prioritization process was that of the computer. With the advent of small and powerful computers, information that is widely distributed and difficult to search, is now readily searchable using any parameter (chemical, taxonomic, or pharmacological) desired. This information has been used in a preselection or prioritization process that does not refer directly to an individual sample. However, it was not until the advent of LC/MS techniques, especially electrospray, that important information could be obtained quickly on the constituents of an extract. While there are many diverse analytical uses for this technology, those discussed here involve the chemical/biological dereplication of plant extracts. The resulting technique represents a merger between the instrumentation of the EIS LC/MS, and the information processing of large databases comprising natural product information.

Mass spectral data, though not unique to a single compound, represents a very powerful piece of information. Together with databases of known natural products, a molecular mass can be used to decrease the possibility from over 80,000 natural products to a few hundred or less. Successive application of UV/VIS information obtained by the photodiode array detector, the genus of the extract under investigation, and finally, the addition of previously reported biological activity reduces these possibilities even further. In the example of *Mesua ferrea*, the projected mass of the active metabolite was 406 amu, which corresponded to 135 compounds in the NAPRALERT database (list I). Compounds that have been previously reported to be active in similar assays (list II) and had the MW of 406 amu numbered only ten (area X and Y). The number of compounds that were reported isolated from the genus *Mesua* totaled fifty (list III). Merging all three datasets together (area X), only one compound was identified that has a molecular weight of 406 amu, was previously isolated from the genus, and was active in a similar assay; that compound was mammeisin.

On subsequent isolation and structure elucidation, the compound that was isolated was identified not as mammeisin, but rather mammea A/AB. Although the method did not suggest the actual compound that was isolated, in this instance, retrospective analysis indicates that this compound had never been isolated from this genus previously and no information on the cytotoxicity had been published. As a result of this investigation, a metabolite has been discovered as having a biological activity (cytotoxicity) not previously reported. More important is that the dereplication process did provide significant, highly specific information to the natural product chemist about the nature of the active principles, namely, the prenylated 4-phenylcoumarins. Additionally, the

chromatographic process was able to assure that the sample could be subjected to fractionation, and that the relative lipophilicity of the active agent was understood. By localizing the activity to two distinct areas, it could be understood that at least two distinct active compounds were present.

The dereplication process did succeed in proposing the specific nature of the active compounds that were subsequently found in the extracts of *Allamanda blanchetii, Ratibida columnifera,* and *Rubia cordifolia*; these were plumericin/isoplumericin, 9α-hydroxy-*seco*-ratiferolide-5α-*O*-angelate, and cyclic peptide RA5, respectively.

In the case of *Eugenia jambos,* mass and UV/VIS information was obtained on the active compounds in the extract. The active compounds were, indeed, flavonoid glycosides and, collision-induced dissociation was able to cleave the sugars and leave the aglycone intact. Information on the aglycone moiety was then achieved for the three compounds. Thus, this method, which is also useful for polar fractions, gives mass and UV/VIS information, and also provides mass information on the nature of the aglycone. This information can then be used to correlate with the information in the database on previously isolated compounds.

The dereplication process described here could form an integral part of many natural product drug discovery programs. Overall effectiveness of such programs can be substantially enhanced when the time and money expended on isolating and identifying known compounds is greatly reduced and the resources that are saved directed towards the identification and characterizication of novel, biologically active natural products.

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Siderophores from Fluorescent Pseudomonas

H. Budzikiewicz

1. INTRODUCTION

In the beginning life on earth developed in a reductive atmosphere where iron was available abundantly in its divalent form. Fe(II) salts are sufficiently water soluble to provide an adequate supply for this element which is essential for many physiological processes. But as a consequence of the photolytic cleavage of water, oxygen was set free and soon with rare exceptions only trivalent iron abounded. Due to the low dissociation constants of its various oxide hydrates, the concentration of free Fe^{3+} at pH-values around 7 is at best 10^{-17} mol/l while about 10^{-6} mol/l would be needed to maintain the necessary supply for living cells. Soil bacteria as well as the ones infecting animals or man (where the supply of iron is limited because it is bound to peptidic complexing substances as, e. g., transferrins), therefore, produce a variety of compounds which can form water soluble Fe(III) complexes, so-called siderophores.

Due to its high charge density, small ion radius and low polarisability Fe^{3+} is a hard Lewis acid and can bind strongly hard Lewis bases such as oxide ions. It forms octahedral d^5 high spin complexes and thus provides six coordination sites which can accomodate three bidentate ligands. The ligand types encountered most frequently among siderophores are catecholate or hydroxamate units. Because of their higher charge density, catecholates form stronger complexes at neutral pH, but tney are more acid-labile than hydroxamate complexes. Mixed systems are not uncommon and other ligand types (e.g., α-hydroxy-carboxylates, see also Section 2.1.3) are observed occasionally. The three bidentate ligands are often connected by aliphatic segments keeping them in place for complexation; this results in an entropic advantage over three non-connected ligands.

The members of the genus *Pseudomonas* are described as straight or slightly bent Gram-negative rods with one or more polar flagella, not forming spores and usually living under aerobic conditions. Many of them are soil bacteria, some are plant

TABLE 1

List of pyoverdins[a,b] from *Pseudomonas* spp.
a. Complete of fairly complete structures

No.	Refs.	Species	Name	Acids
1	(13,15,19)	*aeruginosa*	Py C-E (Pa)	G, S
2	(16)	*aeruginosa*	Py R	S
3	(17)	*aeruginosa*	Py Pa T II[f]	S
4	(18)	*aptata*	Py Pap	S
5	(8,20)	*fluorescens*	Ps	G, Gm, S
6	(21)	*fluorescens*	Py I-III	G, S
7	(22)	*fluorescens*	Py GM	S
8	(23)	*fluorescens*	Py Pf 12	G, S
9	(24)	*fluorescens*	Py Pf 2798	M, S
10	(12,25)	*fluorescens* [i]	Py Pf 13525	G, Gm, M, S
11	(26)	*fluorescens*	Py Pf 17400	S
12	(27)	*putida*	Ps 589A	M
13	(28)	*putida*	Py Pp 1,2	S
14	(29)	*putida*	Py Pp C2,3	M, S
15	(24)	*tolaasii*	Py Pt 2192	G, S
16	(30)	?	Ps 7SR1	M
17	(31)	?	Ps A214	S

b. Partial or Tentative Structures

No.	Refs.	Species	Name	Acids
18	(32)	*aeruginosa* [j]	Py Pa 15152	?
19	(32)	*aeruginosa* [j]	Py Pa UNK	?
20	(9,11)	*fluorescens*	Py SB83	?
21	(11)	*fluorescens*	Py Pf 2392	?
22	(33-35)	*fluorescens*	Py Pf 244	S
23	(9)	*fluorescens*	Py Pf W	?
24	(9-11)	*putida*	Py Pp 12633	?
25	(11)	*putida*	Py Pp 2461	
26	(36)	*putida* [l]	Ps Pp WCS 358	S
	(37)			S
27	(38)	*putida*	Py Pm	?
28	(39)	*syringae*	Py Ps	?
29	(40)	*syringae*	Py PSS	?
30	(9)	?	Py A6	?
31	(9)	?	Py LI	?
32	(9)	?	Py B10	?

Table 1 (continued)

	Peptide Chain[c]	Cycle[d]
1	Ser-Arg-Ser-FoOHOrn-Lys-FoOHOrn-Thr-Thr	Thr-Lys
2	Ser-Dab-FoOHOrn-Gln-Gln-FoOHOrn-Gly	Ser/Dab
3	Ser-FoOHOrn-Orn-Gly-aThr-Ser-cOHOrn	cOHOrn
4	Ala-Lys-Thr-Ser-AcOHOrn-cOHOrn	cOHOrn
5	Lys-OHAsp-Ala-aThr-Ala-cOHOrn	cOHOrn
6	Asn-FoOHOrn-Lys-Thr-Ala-Ala-FoOHOrn-Lys[h]	Lys-Thr[g]
7	Ala-Lys-Gly-Gly-OHAsp-Gln-Ser-Ala-Ala-Ala-Ala-cOHOrn	cOHOrn
8	Ser-Lys-Gly-FoOHOrn-Ser-Ser-Gly-Lys-FoOHOrn-Glu-Ser	Ser-Lys
9	Ser-Dab-Gly-Ser-OHAsp-Ala-Gly-Ala-Gly-cOHOrn	Ser/Dab cOHOrn
10	Ser-Lys-Gly-FoOHOrn-Lys-FoOHOrn-Ser	Ser-Lys
11	Ala-Lys-Gly-Gly-OHAsp-Gln-Dab-Ser-Ala-cOHOrn	Gln/Dab cOHOrn
12	Asp-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOrn[e]	cOHOrn
13	Ser-Thr-Ser-Orn-OHAsp-Gln-Dab-Ser-aThr-cOHOrn	Gln/Dab cOHOrn
14	Asp-BuOHOrn-Dab-Thr-Gly-Ser-Ser-OHAsp-Thr	-
15	Ser-Lys-Ser-Ser-Thr-Ser-AcOHOrn-Thr-Ser-cOrn	cOHOrn
16	Ser-OHAsp-Thr-Ser-AcOHOrn-Ala-Gly-Ser	Ser-Ser
17	Ser-Ala-Gly-Ser-Ala-OHAsp-aThr-AcOHOrn	-

	Peptide Chain	Cycle
18	2 Arg, 2 Orn, 3 Ser, 3 Thr	?
19	Ser-Thr-Ser-Gly-Orn-Orn	?
20	Ala-Lys-Thr-Ser-OHOrn-cOHOrn	cOHOrn
21	OHOrn-Lys-Gly-Thr-Thr-Gly-Gln-Ser-cOHOrn	cOHOrn
22	Ser-Lys-OHHis-aThr-Ser-cOHOrn[e]	cOHOrn
23	2 Ala, OHAsp, 3 Gly, OHOrn, Ser	?
24	Asp-Lys-OHAsp-Ser-Thr-Ala-Glu-Ser-cOHOrn[k]	cOHOrn
25	Asp-Lys-OHAsp-Ser-Thr-Ala-Lys-Thr-cOHOrn	cOHOrn
26	Lys-Ser-Asp-Thr-Ala-Thr-OHAsp-Lys-cOHOrn[l]	cOHOrn
	Lys-OHAsp-Ser-Thr-Ala-aThr-Lys-Asp-cOHOrn[l]	cOHOrn
27	OHAsp, Lys, OHOrn, 2 Ser, 3 Thr	?
28	Lys, OHOrn, 3 Ser, 3 Thr	?
29	2 OHAsp, Lys, 2 Ser, 2 Thr	?
30	Glu, 2 Gly, Lys, 2 OHOrn, Ser, 2 Thr	?
31	Ala, OHAsp, 2 Lys, OHOrn, Ser, 2 Thr	?
32	2 Ala, OHAsp, Lys, OHOrn, Thr	?

pathogens and *Pseudomonas aeruginosa* is amongst the most dangerous excitants of hospitalism (1,2). The rather large genus *Pseudomonas* is commonly divided into five rRNA-homology groups, one of them (I) comprises *i. a.* the so-called "fluorescents" (3). Their name derives from an early observation by *Gessard* (4): When grown under iron deficiency they excrete yellow-greenishly fluorescing substances into the culture medium. Originally these were called bacterial fluoresc(e)ins, but today they are referred to as pseudobactins or more commonly as pyoverdins (occasionally spelled pyoverdines). The genera *Azotobacter* and *Azomonas* produce the same type of siderophores. In view of the present discussion regarding a re-classification of the genus *Pseudomonas* and of related genera the production of pyoverdin-like compounds could be an important characteristic for taxonomic considerations.

Footnotes to Table 1.

[a] In part (a) D-amino acids are underlined; a broken line indicates either that the stereochemistry of the amino acid has not been determined or that a specific amino acid occurs both in the D- and the L-form, but a localization of the two enantiomers has not been effected.

