The Use of Heterocyclic Chemistry in the Synthesis of Natural and Unnatural Products

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Introduction

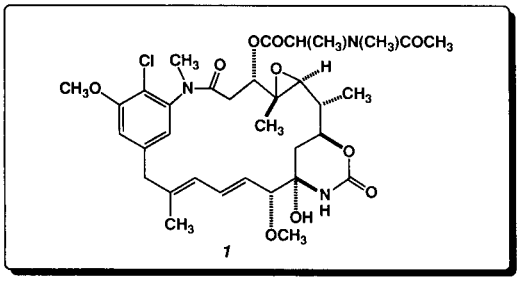
Today's lecture will consist of three topics which illustrate the utility of heterocyclic chemistry for the total synthesis of natural products as well as unnatural products which have been designed with a particular application in mind.

The first topic deals with a [3 + 2] dipolarcycloaddition approach to the total synthesis of the antitumor substance maytansine. We will then turn our attention to the total synthesis of the cytotoxic alkaloidal principle discorhabdin C, isolated from a marine sponge. In the third and final topic, mother nature does not provide the target for our synthetic efforts. Rather, in this exercise, we are concerned with both the design and synthesis of unnatural substances possessing specific biochemical properties which permit these materials to function as reagents for the automated sequencing of DNA.

TOPIC 1: An Intramolecular Nitrile-Oxide/Olefin [3 + 2] Dipolar Cycloaddition Route to the Maytansinoids.

Consider the structure of maytansine (**1**), an antitumor compound active against a wide variety of cancers, exhibiting microgram activity, and possessing a T/C of 220% against P-388 [1].

Maytansine



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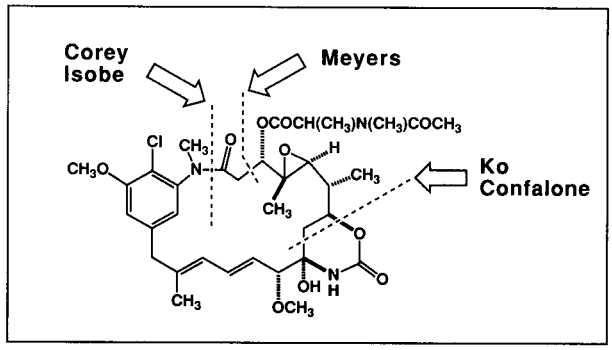
Active against: L-1210, B-16, P-815, etc.
Effective dose range: 0.4-50 micrograms / Kg
P-388: T/C = 220 % @ 25 micrograms / Kg

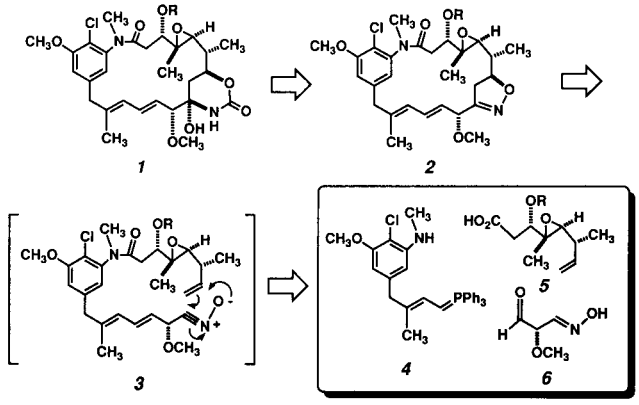
A number of strategic macrocyclic disconnections have been made by previous workers who have completed the total synthesis of maytansine. For example, Corey [2] and Isobe [3] employed a retrolactamization as the key macrocyclic disconnection in their approach to maytansine. Meyers [4], on the other hand, utilized a retroaldol transform in his synthesis of maytansine [5]. Our approach is best illustrated by a disconnection which carves out two of the carbon atoms of the hydroxy cyclic carbamate present in maytansine.

Strategic Macrocyclic Disconnections



This particular retrosynthetic step, which may appear somewhat strange at first glance, actually involves a retro [3 + 2] dipolarcycloaddition disconnection of maytansine to generate the intermediate isoxazoline (**2**). This is the product of the [3 + 2] nitrile-oxide olefin dipolarcycloaddition reaction depicted in structure (**3**). Further disconnection of (**3**) leads to a highly convergent synthesis employing the key intermediates (**4**), (**5**), and (**6**). Of course the chiral fragments (**5**) and (**6**) must be prepared in their correct absolute stereochemical configuration for incorporation into maytansine.

Retrosynthetic Considerations



Conceptually, our approach to the maytansinoids raises three important questions:

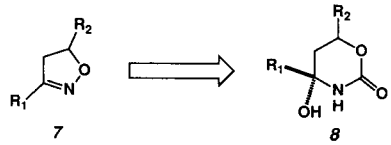
1. Can methodology be developed for the efficient conversion of the isoxazoline heterocycle (**7**) to the hydroxy cyclic carbamate (**8**) found in maytansine?

2. Are the alpha-methoxynitrile oxides required for the macrocyclization step stable to the fragmentation illustrated in structure (**9**), a course which leads to its self destruction rather than to participation in our assault on maytansine?

3. What is the efficiency and scope of such an intramolecular [3 + 2] cycloaddition reaction for ring-closure of a 19-membered ring?

Three Key Issues

1. Conversion of the isoxazoline to the hydroxy cyclic carbamate



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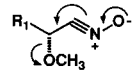
2. Stability of alpha-methoxy nitrile oxides to fragmentation.



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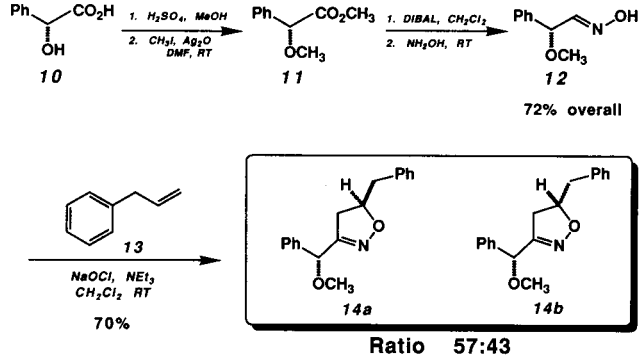
3. Scope and limitation of the intramolecular [3+2] nitrile-oxide cycloaddition for macrocyclization.

Before committing resources to the approach outlined above, a number of model studies were employed to answer some of these questions. Mendelic acid (**10**) was treated sequentially with sulfuric acid and methanol, followed by O-alkylation with methyl iodide and silver oxide in DMF, to furnish the O-methyl methyl ester (**11**). This product was reduced with DiBAL in methylene chloride and the resulting aldehyde treated with hydroxylamine to furnish the oxime (**12**) in 72% overall yield.

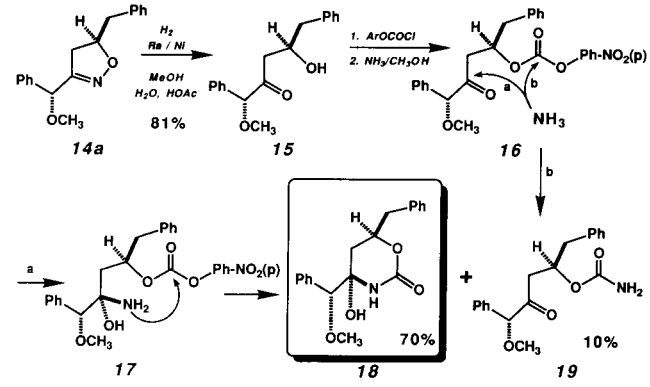
Upon oxidation with sodium hypochlorite in the presence of triethylamine in methylene chloride, the oxime (**12**) was converted *in situ* to the corresponding nitrile oxide. When this was carried out in the presence of allyl benzene (**13**), a smooth nitrile-oxide [3 + 2] intermolecular dipolarcycloaddition reaction ensued, affording a mixture of isomers (**14a**) and (**14b**) in 70% yield in a ratio of 57:43, respectively. Thus, the second question was answered in our favor, namely, that an alpha-methoxy nitrile-oxide is stable to a self-destructive fragmentation reaction, and participates normally in a [3 + 2] dipolarcycloaddition reaction.

Model Study Proving Stability of Alpha-Methoxy Nitrile Oxides



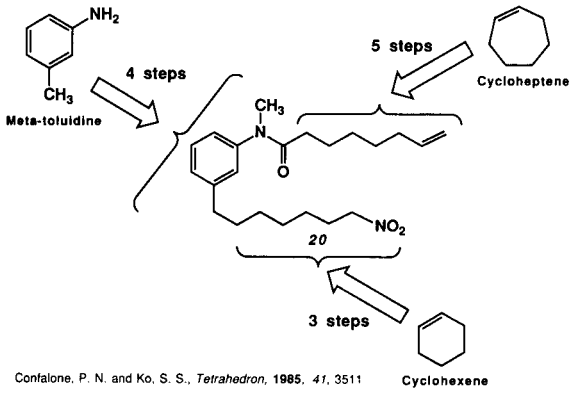
We next turned our attention toward question one, the conversion of isoxazolines (**7**) to hydroxy cyclic carbamates (**8**). The cycloadduct (**14a**) was reduced with hydrogen and Raney nickel in aqueous acidic methanol to afford the beta hydroxy ketone (**15**) in 81% yield [6]. When this substance was treated with the phosgene equivalent p-nitro-phenyl chloroformate, the intermediate (**16**) was generated. Subsequent ammonolysis afforded two products, the desired hydroxy cyclic carbamate (**18**) in 70% yield accompanied by a side-product, the uncyclized isomer (**19**), obtained as a 10% contaminant. Presumably, if ammonia attacked the carbonyl *via* reaction trajectory A, the resulting geminal hydroxy amine (**17**) cyclized to afford the desired product (**18**). If, however, ammonolysis occured via trajectory B, the uncyclized keto carbamate (**19**) was produced and was stable under the reaction conditions. Treatment of (**19**) with DBU successfully converted it to the desired cyclized product (**18**). Thus, this sequence of three steps served to transform isoxazolines to hydroxy cyclic carbamates in overall yields approaching 60%.