[b] Abbreviations: Ps, pseudobactin; Py, pyoverdin; Pa, Pf etc. as part of the name indicates the origin, viz. *Ps. aeruginosa, Ps. fluorescens* etc. - a system not always used; G, 2-ketoglutaric acid; Gm, Glu; M, malic acid (amide); S, succinic acid (amide); OHAsp, *threo*-β-hydroxy-Asp; OHHis, *threo*-β-hydroxy-His; OHOrn, N^5-hydroxy-Orn; Ac(Fo, Bu)OHOrn, N^5-acetyl (formyl, R-β-hydroxy-butyryl) OHOrn; cOHOrn, *cyclo*-OHOrn (3-amino-1-hydroxy-piperidone -2); aThr, *allo*Thr.

[c] Bound to the chromophore **1** via the α-amino group of the N-terminal amino acid except for nos. 5 and 26 (N^6-NH_2 of Lys), and no. 16 (OH of one of the Ser).

[d] E.g., Thr-Lys indicates a large cycle formed by an amide or ester bond between the carboxyl group of the C-terminal amino acid and a side chain functionality of another amino acid; e.g., Ser/Dab refers to the condensation products with the structural unit **6**; for cOrn see Footnote b.

[e] With a N^6-Lys amide bond to the preceding amino acid (see, however, text).

[f] Probably identical with the pyoverdin of *Pseudomonas aeruginosa* ATCC 9027 (41).

[g] Ester bond.

[h] A pyoverdin with Asp instead of Asn was isolated from *Pseudomonas fluorescens* CHAO (42)

[i] The same pyoverdin was isolated from *Pseudomonas chlororaphis* ATCC 9446 (25).

[j] Either the preliminary structural work or the identification of the strains may be questioned since screening of a large number of *Pseudomonas aeruginosa* strains revealed the existence of only three subspecies caracterized by the production of the pyoverdins 1 , 2 or 3 (43).

[k] Probably identical with no. 12 (27)

[l] The two structures were reported differing somwhat in the sequence of the amino acids.

2. PYOVERDINS AND RELATED SIDEROPHORES

2.1 Pyoverdins

The formation of pyoverdins was first described by *Gessard* in 1882 (4). Over the years it was suggested that they were flavins (5), pyrrol derivatives (6) or pteridines (7), and it was not before 1981 that the structure of the first member of this class (see Table 1, no. 5) was established by chemical degradation, NMR- and X-ray studies (8). Sofar, 17 complete or fairly complete (mostly the stereochemistry of the amino acids has not been determined properly) pyoverdin structures were elucidated (Table 1). Usually several pyoverdins are excreted together which possess the same peptide chain, but differ in the nature of the dicarboxylic acid bound to the chromophore (see Section 2.1.3). Partial or tentative structures for further representatives were proposed based on incomplete degradation studies (giving, e. g., the composition but not the sequence of the peptide chain) or just on the fragment ions observed in their FAB mass spectra. Structural conclusions derived from a FAB mass spectrum alone can be dubious: For the pyoverdin Pf 13525 (Table 1, no. 10) a C-terminal sequence Ser-Lys-cOHOrn was deduced from the mass spectrum (9-11) while a structure analysis based on chemical degradation and NMR spectroscopy established the sequence Lys-FoOHOrn-Ser (12) (for the symbols see the footnotes to Table 1).

2.1.1 Production and Isolation of Pyoverdins. *Pseudomonas* strains are preferably grown in an aerated artificial medium (pH 7-8) with Na gluconate as the only carbon source (19,44). Commercial gluconate contains a sufficient amount of Fe^{3+} for the bacterial metabolism, but since the growth rate of the bacteria and the production of pyoverdins are governed inversely by the Fe^{3+} concentration, it is preferable to remove all Fe^{3+} from the gluconate by ion exchange chromatography and to add a definite amount to the culture medium (44).

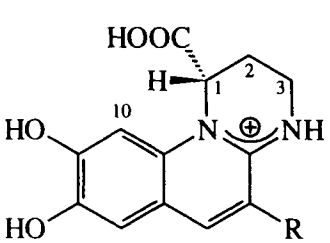
As pyoverdins are sensitive to acid and to O_2 it is advisable to complex them by addition of Fe^{3+} citrate prior to isolation. For the isolation several methods have been described in literature: One of them relies on an extraction with a 1:1 mixture of $CHCl_3$ and phenol or benzyl alcohol (8,45). After addition of ether the Fe^{3+} complex can be transferred back into an aqueous phase. This procedure requires large amounts of solvents. An alternative comprises successive chromatographic steps starting with an adsorption on XAD-4 resin, ion exchange chromatography, separation

and purification of the components by reversed phase HPLC and decomplexation with 8-hydroxyquinoline or K oxalate (19,22,46). A chromatographic separation using Cu^{2+}-chelate adsorbents was also suggested (47). An extraction method combined with a chromatographic purification was described recently for uncomplexed pyoverdins (48).

2.1.2 Physical Properties. Pyoverdins can be easily recognized (and quantitated) (44) by their UV/Vis-absorptions. At pH 7 the Fe^{3+}-complexes show maxima at ~400, 320 and 280 nm related to the quinoline system (19,10), as well as an amide band at ~230 nm. Their reddish-brown color results from the broad charge transfer bands at ~470 and 550 nm. Between pH 3 and 8 the maximum at 400 nm is essentially pH independent for the Fe^{3+}-complexes (16,18,20,22, 23,44). However, it shows a pronounced pH dependance for the free pyoverdins. This effect has been attributed to the degree of deprotonation of the phenolic OH groups: A shift from ~410 nm at pH 9.6 to ~370 nm at pH 3.0 accompanied by a splitting to a double maximum is observed (20,49). Most metal complexes except Fe^{3+} show a bluish-green fluorescence (475-510 nm);this was shown for pyoverdin II (Table 1, no. 6) (21).

The complexing constants of pyoverdins vary between 10^{24} and 10^{26} at pH 7.0 and between 10^{17} and 10^{20} at pH 5.0 (16,22,23,39,40, 49).

2.1.3. Structure of the pyoverdins. Pyoverdins consist of three distinct structural parts, viz. the dihydroxyquinoline chromophore **1**, a peptide chain bound to the carboxyl group of **1** usually (but not necessarily - Table 1, nos. 5, 16, 26) by its N-terminus, and a dicarboxylic acid or its monoamide connected amidically to the NH_2-group of **1**.



1: R=NH_2
2: R=OH

The chromophore. The common structural element of all pyoverdins is the chromophore (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1*H*-pyrimido-[1,2-a]quinoline-1-carboxylic acid (**1**), the catechol unit being one of the binding sites for Fe^{3+}. Only a

careful hydrolysis of a pyoverdin will yield **1** which is easily transformed into **2**, the compound isolated under the conditions usually applied for the degradation of the peptide chain (50,51). The S-configuration at C-1 was established for pseudobactin (Table 1, no. 5) by X-ray analysis (8), and for all pyoverdins isolated in this laboratory the CD-spectrum of **2** obtained by hydrolysis was compared with that of **2** from pseudobactin (20). They all gave identical values ($\Delta\varepsilon$ +0.6 at 365 nm, -0.9 at 296 nm, -1.8 at 253 nm (50)). The S-configuration of **2** could be verified by ozonolyis which yielded L-2,4-diaminobutyric acid (50). A direct correlation of pyoverdins by CD is not reliable (thus, the R-configuration given in (21) for the pyoverdin no. 6 (Table 1) based on the CD-spectrum is wrong (13); the free siderophores and their Fe^{3+} complexes may even give contradicting results). Racemic **1** being protected at all functional groups was obtained by synthesis (52).

The peptide chain. The peptide chain has a two-fold function: It provides two of the ligands for Fe^{3+} in the correct position for complexation and it is responsible for the recognition of the complex at the cell surface. The shortest chains encountered comprise six (e.g., formula **3**, no.5 in Table 1) and the largest ones twelve amino acids (no. 7 in Table 1). About half of the amino acids of a given pyoverdin are D-configurated. The changes in configuration along the peptide chain are necessary for placing the ligands for Fe^{3+} in the correct position. The presence of a large number of D-amino acids prevents a degradation by proteolytic enzymes. The high percentage of polar amino acids provides the pyoverdins water soluble.

The peptide chain is linked to the carboxyl group of **1** in most cases by its N-terminus, but exceptions are known (Table 1, no. 5 - formula **3** - and 26 - ε-NH_2 of Lys, no. 16 - OH of Ser). The amino acids of the peptide chain are connected by peptide bonds (i.e., the α-carboxyl group of one amino acid with the α-amino group of the next one). For no. 12 (Table 1) an amidic linkage between the ε-N-amino group of Lys and Asp was proposed on NMR evidence. A free α-amino group of Lys corresponds with the results of an Edman degradation. However, isolation of Lys dansylated at its ε-amino group after hydrolysis of the dansylated pyoverdin rather suggests a connection via the α-amino group (cf., however, ii-b below). No. 22 (Table 1) is another example for which a Ser-N^6H_2-Lys bond was suggested, but no experimental details are given here.

The methods used for the analysis of the peptide chain can be summarized as follows:

(i) The nature of the amino acids present is established by total hydrolysis, derivatization and identification by GC/MS (e.g. as N/O-trifluoroacetyl *n*-butyl or *i*-propyl esters); their chirality is subsequently determined by GC on a chiral column.

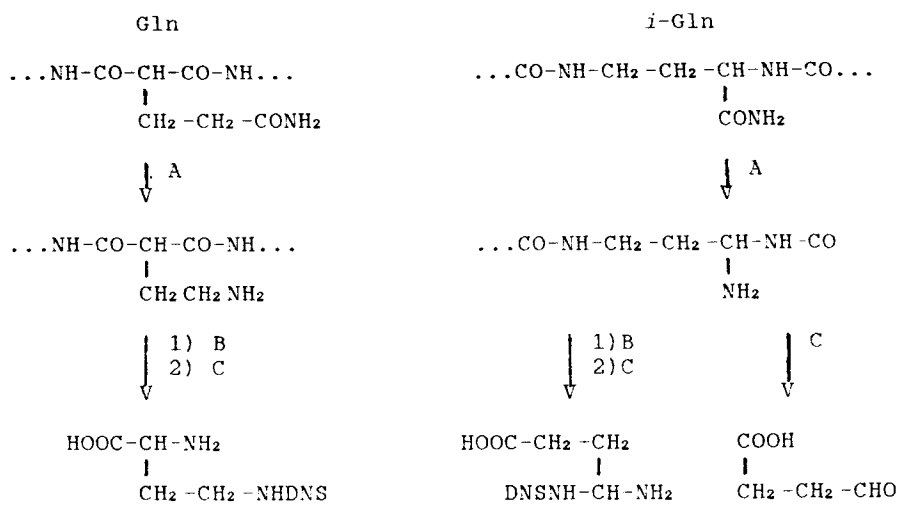
(ii) Additional functional groups of amino acids have to be accounted for. Whether COOH-, NH_2-, or OH-groups are free or not can be shown by chemical or spectroscopic techniques:

(a) Electrophoretic mobility. The number of net charges of (ferri) pyoverdins can be established by paper electrophoresis according to the method of Offord (53) by determining their mobility relative to desferal (+1) with glucose as zero point. At pH 6.5 a ferripyoverdin without any basic or acidic functionalities has a net charge of 0 if Fe^{3+} is complexed by **1** and two hydroxamate units (4+ for **1** protonated at the amidine unit plus Fe^{3+}, 4- for 4 H^+ replaced by the complexation) and a net charge of -1 if Fe^{3+} is complexed by one hydroxamate and one α-hydroxy acid unit (replacement of 5 H^+). Each free carboxyl group adds 1-, each free NH_2- (Lys, Orn) or guanidino group (Arg) 1+ charge. $CONH_2$-groups are neutral. At pH ~3 carboxyl groups with a pK 4-5 will be protonated (16,18,19,21-23). An electrophoretic method ("isoelectrofocusing") was developed for fast screening of bacterial isolates (54).