Conversion of the Isoxazoline Moiety to the Hydroxy Cyclic Carbamate



The third and final question was addressed next, namely, the suitability of a [3 + 2] dipolarcycloaddition reaction for the construction of the 19-membered heterocyclic ring system present in maytansine. To this end, the nitro olefin (**20**) was prepared. Although (**20**) is a vastly simplified version of the projected intermediate (**3**), lacking *inter alia* both the diene system and epoxide present in the natural product - elements which should facilitate the macrocyclization, we felt a demonstration of the desired ring closure was required before any commitment to this approach could be entertained. A facile convergent synthesis of (**20**) was achieved employing meta-toluidine, cycloheptene, and cyclohexene as the key building blocks.

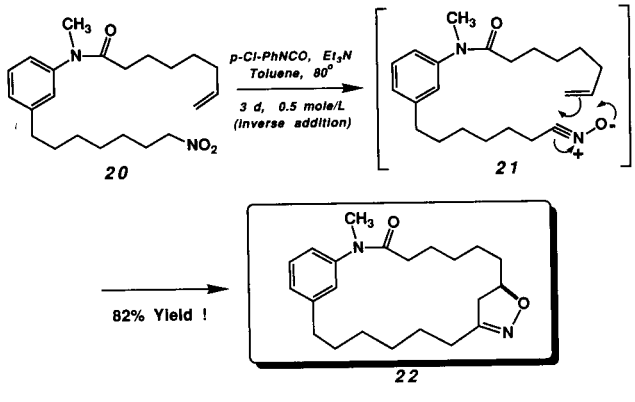
Synthesis of a Simple Model Substrate for Macrocyclization



Confalone, P. N. and Ko, S. S., *Tetrahedron*, **1985**, *41*, 3511

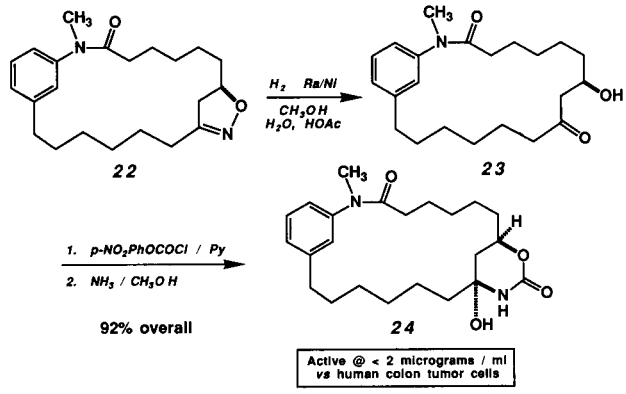
To our delight, inverse addition of the nitro olefin (**20**) to p-chloro-phenylisocyanate and triethylamine in toluene at 80° over a 3-day period afforded the macrocycle (**22**) in 82% yield! This reaction presumably proceeded through the nitrile-oxide olefin intermediate (**21**) and did not require high-dilution conditions. The regiochemistry observed, in which the carbon of the nitrile-oxide becomes attached to the terminal carbon of the vinyl group, is the exact result predicted on the basis of intermolecular reactions employing aliphatic nitrile oxides and dipolarofiles containing a terminal vinyl group. The alternate regioisomer was not detected in the reaction mixture.

Closure of the 19-Membered ring of Maytansine Proceeds Smoothly



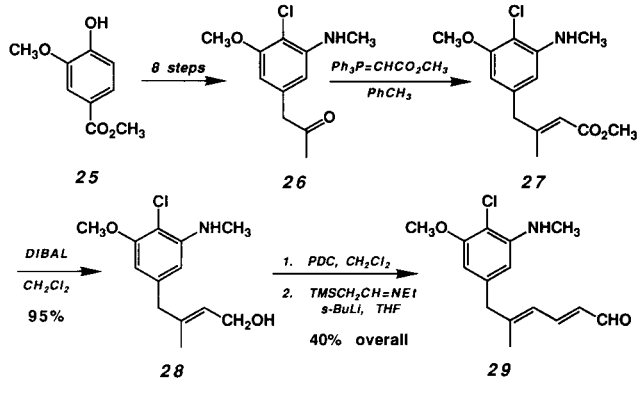
Since large quantities of the macrocyclic isoxazoline (**22**) were now available, we decided to convert this substance to the "parent" analog of maytansine. Treatment of (**22**) under the reducing conditions hydrogen and Raney nickel afforded the beta-hydroxy ketone (**23**) which was then converted by the two-step sequence of p-nitro-phenyl chloroformate followed by ammonolysis into the desired analog of maytansine (**24**). This substance incorporated the hydroxy cyclic carbamate moiety which has been speculated to be the anti-cancer pharmacophore present in the maytansinoids [7]. In our hands, the simple maytansine analog (**24**) exhibited antitumor activity at <2 micrograms/ml *vs* human colon tumor cells in culture.

Conversion of the Product Macrocycle to the "Parent" Analog of Maytansine



Since the three key issues surrounding our proposed synthesis of maytansine were resolved in our favor, we embarked upon the total synthesis of the natural product itself. For the synthesis of the aromatic portion corresponding to (**4**) in our original plan, methyl vanillate (**25**) was converted in eight steps to the aryl acetone derivative (**26**) [8]. This product underwent a Wittig reaction to afford the acrylic ester (**27**), which was reduced with DiBAL in methylene chloride to furnish the allylic alcohol (**28**) in 95% yield. This was oxidized with PDC to yield the corresponding aldehyde, which was converted to the dienal (**29**) [2b] in 40% overall yield.

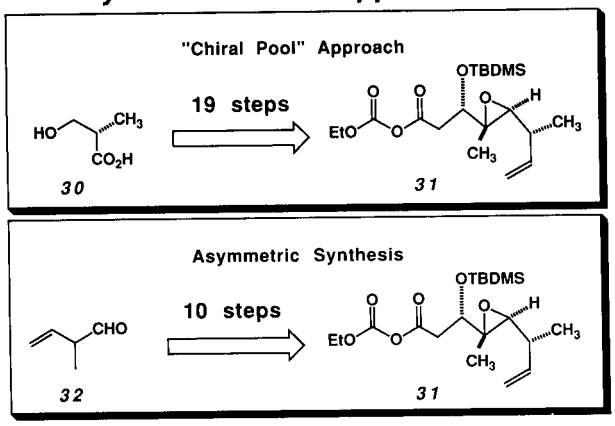
Synthesis of the Aromatic Portion



In the course of our work, two syntheses of the upper chain of maytansine, corresponding to the intermediate (**5**) in our original plan, were developed. The first utilized the chiral pool intermediate, 3-hydroxy-2-methyl propionic acid (**30**) which was converted in 19 steps into the target olefinic mixed anhydride (**31**). Since a more efficient 10 step synthesis of (**31**), starting from the beta-gamma unsaturated aldehyde (**32**) was also generated, I will discuss the latter preparation only.

Reaction of the aldehyde (**32**) and the bromo olefin (**33**) in the presence of magnesium in THF afforded the hydroxy diene (**34**). This was converted to the isomeric diene alcohol (**35**) by consecutive treatment with thionyl chlo-

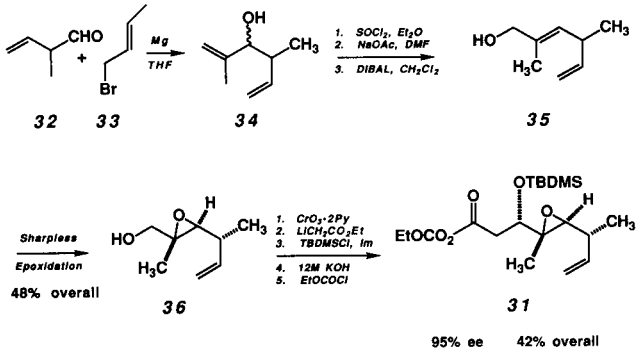
Synthesis of the Upper Chain



ride in ether, and sodium acetate in DMF, followed by deacetylation of the resulting diene acetate with DiBAL in methylene chloride. Sharpless epoxidation of (35) afforded the optically active epoxide (36) in 48% overall yield, a result of a kinetic resolution process. All by-products from this reaction were carried forward and were easily separated by chromatography after the subsequent aldol reaction (two steps later).

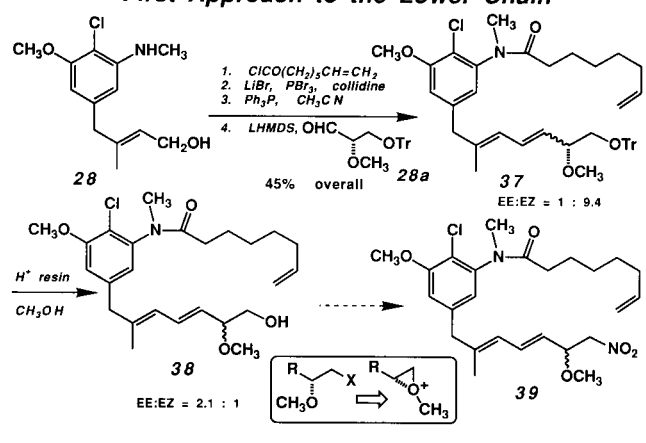
A five step conversion of (36) to the pivotal mixed anhydride (31) required oxidation to the aldehyde with Collins reagent, followed by an aldol reaction employing the lithium enolate of ethyl acetate. The resulting secondary alcohol was silylated with t-butyldimethylsilyl chloride and imidazole to afford the silyl derivative. The resistant ethyl ester was saponified with 12N KOH to afford the free acid, which was converted into the mixed anhydride under standard conditions employing ethyl chloroformate. The resulting optically active olefin (31) was obtained in an overall yield of 42% and an ee of 95%.

Asymmetric Synthesis (Kinetic Resolution) of the Upper Chain of Maytansine



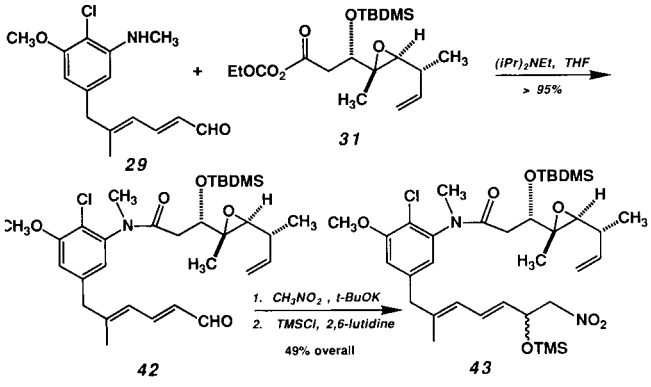
Our first approach to the lower chain of maytansine employed carbohydrate fragments containing the O-methyl substituent attached to C(10) of maytansine in the correct absolute configuration. To this end, the allylic alcohol (28) was acetylated at nitrogen with a model upper chain olefinic acid chloride. The resulting amide alcohol was converted to the corresponding primary bromide which afforded the phosphonium salt upon treatment with triphenylphosphene in acetonitrile. The phosphonium intermediate was converted to the corresponding phosphonium ylide and treated with the optically active O-trityl-O-methyl glyceraldehyde (28a) to afford the desired diene (37), obtained as an EE:EZ mixture in which the undesired EZ isomer predominated by a factor of almost 10:1! Treatment of (37) with acid in methanol not only served to remove the trityl group as expected, but also surprisingly rearranged the diene system, affording the diene alcohol (38) in which the desired EE isomer was now the major product with an EE:EZ ratio of 2:1. Amazingly, a third transformation also occured during the course of this simple treatment of (37) with acidic methanol. The product (38) was obtained in completely *racemic* form - the reaction had destroyed the asymmetric center in a single stroke. The mechanism of this transformation is quite interesting, but beyond the scope of this lecture. An additional difficulty soon arose in the attempted conversion of the primary alcohol (38) to the nitro derivative (39), the key substrate for macrocyclization. Under no conditions could this functional group interchange be achieved. All attempts to activate the alcohol for displacement, yielding intermediates of general structure (40), were complicated by an unpreventable conversion to the oxonium species (41), an event which always occured before the nitro nucleophile could displace the leaving group X. The properties of a reactive species such as (41), which could in principle still afford the desired nitro compound (39), in practice led only to a mixture of useless products.

First Approach to the Lower Chain



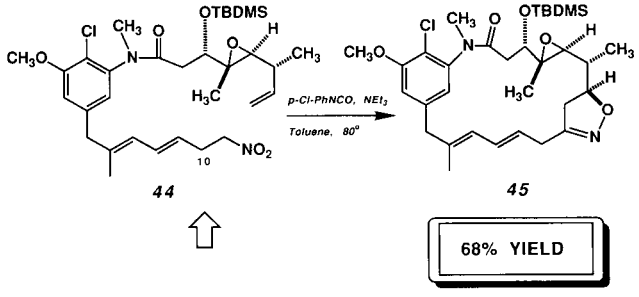
Given this array of difficulties, we retreated to the diene aldehyde (29), which was reacted with the fully elaborated upper chain intermediate (31). In the presence of Hunig's base in THF, a 96% yield of the amide product (42) was obtained. The amide (42) underwent a facile Henry reaction in the presence of potassium t-butoxide and nitromethane. The resulting beta-hydroxy nitro product was silylated with TMS chloride in the presence of 2,6-lutidine to yield the desired nitro compound (43).

Preparation of the Key Intermediate for Macrocyclization



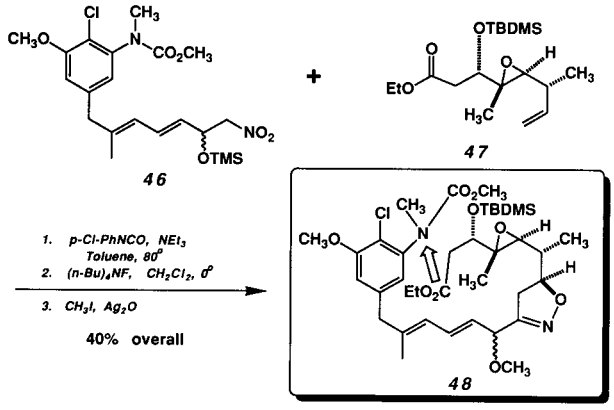
In ancillary studies we also prepared the 10-desmethoxy intermediate (**44**). This substance, now fully configured with the singular exception of the C(10) methoxide, was put to the test. Treatment with p-chloro-phenylisocyanate and triethylamine in toluene at 80° effected a smooth transformation to the macrocyclic isoxazoline (**45**), obtained in 68% yield! This satisfying result indicates that the presence of the constraining epoxide and the diene systems exerts no untoward influence on the course of the macrocyclization. Fortunately as well, the diene does not seem to compete for the transient nitrile-oxide in any intermolecular reaction mode.

Successful Macrocyclization in the 10 - Desmethoxy Series



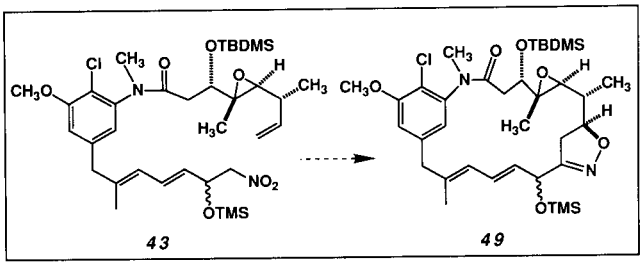
A second encouraging result was the demonstration of a successful *inter*molecular version of the final cyclization we envisioned. In the case of the substrate (**46**), which now contained the required C(10) silyloxy substituent, treatment with p-chloro-phenylisocyanate in the presence of the upper chain ester intermediate (**47**) effected a smooth intermolecular [3 + 2] dipolarcycloaddition. The resulting cycloadduct was further elaborated by a selective desilylation of the C(10) OTMS group in the presence of the O-t-butyldimethylsilyl substituent on the upper chain to afford the desired C(10) hydroxy derivative. In spite of other possible competing nucleophilic sites, the C(10) hydroxy could be selectively alkylated with methyliodide in the presence of silver oxide. This series of transformations afforded the desired product (**48**) in an overall yield of 40%, based on the nitro diene (**46**).

Successful Intermolecular Version of the Final Cyclization



With these encouraging model studies in hand, the fully elaborated intermediate (**43**) has been synthesized. A final [3 + 2] macrocylization of this substrate to the corresponding maytansine precursor (**49**) is required. As I have indicated, our various model studies have shown that the *inter*molecular version of this reaction proceeds smoothly to the desired cycloadduct. Also, the substrate (**44**), lacking only the C(10) OTMS substituent present in (**43**) undergoes *intra*molecular macrocyclization in 68% yield. In addition, we know how to selectively deblock the OTMS substituent in the presence of the t-butyldimethylsilyl group and carry out the required O-methylation of the resulting C(10) hydroxide.

The Final Nitrile-oxide Olefin [3 + 2] Macrocyclization



Finally, we have demonstrated the ready conversion of isoxazoline heterocycles to hydroxy cyclic carbamates. The conditions employed in this latter transformation are believed to be compatible with all functionality present in maytansine itself.

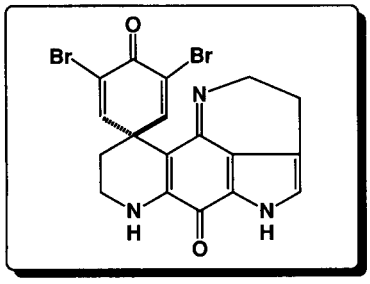
In conclusion, the studies reported above amply demonstrate the validity of an approach to the total synthesis of the maytansinoids based on [3 + 2] dipolarcycloaddition methodology.

TOPIC 2. Approaches to the Total Synthesis of the Anti-Cancer Marine Alkaloid Discorhabdin C

A newly isolated prototype of a novel series of anti-tumor compounds is discorhabdin C, isolated as the active

cytotoxic principle from the sponge *Latruncula* duBocage [9,10]. This substance exhibits extreme toxicity toward L1210 leukemia, with an ED_{50} <100 ng/ml *in vitro*. Discorhabdin C is a highly oxidized indole alkaloid in which the tryptamine side chain has been cyclized onto an indoloquinone to fashion an iminoquinone. In addition, the brominated phenolic substituent, typically found in many marine products, has undergone a biosynthetic phenolic coupling reaction, thereby elaborating a fascinating dibromocyclohexadieneone spirocyclic system.

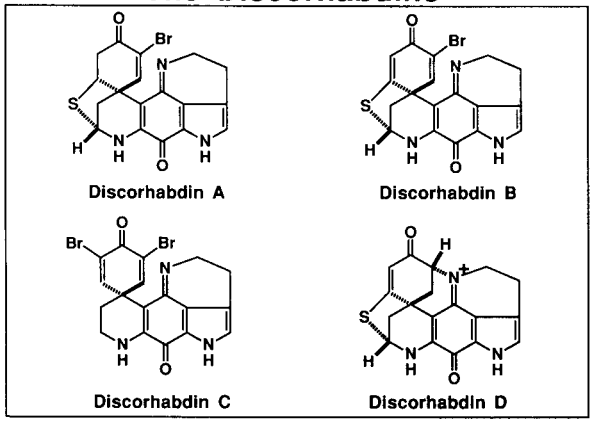
Discorhabdin C



- Active cytotoxic principle from the sponge *Latruncula* duBocage
- Extremely toxic *vs* L1210 leukemia, exhibiting ED_{50} < 100 ng/ml
- Prototype of a novel series of antitumor compounds

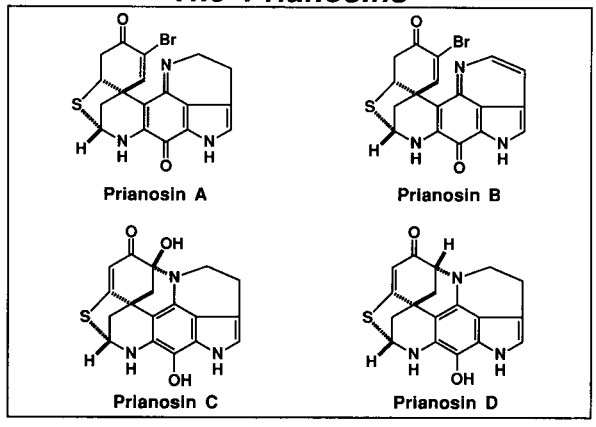
Four discorhabdins have been isolated to date [11,12]. The other three contain additional rings caused by either the internal alkylation of the iminoquinone nitrogen as in discorhabdin D, or by the elaboration of the novel sulfide bridge which spans the cyclohexadieneone moiety and the fused piperdine ring, as in the discorhabdins A and B.