(b) By derivatisation. Free NH_2- groups (Lys, Orn), but not OH- or SH-groups react with maleic anhydride. The mass increase of 98 u for each NH_2 can be determined by mass spectrometry. However, this method does not allow to differentiate between a free α- or terminal NH_2-group. This is possible by dansylation, hydrolysis and comparison of the chromatographic behavior of the dansylated amino acid with that of authentic material (21). The discrepancies for no. 12 (Table 1) mentioned above could be explained (27), if with the solvent system used α- and ε-dansyl-Lys showed the same R_f-values (α-dansyl-Lys was not available).

(c) By degradation. Asn and Gln incorporated in a pyoverdin give by Hofmann degradation 2,3-diaminopropionic and 2,4-diaminobutyric acid, resp., with a free terminal NH_2-group, which upon dansylation can be identified as described in (a) (22) (Scheme 1). Free α-amino groups are amenable to Edman degradation (8). Ester bonds (Ser, Thr) can be cleaved reductively with $NaBH_4$ (21) or by mild hydrolysis (10% aqueous K_2CO_3 + CH_3OH 1:1 at room temperature)

wich does not affect amides or hydroxamic acids (7,21).



Scheme 1. Differentiation between Gln and *i*-Gln incorporated into a peptide chain by Hofmann degradation with [bis(trifluoroacetyl)-iodo]benzene (A), dansylation (B) and hydrolysis with HCl (C) (DNS = dansyl) (22).

(d) By NMR. Both, the ^{1}H- and the ^{13}C-signals of the β-CH_2-(Ser) and the β-CH-group (Thr) are shifted downfield upon esterification (see Table 2). The ^{13}C-resonances of free carboxyl groups (Asp, β-OH-Asp etc.) and of the adjacent α-C-atoms show - not very pronounced (1-2 ppm) - shifts when going from pH 3.0 to 6.8 (29). This can be taken as a corroborating evidence for conclusions drawn from the molecular mass (-COOH and -$CONH_2$ differ by 1 u) and from the net charge (-$CONH_2$ ±0, -COO^- -1 at neutral pH).

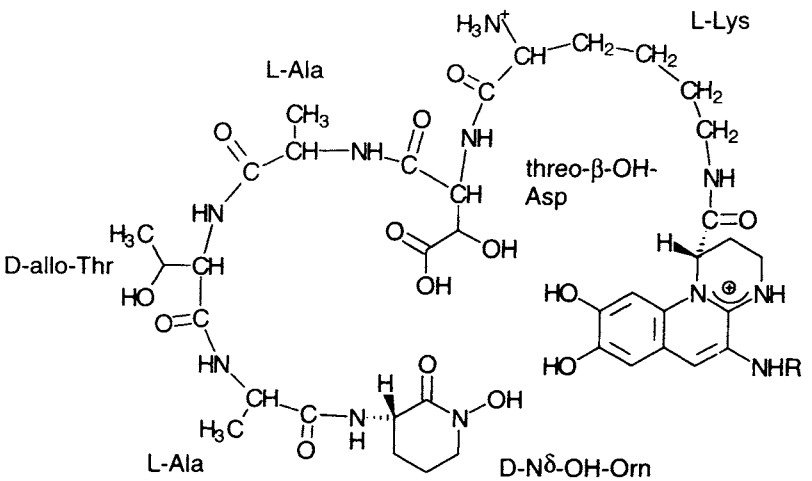
(iii) Sequentional information can be obtained by a combination of FAB-MS, partial hydrolysis and subsequent analysis of the fragments thus obtained, and by two-dimensional NMR studies. The best strategy has to be determined empirically for every new pyoverdin: Sequentionally significant ions may be missing in the FAB-mass spectrum, the same amino acid may occur several times in the chain, NMR signals may coincide, the coiled structure of the chain may give rise to a spatial vicinity and hence to NOE of sequentionally remote groups, etc.

(iv) If the same amino acid occurs in its L- and D-form (as in the case of no. 7 (Table 1) which comprises the sequence L-Ala-D-Ala-D-Ala-L-Ala) partial degradation and isolation of adequate

TABLE 2

^{1}H and ^{13}C chemical shifts (ppm) of $\underline{CH_2}OX$ and $\underline{CH}OX$ of free (X=H) and esterified (X=COR) Ser, Hse and Thr in D_2O (values for the first amino acid bound to the chromphore **1** due to shielding or deshielding effects may differ from the values given below).

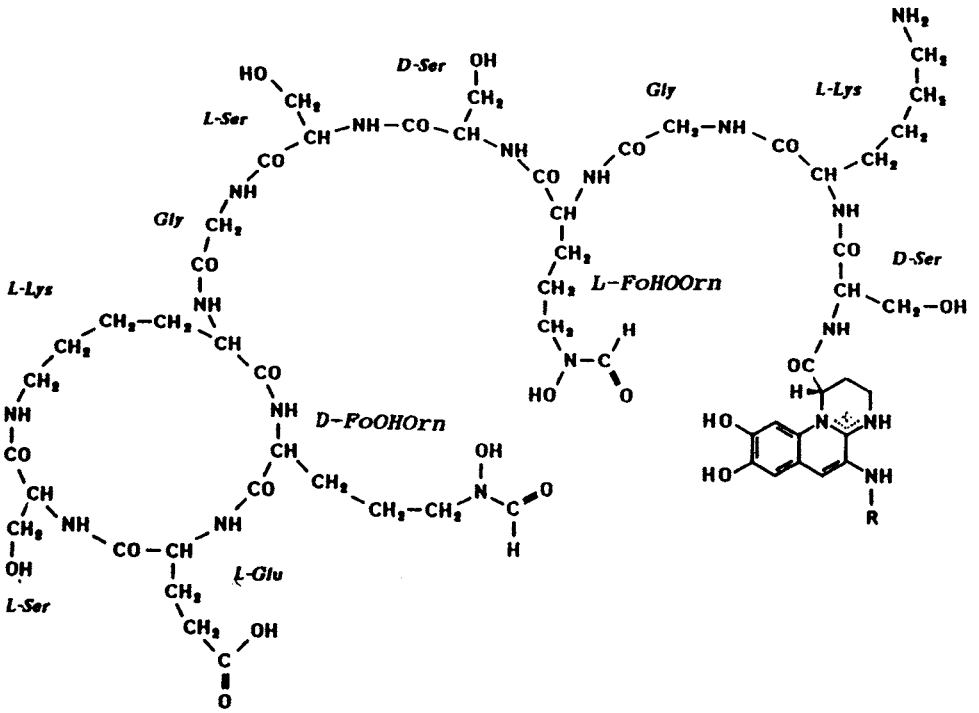
Amino acid	Group	Free OH	Ester	References
Ser	$^{1}CH_2O$-	3.9-4.1	~4.5	13,16,17,22,29,55
	$^{13}CH_2O$-	61.9-62.1	~65.4	
Hse	$^{1}CH_2O$-	3.6	4.5	56
	$^{13}CH_2O$-	58.8-59.2	68.6	
		60.7	70.3	57
Thr	^{1}CHO-	4.3-4.5	5.3	13,21,29
	^{13}CHO-	67.4-69.1	74.2	



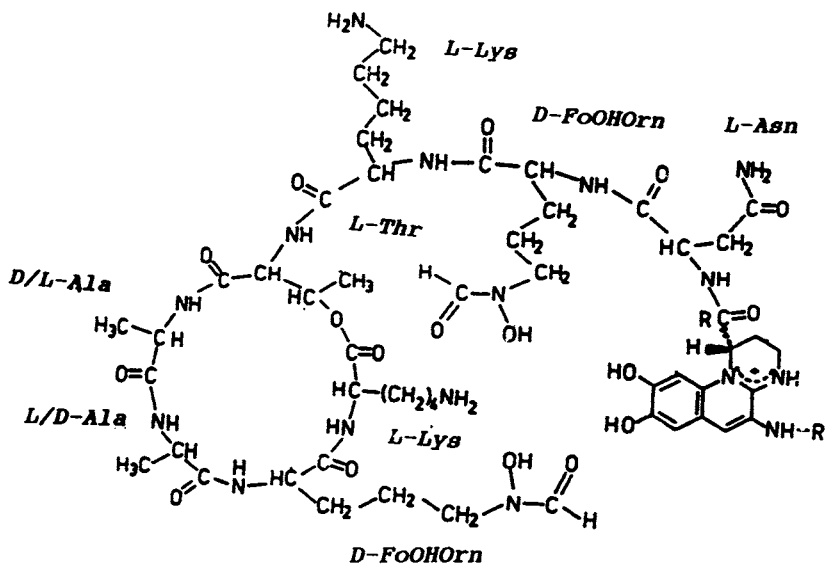
3: R = G or S

fragments becomes inevitable in order to locate their position in the peptide chain.

(v) Parts of the peptide chains of many pyoverdins form cyclic substructures. Most common is a C-terminal *cyclo*-Orn (3-amino-piperidone-2) ring (as in **3**) which will be discussed below. Larger cycles comprising several amino acids can be formed between the C-terminal carboxyl group and either by an amide bond with the ε-amino group of an in-chain Lys (as in **4**) or by an ester bond with Ser or Thr (as in **5**). A lactone ring can be opened reductively with



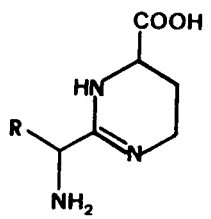
4: R = G, Gm or S



5: R = G or S

$NaBH_4$ (21). The analyis of the reduction product reveals then the structure of the depsipeptide ring. In the case of a lactame ring it will be necessary to isolate the cyclopeptide part by partial hydrolysis and to determine the sequence of the amino acids and the connectivity of the cycle independently (13,23). Also, long-range coupling NMR experiments may be applied (12,25).

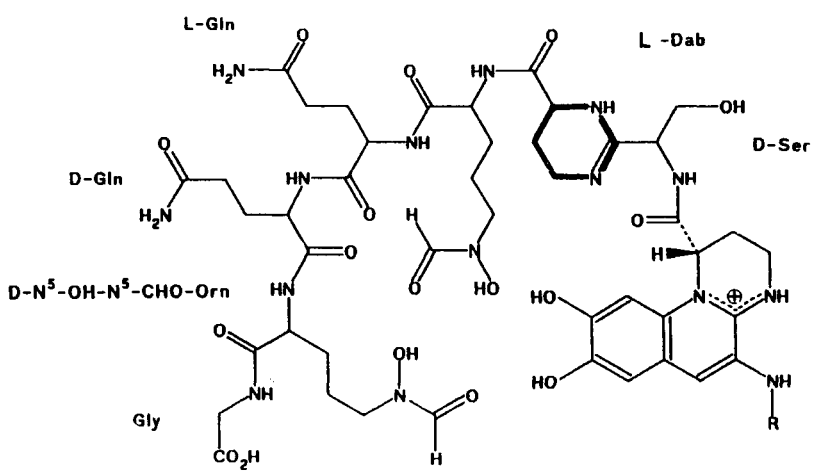
Occasionally condensation products of the two amino groups of 2,4-diaminobutyric acid (sofar only L-Dab) with the carboxyl group of another amino acid are encountered (in Table 1 indicated, e.g., by Ser/Dab, nos. 2 and 9) yielding a tetrahydropyrimidine ring (**6**)



6

as in **7** (cf. also Sections 2.6 and 2.7.3). Extensive NMR (16,24, 26,28,58) and synthetic (59,60) studies were necessary to establish the correct structure of these condensation products.