The Discorhabdins



A closely related structural class, the Prianosins, have also been recently discovered. Prianosin A is identical in structure to discorhabdin A and is the dihydro derivative of prianosin B which contains an additional double bond in the ring formed by the cyclized tryptamine side chain. Prianosins C and D provide still other variations on this theme, including a hydroindoloquinone oxidation state as well as a ring-forming participation of the C(4) nitrogen to generate the same septacyclic ring system found in discorhabdin D.

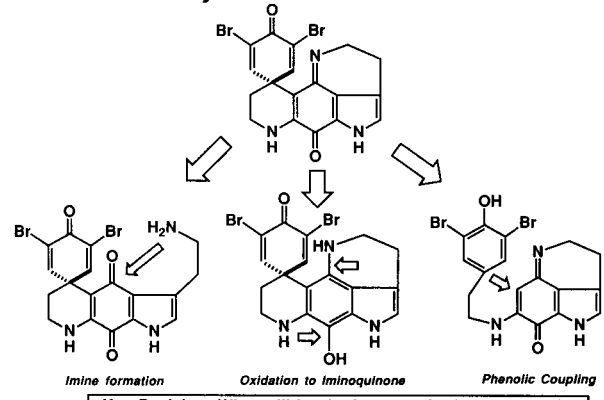
The Prianosins



Consideration of the retrosynthesis of discorhabdin C, clearly indicated that a critical decision needed to be made, namely, what would constitute the final step of the synthesis. One of most imporant aspects of any total synthesis of discorhabdin C is the determination of the precise order of the synthetic transformations required. This sensitivity to the order of chemical steps is a result of the complex series of redox reactions which virtually any preparation of the discorhabdins demands. These transformations must be carried out in the presence of easily oxidized heteroatoms and functionality sensitive to reduction.

One approach required imine formation between the tryptamine nitrogen and the indoloquinone carbonyl to be the final step of the synthesis. A second possibility required that oxidation of a hydroindoloquinone constitute the terminal step. A third option envisioned a preformed iminoquinone and employed a phenolic coupling as the final synthetic transformation.

Retrosynthetic Considerations

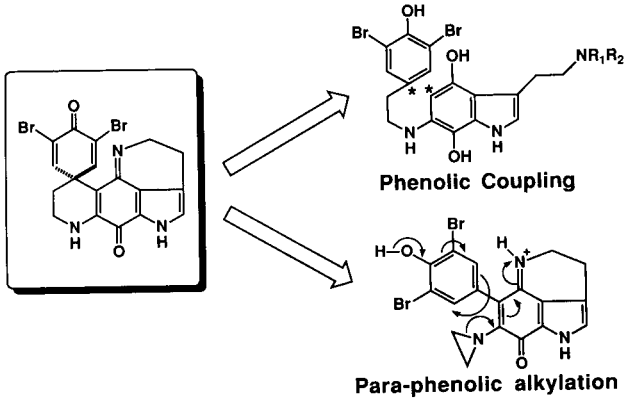


Key Decision: What will be the last step in the synthesis?

In the course of our work, we have studied two approaches to the total synthesis of discorhabdin C. The first features a phenolic coupling of a dibromophenol to the C(5) position of a hydroindoloquinone; the second employs a para-phenolic alkylation and uses an aziridine electrophile (or an equivalent) . In the latter instance, pro-

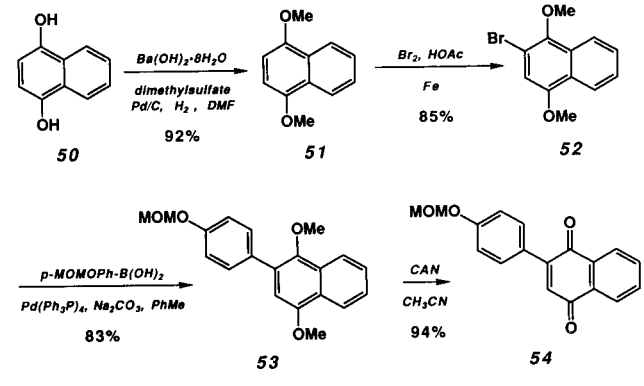
tonation of the iminoquinone nitrogen is expected to facilitate the intramolecular alkylation by activation of the aziridine as shown below.

Two Approaches Studied



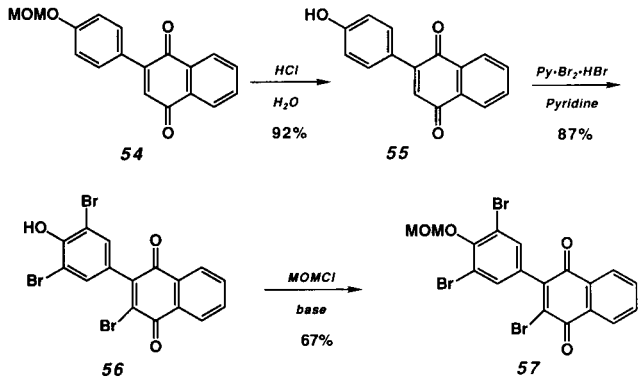
In this lecture I will discuss only the second approach, beginning with the presentation of an important model study in a naphthoquinone series. To this end, 1,4-dihydroxy naphthalene (**50**) was alkylated with dimethylsulphate to furnish the dimethoxy derivative (**51**) in 92% yield. Bromination occurred selectively at the C(2) position to yield the bromo naphthalene (**52**) in 85% yield [15]. A palladium-catalyzed phenyl boronic acid coupling reaction was employed to introduce the protected phenoxy substituent at C(2), affording the aryl naphthalene (**53**) in 83% yield [16,17]. Oxidation of the dimethoxynaphthalene system was achieved with ceric ammonium nitrate in acetonitrile to generate the naphthoquinone (**54**) in 94% yield.

A Model Study - Naphthoquinone Series



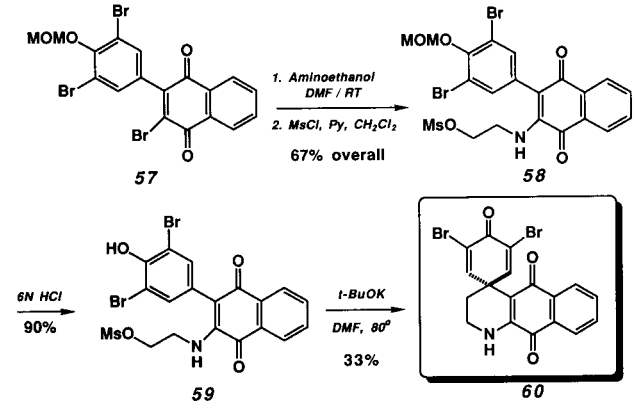
In this model study, the naphthoquinone was to substitute for the indoloquinone and the focus was to develop methodology for the spirocyclization. Deprotection of the MOM group in (**54**) gave the free phenol (**55**) in 92% yield, upon treatment with HCl. After examining a number of brominating conditions, we selected pyridinium bromide per bromide, which smoothly converted the unsubstituted phenol (**55**) to its tribromo derivative (**56**) in 87% yield.

Introduction of the Bromo Substituents



Attempts to introduce aziridine or an equivalent precursor by displacement of the bromoquinone substituent were unsuccessful in the presence of the free phenol. However, reprotection of (**56**) with MOM chloride provided (**57**) which readily underwent an addition-elimination reaction with aminoethanol in DMF at room temperature . The intermediate hydroxy aminoquinone was activated for alkylation by conversion to the corresponding mesylate (**58**) in 67% overall yield. Removal of the MOM protecting group was easily accomplished with 6N HCl without significant hydrolysis of the mesyloxy function, and the dibromophenol mesylate (**59**) was available in 90% yield under these conditions. The validity of the para phenol alkylation approach was demonstrated by the successful conversion of the mesylate (**59**) to the desired tetracyclic dibromocyclohexadieneone (**60**) in an unoptimized yield of 33% upon treatment with potassium t-butoxide in DMF at 80°.