L-N^5-OH-N^5-CHO-Orn



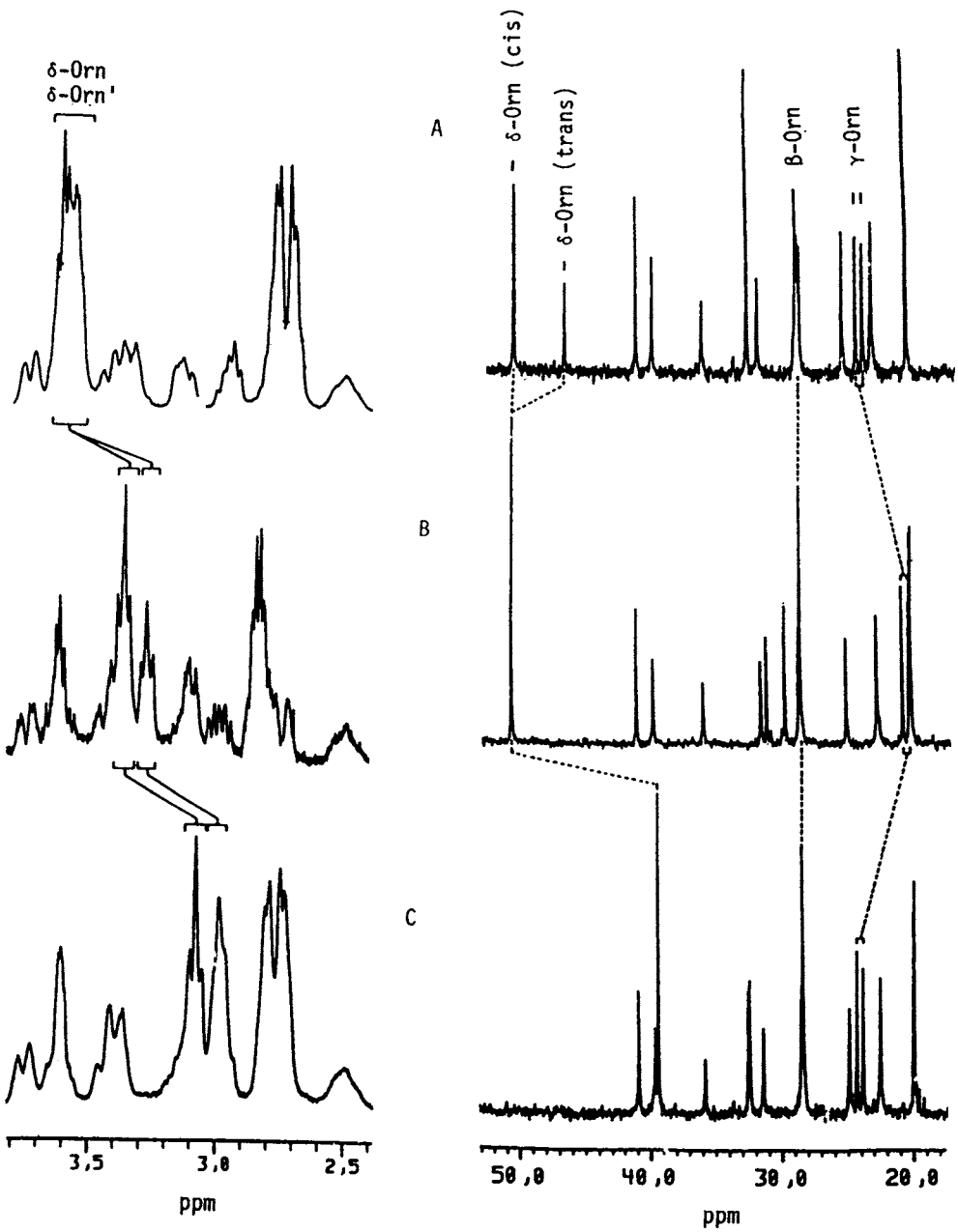
7: R = S

The synthesis of the peptide part of the pyoverdin no. 5 (Table 1) was reported recently (61).

Complexing ligands. One of the binding sites for Fe^{3+} is the catecholate system incorporated in the quinoline chromophore **1**. The other two are located in the peptide chain. They are either one β-hydroxy aminoacid (*threo*-β-hydroxy-Asp or *threo*-β-hydroxy-His) and one hydroxamic acid or two hydroxamic acids derived from Orn. Either the δ-amino groups of two Orn are transformed into N-acyl-N-hydroxy residues (where the acyl group can be formyl, acetyl or β-hydroxybutyryl, see Table 1) or one Orn is cyclized and hydroxylated to 3-amino-1-hydroxy-piperidone (N-hydroxy-*cyclo*-Orn) which forms the C-terminus of the peptide chain as in **3**. All hydroxamic acids are characterized by the facile loss of their acyl group after treatment with diluted acids (21) (in the case of N-hydroxy-*cyclo*-Orn resulting in ring opening (22) to give N-hydroxy-Orn). The hydroxyl group may then be removed reductively (thus HI hydrolysis of pyoverdins leads to Orn, HCl hydrolysis to N^5-hydroxy-Orn, but usually accompanied by some Orn). Alternatively, the hydroxamic acids can be reduced with $TiCl_3$ (22) to give the corresponding amides. For a confirmation of the presence of hydroxamic acids in the course of a structure elucidation these transformations can readily be followed by ^1H- and ^{13}C-NMR (see Figs. 1-3). The ^1H-signals of the formyl (21), the acetyl (18) and the α-CH_2-group of the β-hydroxybutyryl (29) residue are split in a ratio of about 3:1 due to the equilibrium of *cis*- and *trans*-forms (Scheme 2, cf. Fig. 4) (18,21).

The dicarboxylic acids. Usually several pyoverdins are found in a given fermentation broth which have identical peptide chains, but differ in the nature of the dicarboxylic acid bound to the NH_2-group of the chromophore **1**. Sofar Glu, α-ketoglutaric acid, succinic acid (amide) and malic acid (amide) were found. The presence of α-ketoglutaric acid can be shown by the isolation of α-hydroxyglutaric acid after reduction with $NaBH_4$ and hydrolysis with HCl (hydrolysis with HI gives Glu) (21,23). α-Ketoglutaric acid and Glu are bound to the NH_2-group of **1** by their respective γ-carboxyl groups. This was verified by chemical correlation for the pyoverdin no. 8 in Table 1: Its Glu substituent was transformed by treatment with glyoxalic acid/pyridine into an α-ketoglutaric acid side chain (23) which then could be degraded oxidatively with H_2O_2 to a succinic acid side chain (21),

$$\text{Py-NHCOCH}_2\text{CH}_2\text{CH(NH}_2\text{)COOH} \dashrightarrow \text{Py-NHCOCH}_2\text{CH}_2\text{COCOOH}$$
$$\dashrightarrow \text{Py-NHCOCH}_2\text{CH}_2\text{COOH}$$



Figs. 1 and 2. ^{1}H- and ^{13}C-shifts of Orn signals when going from (A) N-formyl-N-hydroxy-Orn in **4** to (B) N-hydroxy-Orn and finally (C) to Orn.

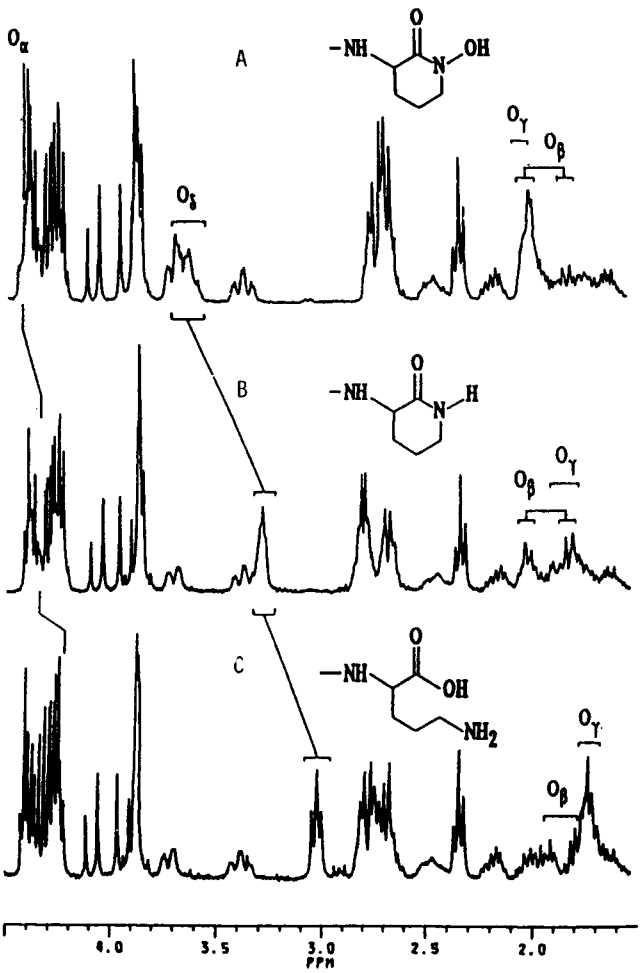
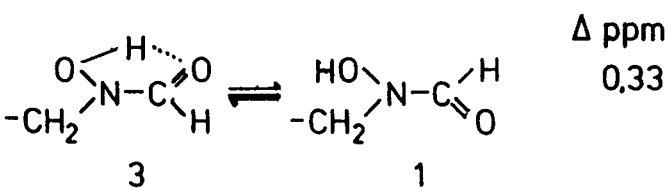


Fig. 3. ^{1}H-shifts of the Orn signals when going from (A) N-hydroxy-c-Orn in **3** (B) to c-Orn and finally (C) to Orn.



Scheme 2. *Cis/trans*-isomers of hydroxamic acids (FoOHOrn)

The ^{1}H- and ^{13}C-shifts in the NMR spectra of pyoverdins possessing a Glu side chain show a better agreement with the values for Gln than with those for *i*-Gln (amide of the α-carboxyl group) as can be seen from Table 3, although the differences are relatively small.

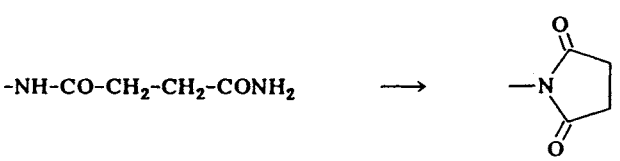
TABLE 3

^{1}H and ^{13}C chemical shifts (ppm) at pH 6.8 of Gln (C-5 amide) and *i*-Gln (C-1 amide) and of pyoverdins no. 5 and 8 (Table 1) carrying a Glu side chain bound with the C-5 carboxyl group to the NH_2 group of the chromophore **1** (19,22).

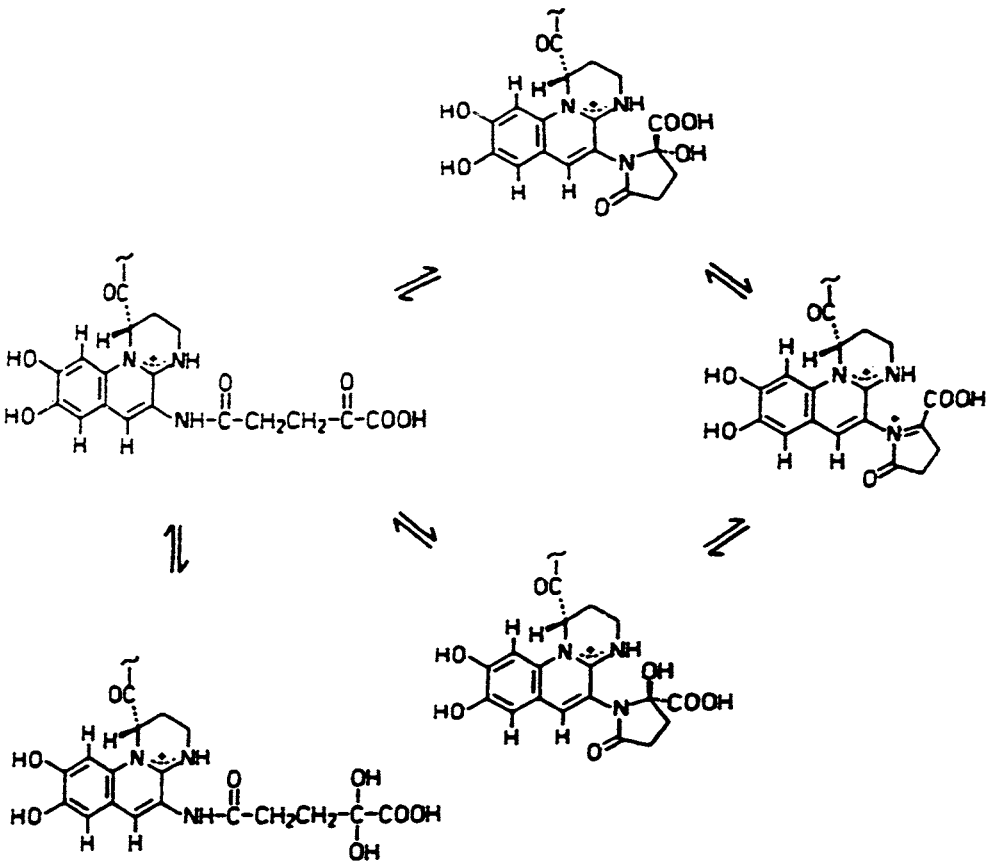
Compound		C-5	C-4	C-3	C-2	C-1
Gln	^{1}H		2.45	2.14	3.77	
	^{13}C	178.8	32.0	27.3	55.3	175.1
i-Gln	^{1}H		2.39	2.13	4.04	
	^{13}C	181.8	34.0	28.7	53.9	173.6
no. 8	^{1}H		2.75	2.25	3.83	
	^{13}C	176.8	32.4	26.7	55.0	175.2
no. 5	^{1}H		2.57	2.17	3.79	
	^{13}C	176.5	32.4	26.7	55.1	175.1

Malic acid is bound to the NH_2-group of **1** by its β-carboxyl group. This was concluded from NMR-evidence: When the ^{1}H-shift values of H_2NCO-*CH_2*-*CHOH*-$CONH_2$ are used for reference the ^{1}H-signal of the CH_2-group of the malic acid amide side chain of pyoverdin no. 16 (Table 1) shows a larger (0.26 ppm) downfield shift than that of the CHOH-group (0.20 ppm). This indicates that the CH_2-group is closer to the deshielding aromatic system of **1** (30). Also, the ^{13}C-signal of the CHOH-group of the malic acid side chain of pyoverdin no. 14 (Table 1) shows a larger shift (1.5 ppm) than that of the CH_2-group when going from pH 6.8 to pH 3.0 (29).

A succinic acid amide side chain is readily recognizable by the pH-independent CH_2-signals at ~2.7 and 2.8 ppm and ^{13}C-signals at 30.9 amd 31.8 ppm. At low pH values it partially cyclizes to a succinimide residue (Scheme 3) which gives rise to an additional CH_2-signal at 3.09 ppm accompanied by a downfield shift of several signals of **1** (13,18,21). The ^{13}C-signals of a succinic acid side chain show a pH-dependence similar to that described above for malic acid.



Scheme 3. Cyclization of succinamide bound to the chromophore **1**.



Scheme 4. Equilibrium structures of α-ketoglutaric acid bound to the chromophore **1**.

An α-ketoglutaric acid side chain forms equilibria between several open chain and cyclic forms (Scheme 4). In neutral solutions two diastereomeric lactams prevail: Instead of the expected CO-signal at ~200 ppm two broadened signals of equal intensity at ~94.3 and 95.5 ppm were observed e.g., for nos. 6 and 8 (Table 1) stemming from the tetrasubstituted C_α) (19,21,23). The effect of the various equilibrium structures on the ^{1}H-signals of the chromophore **1** can be seen in Fig. 4. They are broadened or even split (for numbering see formula **1**).

The various forms can be separated by HPLC to some extent (Fig. 5). *Cave* - re-chromatography of the isolated fractions may result in the same equilibrium mixture as before.

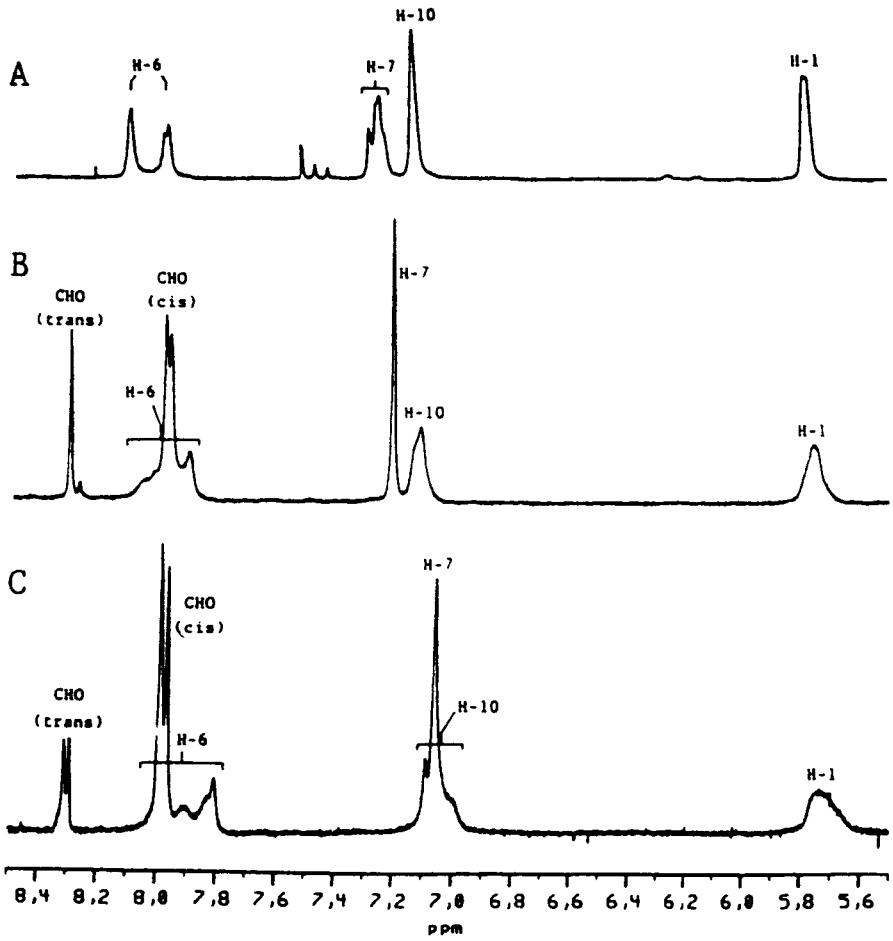


Fig 4. ^{1}H-signals of the chromophore **1** of desformyl-**4** (A) at pH 1.0, (B) at pH 3.0, and (C) of **4** at pH 6.8 showing the influence of the changing equilibrium structures of α-ketoglutaric acid (cf. Scheme 4 and Fig. 5).

2.1.4 Three-dimensional Structure of the Fe^{3+} Complexes

Theoretically an octahedral Fe^{3+} complex with three different unsymmetrical ligands (catecholate, two hydroxamate or one hydroxamate and one α-hydroxycarboxylate unit, see Section 2.1.4) can form 16 different coordination isomers (see Fig. 6), but for sterical reasons not all of them can be formed when the ligands are connected by an aliphatic chain. An X-ray analysis of the Fe^{3+} complex of no. 5 (Table 1) indicated a Λ-1 structure according to the numbering in Fig. 6 (8). To establish the solution structures of the Ga^{3+}-complexes of two further pyoverdins CD- and NMR-

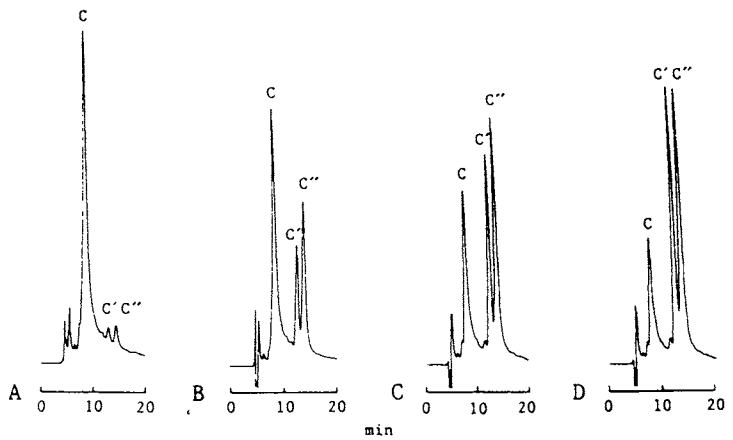


Fig. 5. HPLC chromatograms of **4** (R = G) showing th separation of equilibrium forms (c, c', c") of α-ketoglutaric acid (cf. Scheme 4) at different pH values (A: H_2O; B: H_2O/CH_3COOH 8:2; C: H_2O/CH_3COOH 4:6; D: H_2O/CH_3COOH 1:9).

experiments were performed. For no. 7 the conformation Λ-6 (62) and for no. 3 Δ-1 (63) was determined in this way. (Ga^{3+} has about the same ion radius as Fe^{3+}, but it is not paramagnetic and hence it allows NMR studies).

2.2 Dihydropyoverdins

Especially when the oxygen supply of the bacteria is somewhat limited during cultivation, in addition to the pyoverdins their 5,6-dihydro derivatives (chromophore **8**) may be found in the cultural broths. Co-occurring dihydropyoverdins and pyoverdins have the same peptide chain; sofar, Glu and succinic acid amide were reported as side chains. The dihydropyoverdins are colorless and do not show any fluorescence. They have absorption bands at ~225 and ~300 nm. Their Fe^{3+}-complexes are characterized by a pH-dependent broad charge transfer band responsible for their color (pH 3.2, 555 nm, bluish violet; pH 6.4, 530 nm, reddish violet; pH 11.6, 580 nm, blue). The 5,6-dihydro derivatives of nos. 5 and 13 (Table 1) are described in the literature (20,28,64). Dihydropyoverdins can be oxidized easily to the corresponding pyoverdins. Provided, 5,6-dihydropyoverdins are intermediates in the formation of **1** from **12** (see Section 2.7.3) and no configurational change occurs during biosynthesis C-5 should have R-configuration.

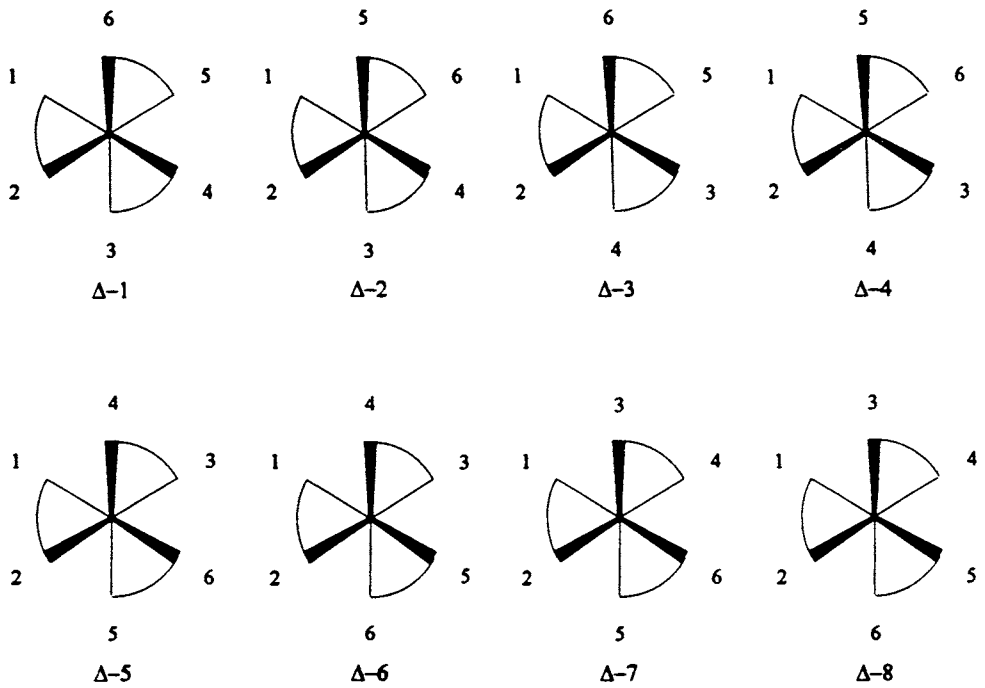
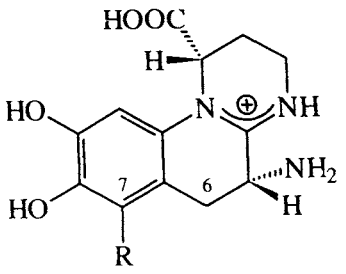


Fig. 6. The 8 possible Δ-coordination isomers of Fe^{3+}. The 8 Λ-isomers can be obtained as their mirror images by reflection on the paper plane.