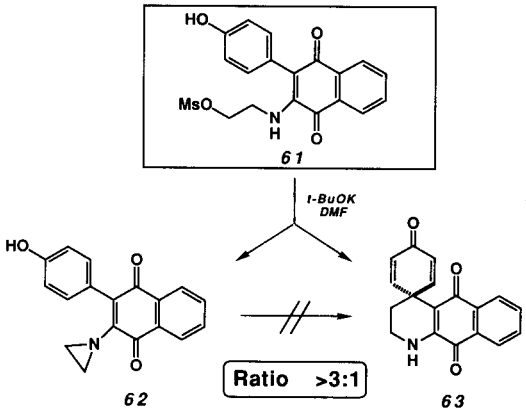
Spirocyclization to the Dibromocyclohexadienone System in the Naphthoquinone Model Series



The possibility that the mesyloxyamino substituent is cyclizing to an aziridine prior to alkylation was examined. In the case of the unbrominated analog (**61**), a significant amount of the aziridine (**62**) was produced in addition to the desired cyclohexadienone (**63**) in a ratio 0f >3:1. The aziridinoquinone (**62**) was also independently synthesized by the direct addition of aziridine in dimethylformamide to

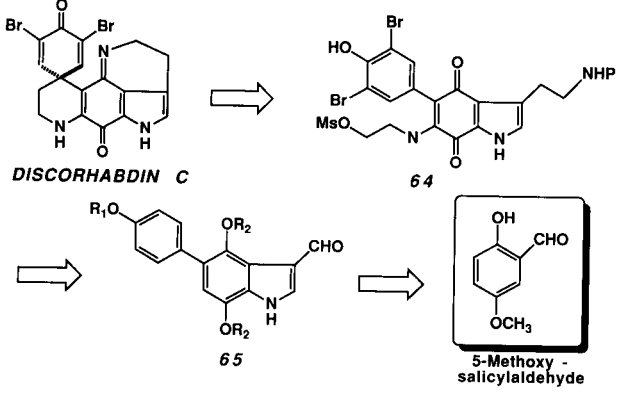
the phenolic naphthoquinone (55) followed by air oxidation. When a pure sample of the aziridine (62) was placed under the identical reaction conditions no trace of the spirocyclic product (63) could be detected, indicating that the aziridine derivative is probably *not* an intermediate in the cyclization process, presumably a result of its inability to present suitable reaction trajectories to the developing nucleophile, a limitation not shared by the less constrained acyclic precursor (61)

Aziridine Derivative is NOT an Intermediate



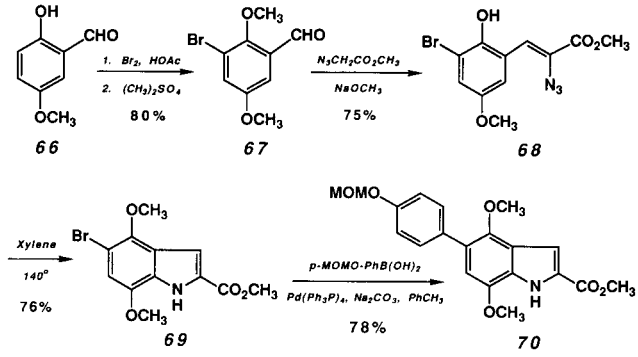
Given the success of this model study, our approach to discorhabdin C now required an intermediate such as the indoloquinone (64) which might be readily prepared from a 5-aryloxy-4,7-dialkoxy-indole-3-carboxaldehyde (65). A reasonable starting material for the synthesis of compounds such as (65) is 5-methoxy-salicylaldehyde.

Retrosynthesis of Discorhabdin C Based on the Para-Phenolic Alkylation Approach



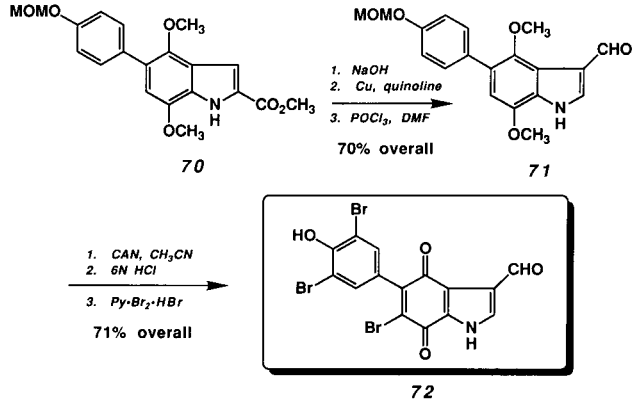
Bromination of 5-methoxy-salicylaldehyde (66) in acetic acid selectively inserted bromine ortho to the phenolic group and yielded the bromodimethoxy benzaldehyde (67) in 80% yield after a subsequent methylation with dimethyl sulfate [18,19]. For construction of the indole, methodology based on thermolysis of azidocinnamates was selected [19]. Therefore, treatment of the benzaldehyde (67) with methyl azidoacetate in the presence of sodium methoxide yielded the azido methylester (68) which was heated in xylene at 140°, affording 5-bromo-4,7-dimethoxyindole-2-carboxylic acid, methyl ester (69) in 76% yield. This product underwent a smooth arylboronic acid coupling reaction to produce the aryloxy derivative (70) in 78% yield.

An Efficient Synthesis of 5-Aryl-4,7-Dimethoxyindoles



A three-step conversion of (70) to the 3-carboxaldehyde derivative (71) was achieved in 70% overall yield by saponification of the ester, followed by decarboxylation and a Vilsmeier reaction. Further elaboration of the indole carboxaldehyde (71) to the desired tribromo indoloquinone (72) was achieved in 71% overall yield by oxidation with ceric ammonium nitrate in acetonitrile, hydrolysis with 6N HCl to remove the MOM group, and tribromination with pyridinium bromide per bromide.

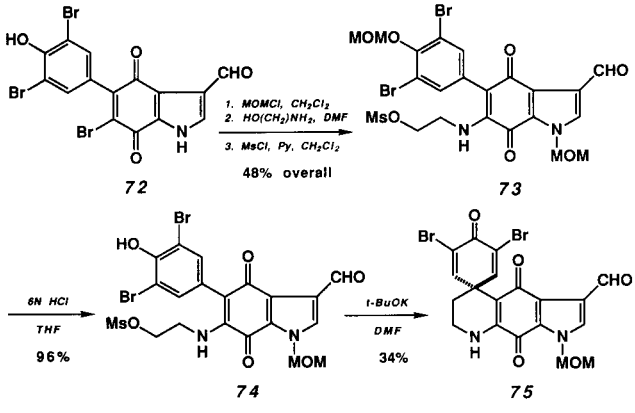
Synthesis of the Tribromo Indoloquinone



As observed in the model study, displacement of the 6-bromoquinone was not achievable as long as the free phenol was present. Therefore, the phenol (72) was first reprotected with methoxymethylchloride. The resulting bis-MOM derivative underwent a smooth displacement with ethanolamine in DMF to provide the aminoquinone product which was treated with methanesulfonyl chloride in pyridine to afford the desired mesyloxy aminoquinone (73) in 48% overall yield. The phenolic MOM group was selectively removed in 96% yield by treatment of (73) with 6N HCl in THF. The key spirocyclization reaction proceeded in 34% yield upon treatment of the mesylate (74)

with potassium t-butoxide in dimethylformamide, affording the desired spirocycle (**75**).

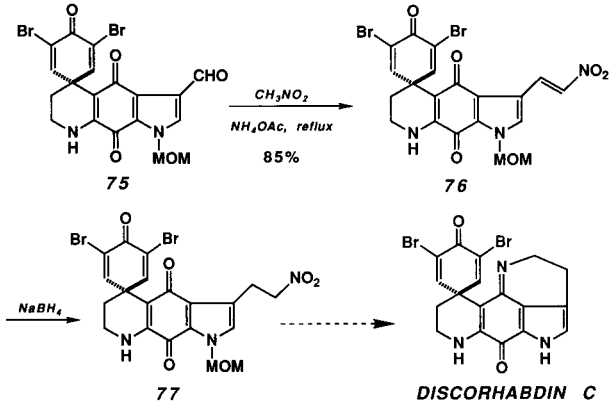
Synthesis of the Spirocyclization Product in the Indoloquinone Series



Condensation of the aldehyde (**75**) with nitromethane yielded the nitroethylene derivative (**76**). We now faced the difficult problem of carrying out a selective reduction of the nitro olefin to the tryptamine side chain in the presence of a number of other easily reduced functionalities. Fortunately, the carbonyl of the dibromocyclohexadienone system is deactivated enough to resist the action of sodium borohydride long enough for hydride to add to the nitro olefin, affording the advanced intermediate, the nitroethyl indole (**77**) in 80% yield.

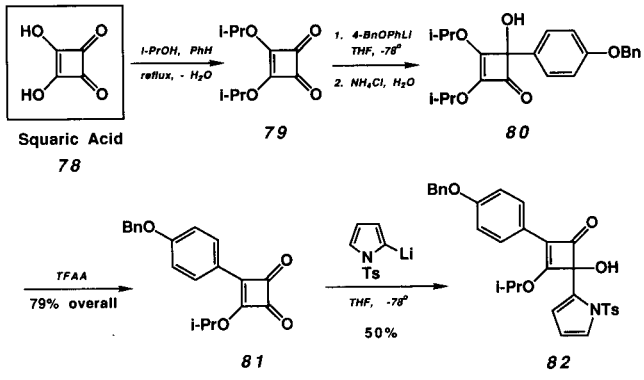
We are presently examining an array of reducing agents designed to convert the primary nitro group of (**77**) to an amine and also studying the final cyclodehydration in model systems. A successful execution of this two-step sequence will complete the total synthesis of discorhabdin C.

Synthesis of the Penultimate Intermediate



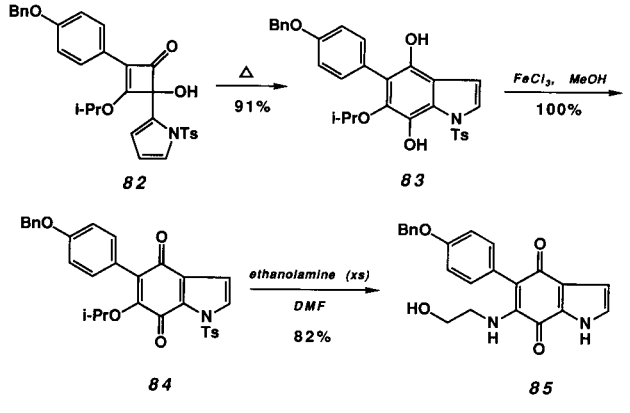
An alternative approach to the synthesis of discorhabdin C which avoids the problem of a selective reduction of nitroolefins is based on some novel chemistry of squaric acid (**78**) [20,21]. Conversion of squaric acid to its diisopropoxy derivative (**79**) was followed by treatment with 4-benzyloxyphenyl lithium in THF. After an acid quench, the aryl alcohol (**80**) was obtained which was treated with trifluoroacetic acid anhydride to furnish the squaric acid derivative (**81**). Treatment with 2-lithio-N-tosylpyrrole in THF at -78° selected the more reactive carbonyl of (**81**) and produced the hydroxypyrrole (**82**) in 50% yield.