2.3 5,6-Dihydropyoverdin-7-sulfonic acids

Compounds with chromophore **9** were discovered only recently accompanying the pyoverdins from strains of *Pseudomonas aptata* (no. 4 in Table 1) and *fluorescens* (no. 10). Their peptide chains are identical with those of the pyoverdins. Glu or succinic acid amide side chains were observed. The maximum of the charge transfer band of their Fe^{3+}-complexes is pH-dependent (cf. Section 2.2), and it is shifted to shorter wavelengths in comparison to dihydropyoverdins (pH 3.0 480 nm; pH 5.0 516 nm; pH 6.8 521 nm; pH 9.9 511 nm). The net charge at pH 6.5 is more negative (-1 due to the $-SO_3^-$ group) relative to that of the corresponding pyoverdin. The NMR spectra are highly characteristic (values for the compound derived from no. 12 in comparison with the 5,6-dihydropyoverdin derived from no. 13): The aromatic H-7-signal is missing, H-5 is shifted to higher field (5.04 instead of 5.50 ppm), H-6 to lower field (3.16 and 3.60 instead of 3.03 and 3.06 ppm). The ^{13}C-6-signal is observed at 26.5 instead of 29.1, ^{13}C-7 at 126.1 instead of 117.6 ppm (46).



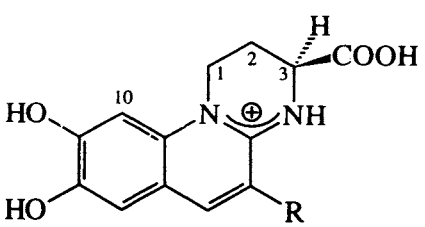
8: R = H
9: R = SO_3H

Aliphatic sulfonic acids are rather common in nature. Many of them are derived from cysteic acid, the oxidation product of Cys. Aromatic sulfonic acids have been unknown sofar the only exception being a phenazine derivative isolated from *Pseudomonas aeruginosa* (65).

2.4 Isopyoverdins

Pseudomonas putida BTP 1 produces an interesting structural variation to "standard" pyoverdins. Its chromophore carries the carboxyl group not at C-1 (**1**) but rather at C-3 (**10a**). The ^1H-NMR shifts of the aliphatic part of the cnromophore clearly differ (see Table 4). Most revealing are the cross peaks between the H-10-signal with *two* protons of a CH_2-group (3.97 and 4.45 ppm) instead with *one* lowfield proton of the C^{1}H-CONH-unit of **1**. From biogenetic considerations (see Section 2.7.3) C-3 should be S-configurated (66). The peptide sequence of isopyoverdin BTB 1 (for abbreviations see footnotes to Table 1) is

L-Asp-L-Ala-L-Asp-D-AcOHOrn-L-Ser-L-cOHOrn.



10a: R = NH_2
10b: R = OH

A second example is the siderophore isolated from cultures of *Azomonas macrocytogenes* named azoverdin. Intially (67) a pyoverdin structure (chromophore **1** with a succinamide side chain) had been assigned (68) with the peptide unit (Hse ... homoserine, cDab ... 2,4-diaminobutyric acid lactame)

L-Hse-D-AcOHOrn-D-Ser-L-AcOHOrn-D-Hse-L-cDab

which was subsequently corrected as to carry a C-terminal Hse/Dab cycle (**6**, R = CH_2CH_2OH) (69):

L-Hse-D-AcOHOrn-D-Ser-L-AcOHOrn-D-Hse/L-Dab.

Independent studies (70) demonstrate, however, the presence of an isopyoverdin chromophore (**10a**, succinamide - azoverdin, succinic acid - azoverdin A - and glutamic acid - azoverdin G - side chains) and a different arrangement of the amino acids in the peptide unit:

L-Hse-D-Hse/L-Dab-D-AcOHOrn-D-Ser-L-AcOHOrn.

In addition to the main pigments a 5,6-dihydro-azoverdin G (cf. Section 2.2) was found (71).

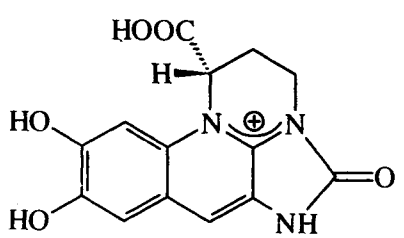
TABLE 4

^1H NMR data (ppm) the chromophores of pyoverdin no. 14 (Table 1) and isopyoverdin BTP 1 (in D_2O) as well as of **2** and of **10b** (in d_6-DSMO) (66).

Compound	H-1	H-2	H-3	H-6	H-7	H-10
no. 14	5.68	2.44/2.70	3.38/3.72	7.93	7.18	6.98
BTP 1	3.97/4.45	2.45/2.68	4.57	7.88	6.95	6.97
2	5.63	2.29/2.57	3.17/3.61	7.37	7.08	6.99
10b	3.96/4.50	2.42/2.42	4.39	7.45	7.11	7.21

2.5 Azotobactins

Azotobactins differ from pyoverdins to the extent that the amino group of the chromophore **1** is not bound to a dicarboxylic acid side chain. Instead, carbonic acid forms an additional ring by condensation to a urea structure (**11**, (1S)-8,9-dihydroxy-4-oxo-2,3,4,5-tetrahydro-1*H*,10c*H*-3a,5,10b-triazaphenantrylene-1-carboxylic acid). The configuration at C-1 was determined by comparison of the CD-spectrum of **11** with that of the chromophore **2** from no. 5 (Table 1) (56) (cf. Section 2.1.3). The UV/Vis absorptions of the free



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azotobactins and of their Fe^{3+}-complexes resemble those of pyoverdins (Sectiom 2.1.2). The free compounds show an intense green fluorescence differing clearly from the more yellowish one of

pyoverdins. Azotobactins were first isolated from *Azotobacter vinelandii*. For the analysis of the peptide chain the same strategies can be used as described in Section 2.1.3 for pyoverdins. The following peptide sequences were encountered (for abbreviations see footnotes to Table 1; Hse ... homoserine, Cit ... citrulline):

Az D: **7**-L-Asp-D-Ser-L-Hse-Gly-D-*threo*-OHAsp-L-Ser-D-Cit-L-Hse-D-AcOHOrn-L-Hse (57)

Az 87-I: **7**-L-Ser-D-Ser-L-Hse-Gly-D-*threo*-OHAsp-(1D;2L)Hse-Hse-Hse-D-R-BuOHOrn-L-Hse (56).

Azotobactins D and 87-I are accompanied by compounds with a C-terminal Hse-lactone (Az δ and A 87-II) which are most likely artefacts from the workup. A somewhat spurious azotobactin O from the same source was described (73). A quantitative analysis of amino acids was not performed and no molecular mass was determined. All structural information regarding the peptide chain stems from Edman degradations of peptide fragments isolated after partial hydrolysis. From these results the following most likely structure can be deduced (72):

Az O **7**-Asp-Hse-Ser-Hse-Cit-Ser-Gly-OHAsp

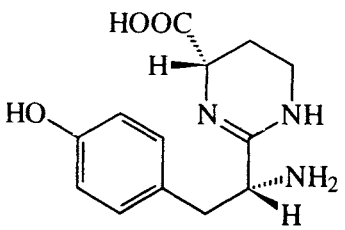
which has only 2 complexing sites for Fe^{3+}. It is, however, possible that parts of the peptide chain were not detected in the hydrolysate (in an earlier amino acid analysis (74) small amounts of Orn were found which were attributed (73) to a partial degradation of Cit; if they actually stem from an N-acyl-N-hydroxy-Orn unit a complete siderophore could be constructed). Two other pyoverdin type siderophores (probably azotobactins) from *Azotobacter vinelandii* UW were reported at a conference (75) containing OHAsp, Gly, Hse, FoOHOrn, Ser in the ratios 1:2:3:1:4 and 1:1:2:0:2, but no further data have been published since.

Recently azotobactins were also described which accompany pyoverdins having the same peptide chain as the latter, viz. no. 9 (Table 1) from *Pseudomonas fluorescens* (24) and no. 10 from *Ps. chlororaphis* (25).

2.6 Ferribactins

In the original publications (76,77) the name ferribactin was actually used for the Fe^{3+}-complex and the free ligand was referred to as desferri-ferribactin, but recently also the free ligands have been described as ferribactins. Their characteristic feature is the chromophore **12** which upon hydrolysis yields D-Tyr and L-Dab . Its structure was established by a careful NMR analysis

(58) (cf. also the condensation products **6** of the two amino groups of Dab with the carboxyl group of another amino acid discussed in Section 2.1.3). The Fe^{3+}-complexes (as do the free ligands) show the Tyr absorptions at 275 nm and in addition a charge transfer band whose maximum is strongly pH dependant (pH 3.0 ... 455 nm; 5.0 ... 430 nm; 6.5 ... 420 nm; 10.0 ... 380 nm; 12.0 ... absent). Accordingly the color changes from red to yellow and disappears at pH 12.0. In all ferribactins described sofar the NH_2-group of **12** is bound amidically to the γ-carboxyl group of Glu. Ferribactins were found to be minor components of the siderophore mixtures of *Pseudomonas* spp. having the same peptide chains as the accompanying pyoverdins. Those corresponding to the pyoverdins nos. 4 (18) and 10 (Table 1) (58,77,78) are reported in the literature.



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2.7 Biogenesis of Pyoverdins and Their Congeners

2.7.1 The Peptide Chain

Evidence has been presented (79,80) that the synthesis of the peptide chain occurs not via the classical ribosomal pathway, but rather through a multi-enzyme thiotemplate mechanism involving peptide synthetases. They activate the constituent amino acids as their adenylates (80) and are also responsible (81) for the L/D-isomerisation, as it had been shown for peptide antibiotics (82). L/D-isomerisation is suggested by the observation that the production of the pyoverdin no. 27 (Table 1) from non-producing mutants of *Pseudomonas putida* could be induced by auxotrophic Phe (cf. Section 2.7.3). The L- and the D-form (which is actually incorporated into **1**) are equally effective (83). High molecular mass (estimated as 180 to 600 kD) cytoplasmatic proteins only present in iron-starved pyoverdin-producing *Pseudomonas* spp. seem to be specifically involved in the synthesis (79). The only other piece of evidence regarding the biosynthesis of the peptide chain is the observation that the hydroxamic acid parts derived from Orn (OHOrn, AcylOHOrn) are preformed and incorporated as such into

the peptide chain (84).

2.7.2 The Dicarboxylic Acid Side Chains

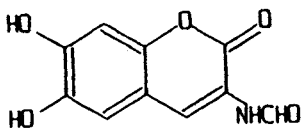
The dicarboxylic acids bound amidically to the NH_2-group of **1** (Glu, α-ketoglutaric acid, succinic and malic acid amide) are all elements of the citric acid cycle. Detailled studies (44) with respect to the relative amounts of the various acids and amides present during the growth period of *Pseudomonas fluorescens* showed that Glu and α-ketoglutaric acid form an equilibrium. Their ratio changes during the growth. Succinic acid amide is present from the start of pyoverdin production (in accordance with the isolation of 5,6-dihydropyoverdins - the probable biogenetic precursors of pyoverdins, cf. Section 2.7.3 - with this side chain). Free succinic acid is a hydrolysis product of the amide in the culture medium. Pyoverdins with a malic acid amide side chain appear with those with a succinic acid amide substituent in accordance with the progress in the citric acid cycle.

It is not clear how the azotoverdin chromophore **11** fits into this pattern. CO_2 formed during the oxidative decarboxylation of α-ketoglutaric acid to succinic acid might be the source for the urea structure.

2.7.3 The Chromophore **1**

In contrast to the rather scarce experimental data presented in Sections 2.7.1 and 2.7.2 a fair amount of evidence has been assembled regarding the formation of **1**. The isolation of ferribactins and 5,6-dihydropyoverdins as well as of a 5,6-dihydro-isopyoverdin (see Sections 2.3, 2.4 and 2.6) along with the corresponding pyoverdins (isopyoverdin) suggested that the former ones are actually biogenetic precursors of the (iso)pyoverdins. The ferribactin chromophore **12** was shown to be a condensation product of D-Tyr and L-Dab (58). The following feeding experiments correspond with this observation: [^{14}C]-Tyr is incorporated into **1** of no.2 (Table 1) from *Pseudomonas aeruginosa* (85) and [3-^{14}C]-Tyr into the pyoverdin no. 5 from *Ps. fluorescens* (86). The incorporation of Tyr into the chromophore **1** could be demonstrated with [2,3,3-$^2H_3$]-Tyr: ^{2}H was located by ^{2}H-NMR at C-6 of **1**. No incorporation was observed with [2',5',6'-$^2H_3$]-3,4-dihydroxy-Phe (DOPA). This indicates that the second hydroxyl group of **1** is introduced *after* the formation of **12** (86). Production of pyoverdin no. 27 from a non-producing mutant of *Ps. putida* could be induced by auxotrophic Phe (but not Tyr or DOPA) and [2-^{14}C]-Phe was incorporated into the pyoverdin (83). Phe-hydroxylase activity

was observed both for *Pseudomonas aeruginosa* (85) and *putida* (84).



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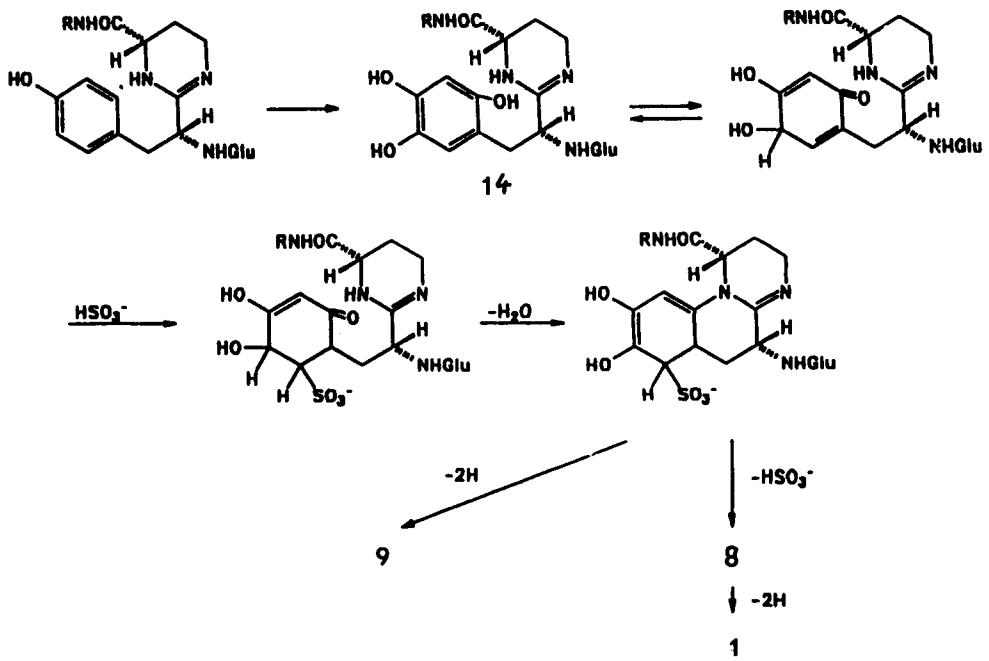
12 comprises all structural elements necessary for the formation of **8** by ring closure and introduction of the second hydroxyl group. This also explains the formation of isopyoverdins (Section 2.4) since ring closure should be possible with either of the nitrogen atoms present in the tetrahydropyrimidine ring. Of interest is the observation that a genetically modified non-fluorescent *Pseudomonas aeruginosa* mutant produces **13** with three hydroxyl groups in the benzene ring (87) (incorporation of ^{14}C-Tyr was also shown here (85)). It suggests that **12** (incorporated into the ferribactin molecule) is first hydroxylated to give **14**. One of its nitrogen atoms then attacks the carbon atom of the phenyl ring carrying the 2'-hydroxyl group. The recent discovery of dihydropyoverdin sulfonic acids with chromophore **9** (Section 2.3) suggests that ring closure occurs by the mechanism of a *Bucherer* reaction as depicted in Scheme 5.

A completely different scheme for the biosynthesis of **1** in pyoverdin no. 27 (Table 1) was suggested by a research group from Minsk (88,89). It is based on the observation that for non-fluorescent mutants of *Pseudomonas putida* M whose pyrimidine biosynthesis was blocked at different stages the production of the pyoverdin was restored by auxotrophic dihydroorotate. From this it was concluded that the latter (and, therefore, Asp rather than Dab) is the precursor of the tetrahydropyrimidine part of **1**, and that the carboxyl group of Phe is lost. The sequence shown in Scheme 6 was proposed were steps 4 and 5 should take place spontaneously (and not enzyme-catalyzed). This, however, would require a nucleophilic attack of the imine nitrogen on the benzene ring and a hydride shift, reactions not in agreement with the general rules of aromatic substitution. In any case, since dihydroorotate is the precursor of the nucleotide bases, its auxotrophic effect on the formation of the pyoverdin may lie on a competely different level (cf., e.g., Section 2.7.1).



Scheme 5. Proposed biogenesis of the pyoverdin chromophore **1** starting from the ferribactin chromophore **12**.

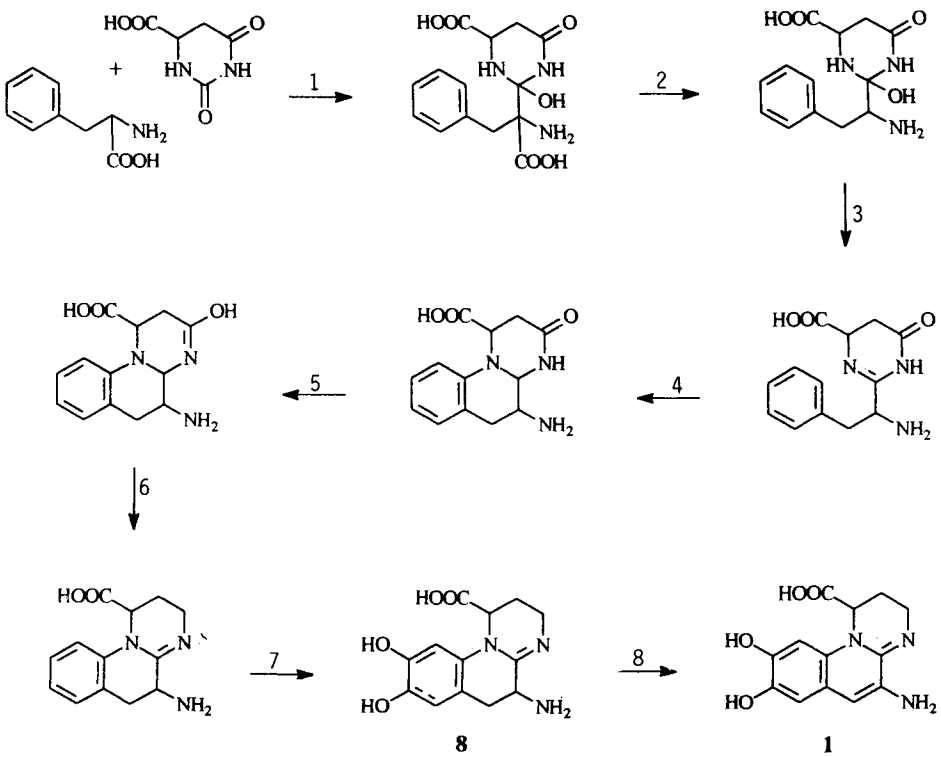
The opinions differ whether the peptide chain and the precursors of **1** are formed independently or not, but only circumstantial evidence could be adduced regarding this question (90).

3. OTHER IRON TRANSPORT SYSTEMS

3.1 Pyochelin and Related Compounds

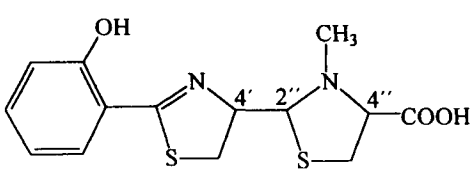
Pyochelin **15** (91) is an interconvertible mixture of the two epimers pyochelin I and II. It usually accompanies the pyoverdins and it is responsible for a second iron-transporting system. The relative and absolute stereochemistry of **15** was established (I: 4'R,2"R,4"R; II: 4'R,2"S,4"R) (92). A synthesis (condensation of salicylonitril with L-Cys, reduction of the carboxylic acid to an aldehyde function and condensation of the latter with N-methyl-L-Cys) was reported (92). **15** forms a red Fe^{3+} complex whose structure is not known. By feeding a mutant of *Pseudomonas aeruginosa* blocked in the salicylic acid biosynthesis with salicylic acid derivatives mutasynthetic analogs of **15** could be obtained (93).

Biogenetically **15** is a condensation product of salicylic acid and two molecules of Cys and thus its precursor is probably



Scheme 6. Proposed biogenesis of the pyoverdin chromophore **1** starting from dihydroorotic acid and Phe.

aeruginoic acid **16** first isolated from *Pseudomonas aeruginosa* (94). Subsequently also its decarboxylation and reduction products (aldehyde, alcohol) **17-19** (95,96), the dihydro products (+)S-dihydroaeruginoic acid (97) and the alcohol (+)aerugin (98) were found in various *Pseudomonas* species.



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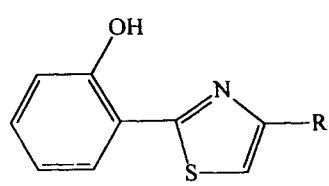
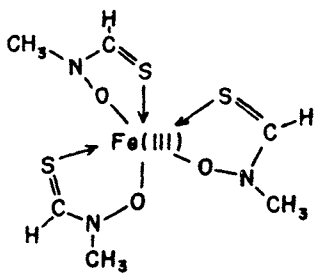


16: R = COOH
17: R = CHO
18: R = CH_2OH
19: R = H

3.2. Various Small Molecules

Salicylic acid (the biogenetic precursor of **15**) could be isolated from *Pseudomonas aeruginosa, cepacia* and *fluorescens* cultures. It acts as a siderophore for these species (99,100,101). N-Methyl-N-thioformyl hydroxylamine was obtained from the culture broth of *Pseudomonas fluorescens* KY 4032 in the form of its Cu^{2+} (two ligand molecules, fluopsin C) and Fe^{3+} (three ligand molecules, fluopsin F, **20**) complexes (102). An interesting condensation product of histamine, Thr and salicylic acid (**21**) was isolated from *Pseudomonas fluorescens* AH2 (100).