An Alternative Approach to the Synthesis of Discorhabdin C Based on Squaric Acid



Thermolysis of the hydroxypyrrole (**82**) effected a dramatic rearrangement to afford the dihydroxyindole tosylate (**83**) in 91% yield. Oxidation of (**83**) with ferric chloride in methanol produced the indoloquinone (**84**) in quantitative yield. The isopropoxy substituent fortuitously carried through from the initial squaric acid intermediate is properly positioned and sufficiently reactive for the addition-elimination of ethanolamine. In the presence of an excess of ethanolamine, the isopropoxy substituent of (**84**) was cleanly displaced to yield the aminoethanol adduct; the reaction conditions also cleaved the N-tosyl group to afford the indoloquinone (**85**) in 82% yield. This critical observation is very important to the final phase of our projected synthesis

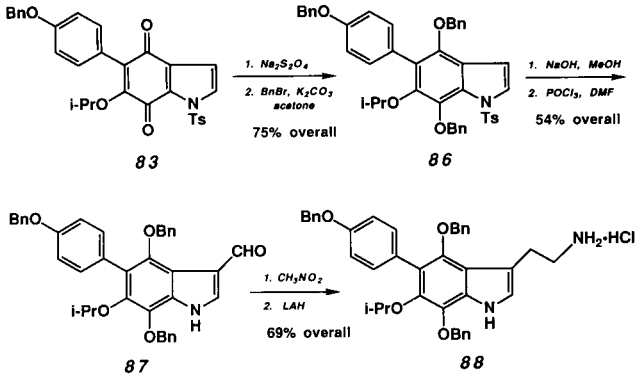
Further Elaboration to an Indoloquinone



The readily available intermediate isopropoxy indoloquinone (**83**) was converted in a two-step sequence of sodium dithionite and benzyl bromide alkylation to the desired tribenzyl derivative (**86**) in an overall yield of 75%. Introduction of the 3-carboxaldehyde was achieved by first

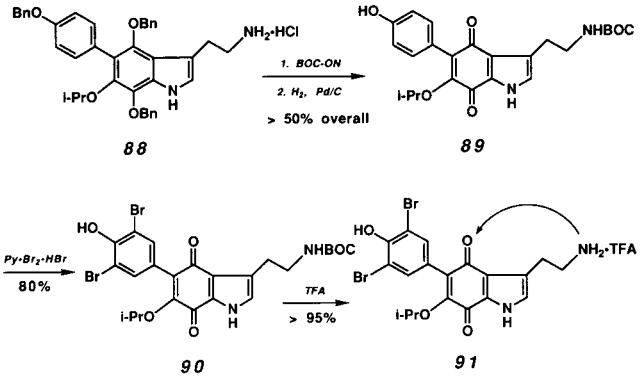
N-detosylation under basic conditions followed by a Vilsmeier reaction, yielding the indole carboxaldehyde (87) in 54% yield. A Henry reaction employing nitromethane followed by lithium aluminum hydride reduction of the intermediate nitroethylene converted the aldehyde (87) into the tryptamine hydrochloride (88) in 69% overall yield. The intermediate (88) is readily obtained in large quantities from squaric acid and is an ideal intermediate for the synthesis of discorhabdin C.

A Successful Conversion to the Tryptamine Derivative



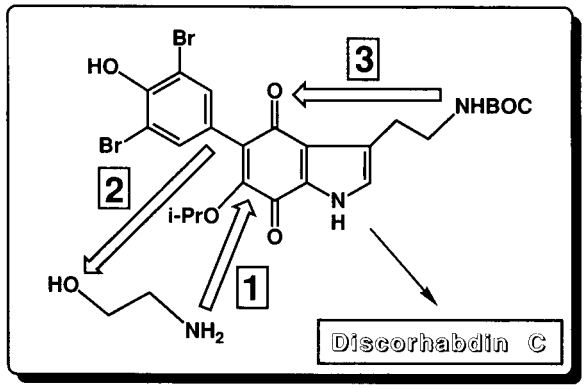
Treatment of the tryptamine hydrochloride (88) with BOC-ON selectively acylated the tryptamine nitrogen. Palladium catalyzed hydrogenation removed the three benzyloxy groups, yielding a transient intermediate triphenol which rapidly underwent air oxidation to afford the desired indoloquinone (89) in an overall yield of >50%. Bromination of (89) yielded the dibromophenol (90). The current status of this approach is at the indoloquinone tryptamine TFA salt (91), obtained in high yield by TFA deprotection of the precursor (90). The cyclodehydration of (91) as well as model studies in this regard are under intensive study.

Current Status of the Squaric Acid Approach



Thus, the squaric acid approach to discorhabdin C is only three transformations from completion. Starting from the dibromophenol (90), the first step is expected to be the addition-elimination displacement of the isopropoxide by aminoethanol. The second transformation is the para phenolic alkylation sequence, and the third is projected to be deprotection and cyclodehydration of the N-BOC side chain.

Probable Order of the Final Three Steps in the Squaric Acid Approach to Discorhabdin C



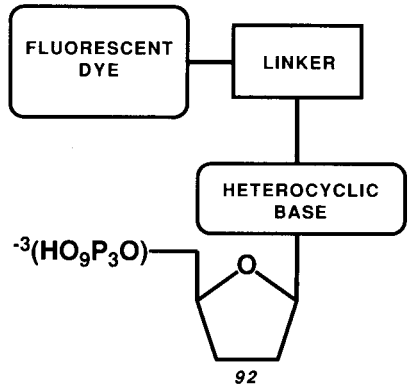
I note with some amusement that both of our approaches to discorhabdin C currently require the same last step, a cyclo*dehydration* - perhaps a suitable conclusion to any synthesis of a product initally elaborated in the world's oceans!

TOPIC 3. The Design and Synthesis of Novel Fluorescent Reagents for the Automated Sequencing of DNA.

As part of a multidisciplinary effort to develop an instrument which readily determined DNA sequence information, we needed to design and synthesize a fluorescently labelled DNA terminator of general structure (92). Compounds such as (92) were envisioned to be biochemical reagents for an automated version of Sanger DNA sequencing methodology [22].

A unique fluorescent dye was to be used as a reporter group for each of the four bases of DNA. The nature of the chemical linker as well as the specific point of attachment of the dye-linker assembly to the nucleotide fragment was unknown at this point, although linkage to the heterocyclic bases seemed likely. In addition to certain

Synthetic Targets: DNA Terminators Labeled with a Fluorescent Reporter Group

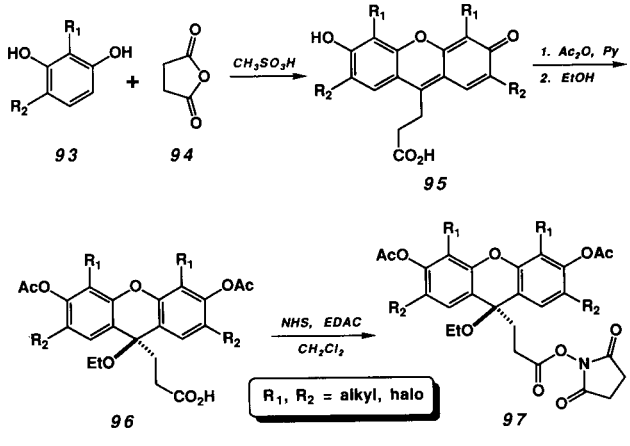


biochemical and physical properties, the absolute requirement for the reagents (92) was that they were substrates for DNA polymerase or any of the other enzymes used in Sanger sequencing.

A careful study of both commercially available and readily available fluorescent dyes failed to identify a class which met our specifications. The key requirement was that the set of four dyes span a wavelength from 480 to 510 nanometers, allowing excitation by an inexpensive argon laser. As a consequence, the lambda max of the individual dyes could differ by no more than 5 -6 nanometers.

The dye set finally chosen was from the class of succinyl fluoresceins and were prepared by the reaction of resorcinols such as (93) with succinic anhydride (94) in the presence of methanesulfonic acid, which yielded the tricyclic acids (95), highly fluorescent insoluble substances. Protection of the acids (95) with acetic anhydride in pyridine followed by ethanol addition afforded the ethoxy diacetates (96), which exhibited reasonable properties of solubility and chromatographic behavior. The acids (96) were activated as their NHS esters, yielding the labeling reagents (97).

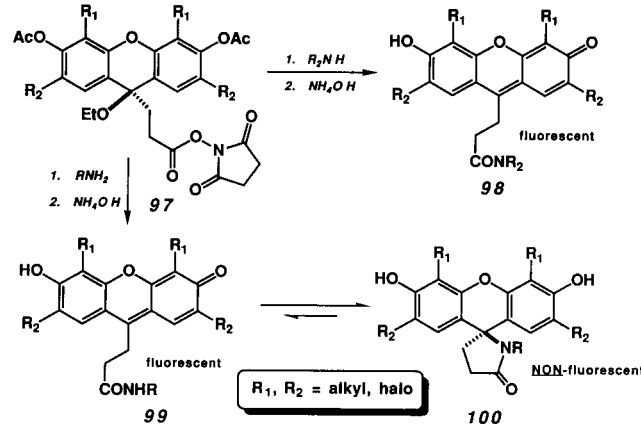
Preparation of the Succinyl Fluorescein Dyes



Since our construct required coupling of these activated acids (97) with a primary amino function on the nucleotide, a few model studies were carried out which revealed a devastating property of these substances. For example, reaction with a secondary amine followed by removal of the protecting groups with ammonium hydroxide afforded a nicely fluorescent carboxamide (98). On the other hand, reaction of (97) with a primary amine followed by deprotection yielded initially a fluorescent substance (99) which rapidly converted to a cyclized isomer (100) which did *not* exhibit fluorescent properties.