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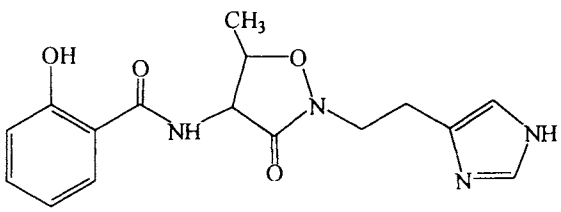

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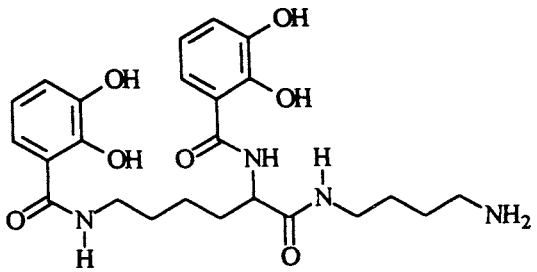
3.3. Catecholate Siderophores

The typical catecholate siderophores are derived from 2,3-dihydroxybenzoic acid attached to amino acids, amino alcohols or aliphatic diamines. Catecholate siderophores have only rarely been encountered with *Pseudomonas* spp. (a catecholate containing Arg from *Pseudomonas stutzeri* (103) and cepaciachelin **22** from *Pseudomonas* (*Burkholderia*) *cepacia* (104)), but not in the fluorescent group (cf., however, Section 3.4). This is in contrast to *Azotobacter vinelandii* where catecholates were found in addition to azotobactins (Section 2.5). Which type of siderophore is actually produced depends on the amount of Fe^{3+} available (105): At concentrations >7 µmol 2,3-dihydroxybenzoic acid with its rather low complexing constant seems to be sufficient for iron procurement. Between 7 and 3 µmol mono- (aminochelin (106), **23**), di- (azotochelin (107), **24**) and tricatecolates (protochelin (105,108), **25**) are produced, and finally at Fe^{3+} concentrations <3 µmol the high-affinity azotobactins are resorted to.



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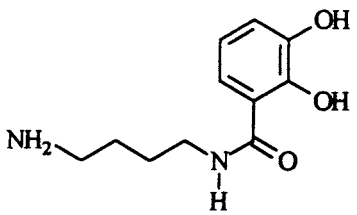

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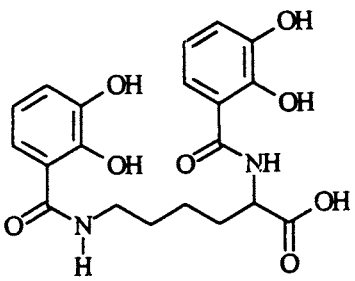

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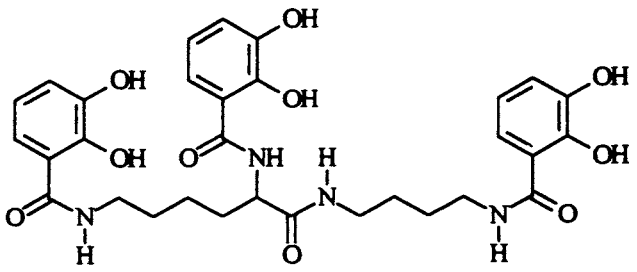

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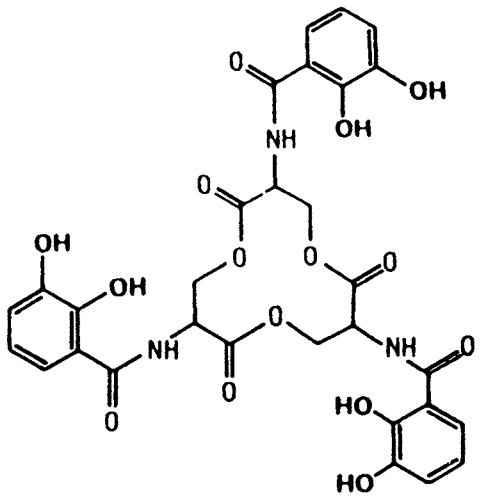
3.4. "Foreign" Siderophores

Although *Pseudomonas* spp. and even strains ("siderovars") usually excrete specific pyoverdins which are recognized only by the producing strain (cf. Section 4) occasionally the formation of a receptor for the pyoverdin of another species can be induced. Thus, *Pseudomonas putida* WCS358 can use pseudobactin (no. 5 in Table 1) from *Pseudomonas fluorescens* (109; cf. also 66). Even more interesting is the observation that also siderophores from other bacterial genera can be alienated. As stated in Section 3.3 fluorescent pseudomonads do not produce catecholates, but *Pseudomonas aeruginosa* can use enterobactin **26** produced by *Escherichia coli* (110) as well as its biosynthetic precursors 2,3-

dihydroxybenzoic acid and N-(2,3-dihydroxybenzoyl)-L-Ser **27** (111). Also desferri-ferrioxamine B **28** from *Streptomyces* spp. can be utilized (112). Other examples are *myo*-inositol hexakisphosphate (= phytic acid) **29** found in the soil and in most, if not all, plant and animal cells (113), and citrate (114,115).



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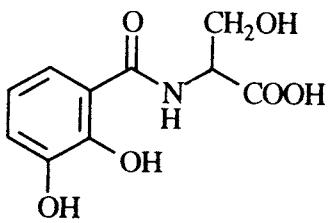

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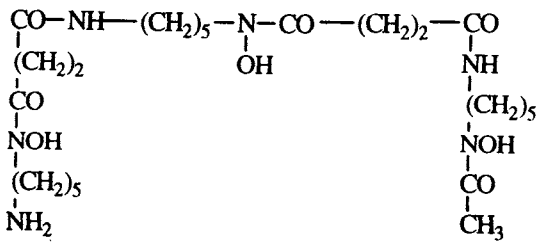

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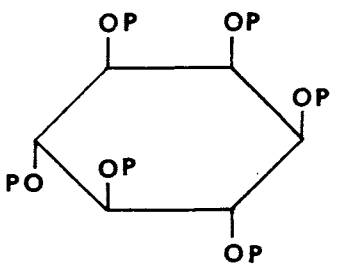

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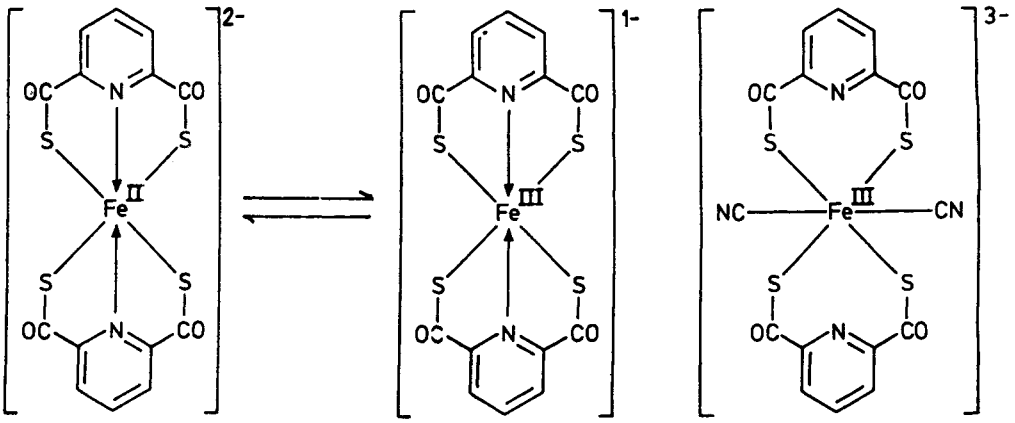
Even artificial siderophores may be used: Nitrilotriacetic acid, i.e. $N(CH_2COOH)_3$, can act as such for several *Pseudomonas* spp., but a specific receptor could not be detected. Related compounds as e.g., ethylenediamine tetraacetic acid (EDTA) were found to be inactive (116).

3.5 Pyridine-di(monothiocarboxylic) Acid and Related Compounds.

From the culture medium of *Pseudomonas putida* when grown under iron deficiency pyridine-2,6-di(monothiocarboxylic acid) was isolated. It forms a blue Fe^{2+}- and a brown Fe^{3+}-complex (**30**/**31**) (117). **30** may be accompanied by another complex carrying two

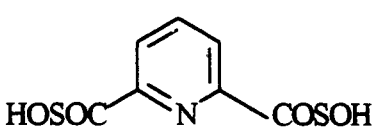
additional cyanide ions (**32**) (118) (CN^- is actually a genuine metabolite of *Pseudomonas* spp. (119,120) derived from the α-C of Gly).



30 **31** **32**

In addition to pyridine-2,6-di(monothiocarboxylic acid), compounds were found with hitherto unknown carbonyl sulfenic acid (-CO-SOH) groups in 2,6-position (**33**) (121, 122). As shown by labelling studies (123) they are the biosynthetic intermediates in the sequence -COOH ⟶ -CO-SOH ⟶ -COSH.

From iron-free cultures of *Pseudomonas fluorescens* 8-hydroxy-4-methoxy-monothiochinaldic acid **34** together with the chinaldic acid **35** could be isolated (124).



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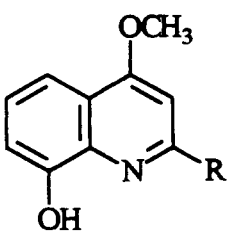

34: R = COSH

35: R = COOH

31 and **32** together with their Fe^{2+}-complexes form redox systems (125). Cyclovoltametric studies gave for **31** a redox potential E_0 = +0.381 V and for **32** +0.400 V relative to a hydrogen

electrode at pH 7.0. These values are comparable with those of certain cytochromes (117,118). It is unknown whether these complexes serve as siderophores or play a role in the reductive release of Fe^{3+} from the ferri-pyoverdin complexes (cf. Section 3).

A further way of securing iron consists in transcomplexation by making use of the high complexing constants of pyoverdins. Thus, the growth rate of *Pseudomonas aeruginosa* is increased dramatically when its own ferri-pyoverdin is added to the culture solution; it is enhanced with considerable delay when the ferri-pyoverdin of *Pseudomonas fluorescens* is added: The latter one is not recognized at the cell surface, but when production of the proper pyoverdin reaches a sufficient level, iron can be secured by transcomplexation (126).

4. SIDEROPHORE MEDIATED IRON UPTAKE

As mentioned before, pseudomonads possess different iron uptake systems, but the pyoverdin mediated one has been studied most extensively. It will be summarized here exemplarily (127). The variations in the peptidic chain of pyoverdins produced even by different strains of the same *Pseudomonas* species (see Table 1) suggest that a highly specific recognition mechanism is operating: e.g., *Pseudomonas aeruginosa* can be divided into three subspecies differing in their pyoverdins (43,128). As the structural requirements for the recognition of a pyoverdin at the receptor site of the cell surface are not known the degree of specificity is still a moot point (66). In any case, recognition seems to be associated with 80 to 90 kDa outer membrane proteins (37,112,129,130). ^{55}Fe- and ^{14}C-labelling studies indicate that ferri-pyoverdins do not enter the cytoplasm as an entity, i.e., that Fe is set free in the periplasmatic space (131) and is transferred to a bacterioferritin (132). The separation of Fe from the pyoverdin probably takes place by reductive dissociation: The redution potential for ferri-pyoverdin no. 1 (Table 1) is -510 mV relative to a normal hydrogen electrode well within the realm of cellular redox systems, and the stability of the Fe^{2+}-complex is about 20 orders of magnitude lower than that of the Fe^{3+}-complex (133) (cf. also Section 3.5). Ferri-pyoverdin reductase activity could be demonstrated for *Pseudomonas fluorescens* (134, cf. 135).

5. CONCLUSION

It was mentioned in the Introduction that various *Pseudomonas* spp. are associated with higher plants. Some are plant-pathogens

while the presence of others especially in the rhizosphere helps to suppress other plant-deleterious microorganisms (136,137) and/or increases the plant growth directly (138-140). Antibiotic as well as siderophore activities (by depriving competing bacteria of the essential iron as well as by providing it to the higher plants directly) seem to play a role likewise (141-143). Hospital infections by *Pseudomonas aeruginosa* were also mentioned (1) which affect especially severely injured patients and those whose immune system is weakened. An extremely critical situation exists for persons suffering from mucoviscidosis (cystic fibrosis) when *Pseudomonas aeruginosa* or *Ps. cepacia* infect the bronchial tubes (144). *Pseudomonas aeruginosa* is resistant against many of the common antibiotics (145,146). New therapeutic strategies may possibly be opened by using the tranport system of the siderophores as reduced membrane permeability is one of the reasons for resistance, or by interfering with the transport system: The action of siderophores seems to be essential for the virulence (147,148). A wide field of research is still open.

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