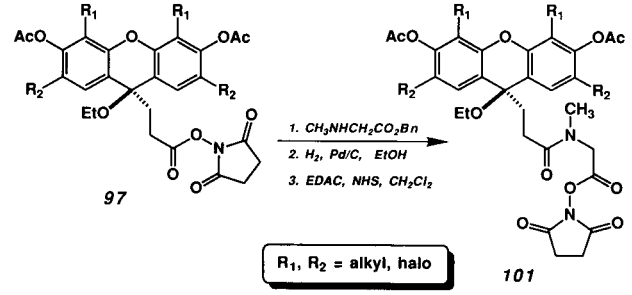
The solution was to insert a sarcosine "spacer" into the dye reagent which prevented the amide cyclization. To this end, the NHS ester (97) was treated with sarcosine benzyl ester and then hydrogenated. The resulting free acid was converted to its NHS ester, yielding the modified reagent (101) which had no tendency to cyclize but still re-

Fluorescence and Amide Equilibria



tained an activated acid for coupling purposes. Of course, the reagents (97) and (101) are capable of labeling other biological agents such as proteins, etc. with fluorescent reporter groups. This novel collection of succinyl fluoresceins exhibit strong absorption and efficient emission properties, are easily prepared, and the specific lambda max of the reagent set is selected by a judicious choice of R_1 and R_2.

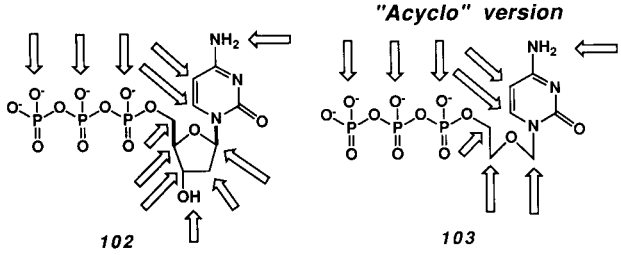
The Solution to the Amide Cyclization Problem is the Insertion of a Sarcosine Spacer



The next key question was: Which of the many sites of attachment of the linker and dye assembly on the nucleotide fragment afford a stable, competitive substrate? Consider structure (102) which illustrates the many diverse attachments sites possible. In addition, acyclo versions such as (103) offer an additional number of options. We synthesized many potential reagents arising from dye attachment at a number of these sites in both the natural and acyclo series. Unfortunately, not a single example was found to be a substrate for DNA polymerase or any of the other sequencing enzymes.

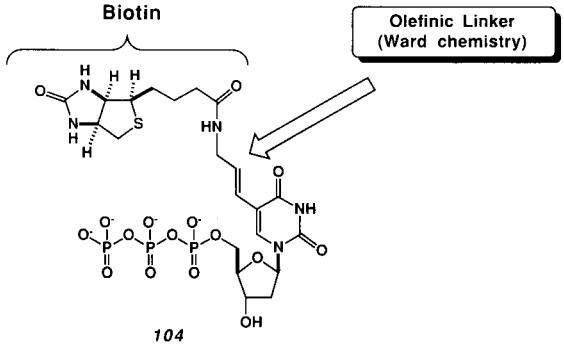
A report that the biotinylated thymidine triphosphate (104) was an acceptable substrate for DNA polymerase indicated that attachment at that site allowed covalently bound biotin to be accommodated by the enzyme [23].

The Key Question: ***Which of the many sites of attachment of the linker and dye assembly will afford a stable, competitive substrate?***



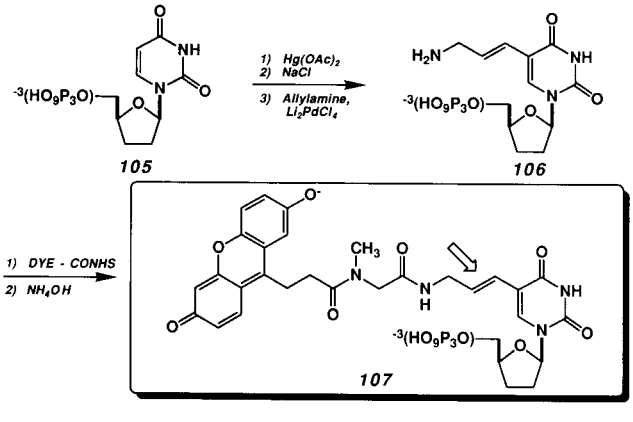
The relationship of the biotin system with its side chain attached to a nucelotide by an amide linkage and our newly prepared fluorescent dye with its corresponding spacer and amide linkage was not lost upon us.

Biotinylated Thymidine Triphosphate is a Known Substrate for DNA Polymerase



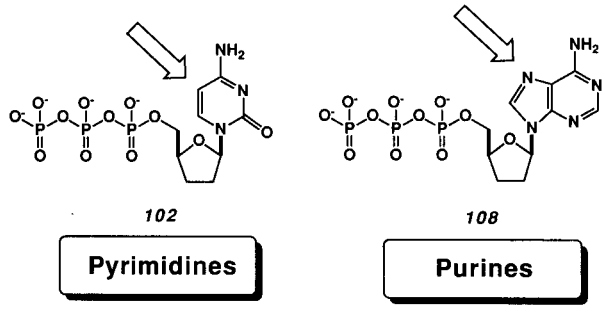
The validity of this relationship was easily tested by conversion of dideoxyuridine triphosphate (**105**) to its corresponding allylamine (**106**) by the three step sequence of mercuration, followed by conversion of the intermediate acetoxy mercury compound to the chloro derivative, and palladium catalyzed addition to allylamine. Coupling of (**106**) with the NHS ester of one of our fluorescent dyes followed by aminolysis afforded the dye coupled amide (**107**) which proved to be a substrate for DNA polymerase!

Synthesis of the Olefin-linked Dye-labeled T Terminator



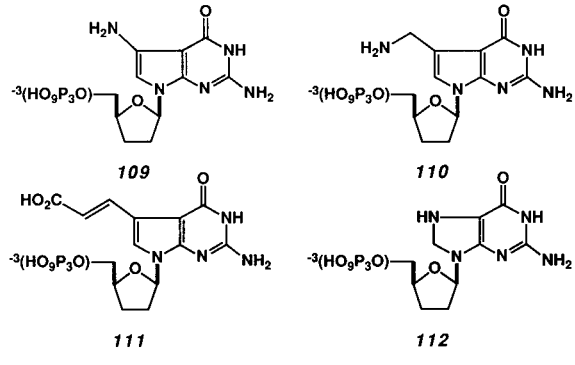
A complication immediately arose since the site in purines (**108**) corresponding to that found acceptable in the pyrimidines (**102**) has a nitrogen atom whose alkylation or acetylation would yield a very unstable system.

Selected Attachment Sites



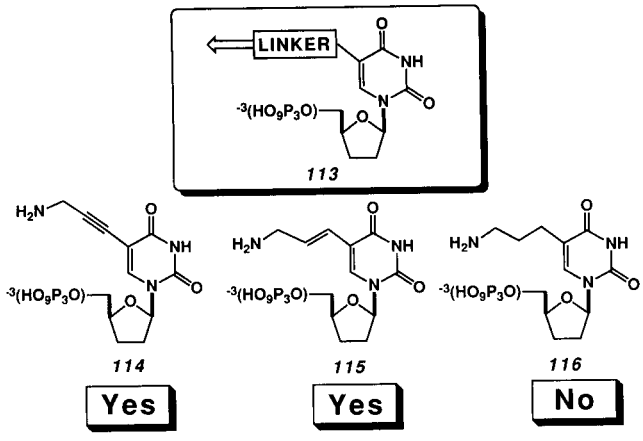
Several potential solutions to this problem are presented in structures (**109**) to (**112**). In the end, an opportunistic approach based on the availability of a deazapurine from a fermentation source was selected.

Several Potential Solutions to the Purine Problem



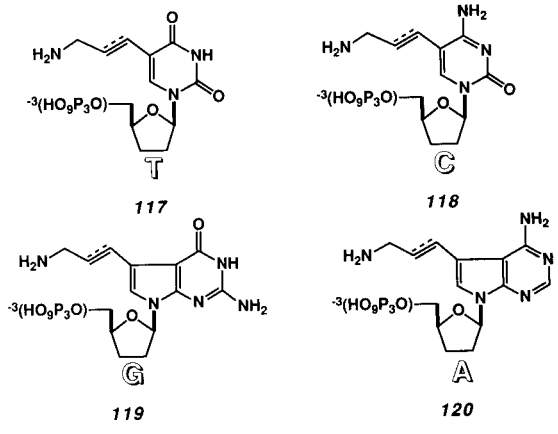
In the course of our work, we synthesized a number of pyrimidine nucleotides with various linkers (**113**) and examined the substrate specificity of DNA polymerase. The propargylamine (**114**) and the allyl amine (**115**) were substrates, whereas the reduction product (**116**) was not, indicating a possible requirement for an sp^2 hybridized carbon at the alpha position.

Linkers and DNA Polymerase Specificity



The results of this study dictated the design of the four DNA terminators. Therefore, propargyl and allylic aminonucleotide triphosphates (**117**) to (**120**) were targeted for synthesis and biochemical evaluation.

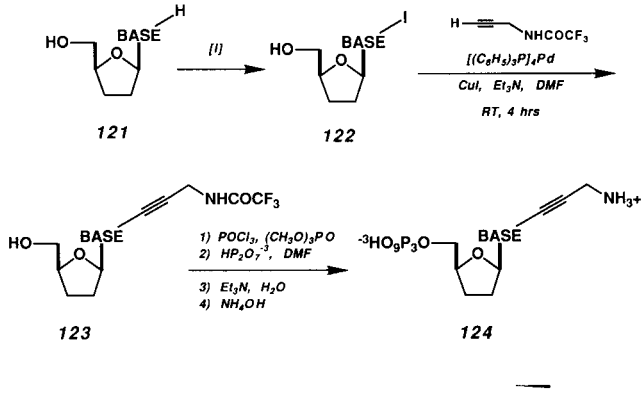
Olefinic / Acetylenic Targets



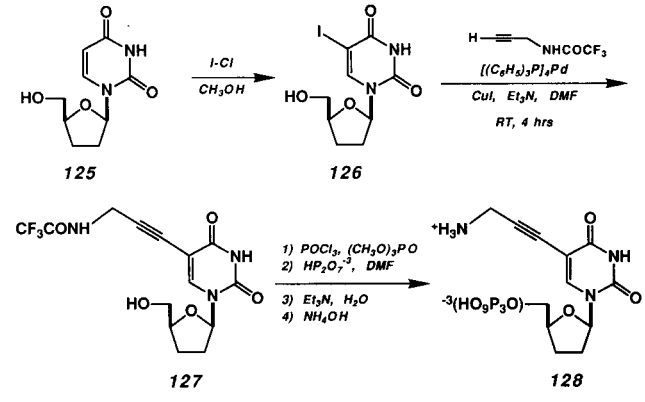
I will discuss only the acetylenic linkers since they are more generally applicable [24,25]. The synthesis of this class begins with the dideoxynucleosides (**121**) which were converted to the iodinated [26,27] derivatives (**122**). Palladium catalyzed addition [28] to allyl-N-trifluoroacetamide yielded the acetylenes (**123**) which were converted to their triphosphates using standard methods [29], affording the ammonium triphosphates (**124**) after ammonolysis of the trifluoroacetamide group. The ammonium triphosphates are the key building blocks for construction of the final DNA sequencing reagents.

For the synthesis of the T (thymidine) terminator, dideoxy uridine (**125**) is specifically iodinated at the desired position by iodonium chloride in methanol to afford the iodouridine (**126**). The coupling reaction yielded the expected trifluoroacetamide (**127**) which was smoothly converted to the targeted ammonium triphosphate (**128**) as outlined.

General Strategy for the Preparation of Acetylenic Nucleoside Triphosphates

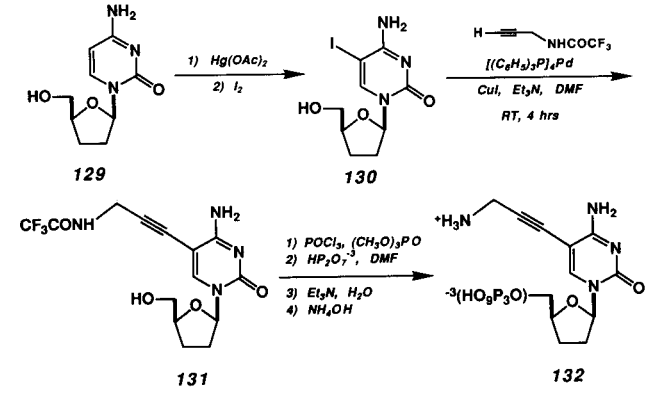


Synthesis of the Ammonium Triphosphate Intermediate in the Thymidine Series



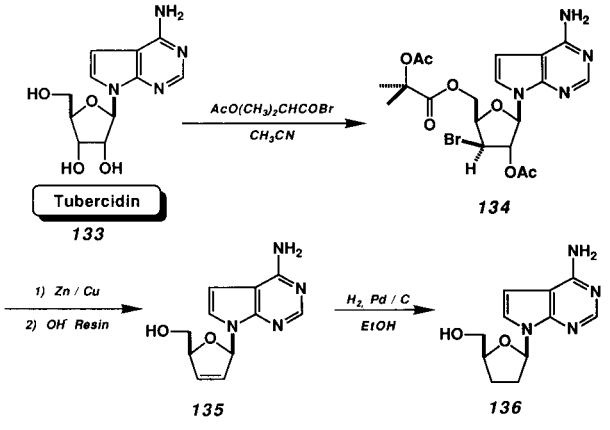
The preparation of the C (cytidine) terminator began with dideoxy cytidine (**129**) which was specifically mercurated and subsequently iodinated to afford the iodo derivative (**130**). The remaining chemistry paralleled the thymidine example above and afforded the acetylene (**131**) and the ammonium triphosphate (**132**).

Synthesis of the Ammonium Triphosphate Intermediate in the Cytidine Series



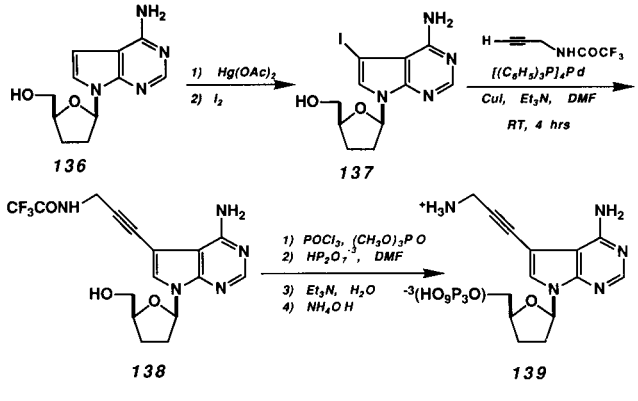
For the A (adenine) terminator, the first of the purine examples we studied, Mother Nature provided an excellent starting material, turbercidin (**133**). Removal of the unwanted 2',3'-dihydroxy groups was accomplished by treatment with 2-acetoxy isobutyric acid bromide in acetonitrile to afford the bromo acetate (**134**). Conversion to the olefin (**135**) was accomplished by treatment with zinc/copper couple followed by saponification. Hydrogenation of the olefin (**135**) yielded the desired dideoxydeazaadenosine (**136**).

Deoxygenation of Tubercidin



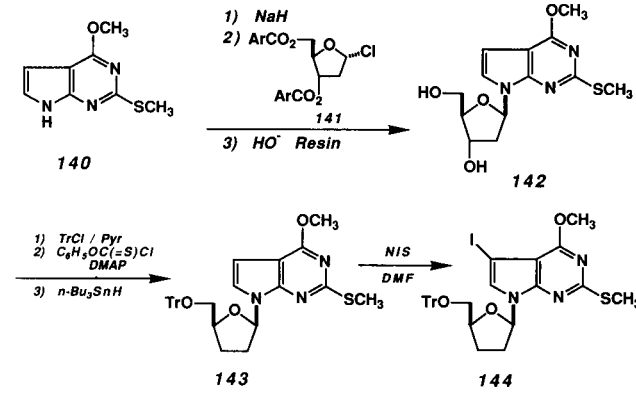
Mercuration/iodination of (**136**) afforded the iodo derivative (**137**) which was converted to the acetylene (**138**) and the ammonium triphosphate (**139**), the key intermediate in the adenine series.

Synthesis of the Ammonium Triphosphate Intermediate in the Deaza-adenosine Series



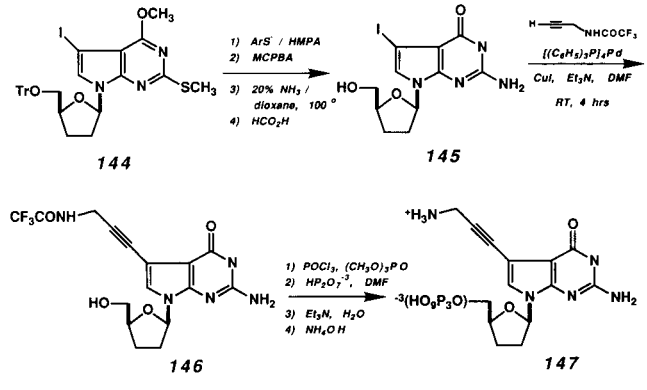
Nature was much less cooperative in the case of the G (guanine) terminator, failing to provide any advanced intermediates. Therefore, the diazaadenine derivative (**140**) was prepared [30] in five steps and deoxyribosylated with the chloride (**141**). Saponification to remove the aryl esters yielded the 2'-deoxy ribonucleoside (**142**). Selective protection of the 5'-hydroxy group was achieved with trityl chloride and the 3'-hydroxyl group was removed using Barton deoxygenation chemistry to yield the dideoxy derivative (**143**). N-iodosuccinimide in dimethylformamide selectively iodinated the substrate (**143**) to afford the required iodo derivative (**144**). This latter reaction solved a very difficult problem in our synthesis since only the intermediate (**143**) underwent a selective iodination at the desired position. In all other substrates, the adjacent C(2) position was the predominant, often exclusive site of iodination.

Preparation of the Iodinated Dideoxyguanosine Intermediate



The pivotal intermediate (**144**) was converted to the iodinated deazadideoxyguanosine (**145**) by the sequence: demethylation, oxidation of the sulfide to the sulfoxide with MCPBA, ammonolysis in dioxane at 100°, and detritylation with formic acid [31]. Conversion of (**145**) to the acetylene (**146**) and the ammonium triphosphate (**147**) was uneventful.

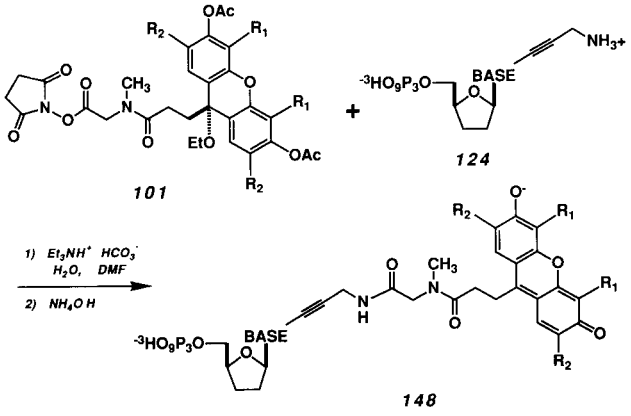
Synthesis of the Ammonium Triphosphate Intermediate in the Deaza-guanosine Series



Acylation of the ammonium phosphates (**124**) with the NHS esters (**101**) proceeded smoothly to yield the fully elaborated fluoroscein dye-labelled dideoxynucleosidetriphosphates (**148**). Since there are four bases in DNA and a large number of options for R_1 and R_2, a significant number of candidate reagents of general structure (**148**)

have been prepared and their fluorescent characteristics and biochemical properties determined. From this collection, a set of the best four reagents has been selected. They are currently an integral part of our commercial DNA sequencing instrument [32].

General Synthesis of Dye-labeled Terminators



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