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BIOACTIVE COMPOUNDS FROM AQUATIC AND TERRESTRIAL SOURCES[1]

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ABSTRACT.—The world of nature provides a never-ending set of fascinating problems for the chemist. Many of the most intriguing problems, however, concern compounds available in only truly minute quantities. One solution is to focus on bioassay-guided separations. In so doing one can isolate compounds with novel structures or unsuspected activities from almost any phylum, including tunicates, sponges, insects, or even the much-studied terrestrial plants, as exemplified in several recent studies in our laboratory involving activities ranging from antiviral and antimicrobial activity to cytotoxicity and immunomodulation.

Moreover, newer spectroscopic techniques, especially fast atom bombardment mass spectrometry and tandem mass spectrometry, enhance one's ability to study compounds present in minute quantities, including those of importance to the host organism, such as neuropeptides in insects or marine invertebrates.

These are exciting times for organic chemists. Classical organic natural product research usually involved isolating the most abundant compound, assigning its structure, and then hoping to find a use for it. Today we are more likely to be guided by biology, that is, by bioassay, to direct our attention to the truly important compounds present in a species. Most bioassays are exceedingly sensitive, so that the active compound may be present in only minute quantities. Fortunately, modern structural techniques require only miniscule quantities of the material. X-ray crystallography is an obvious choice, but in the absence of a suitable crystal or even a pure compound the structure can often be established by mass spectrometry combined with other spectroscopic studies. The present discussion will provide examples of newer bioassay methodology as well as of the latest techniques for structure elucidation.

ANTIVIRAL COMPOUNDS FROM PLANTS AND INSECTS.—We initiated our search for antiviral natural products with our Alpha Helix Caribbean Expedition in 1978 (1), when some 1000 samples of marine plants and animals were assayed for activity against *Herpes simplex* virus, type 1 (HSV-1, a DNA virus). The eudistomins **1–4**, tetrahydro-β-carbolines fused to a unique oxathiazepine ring (2,3), and the didemnins (4–9) (cf.

[1]The 1989 Research Achievement Award address by K.L. Rinehart at the Thirtieth Annual Meeting of the American Society of Pharmacognosy, San Juan, Puerto Rico, August 6–10, 1989.

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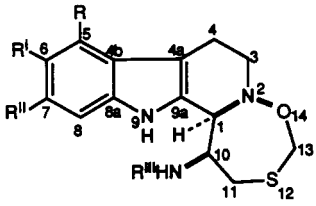
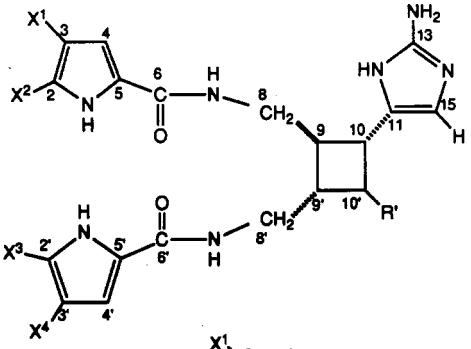
below), from marine tunicates (sea squirts), were the major antiviral discoveries of that effort, but a number of other species, including *Agelas* sponges (10–12), have provided antiviral compounds [e.g., in the sceptrin **5–7** (13), oxysceptrin **8**, **9**, and ageliferin **10–12** series (10–12, 14)].

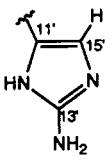
More recently, we began extensive assays of terrestrial plant and insect phyla, employing HSV-1 and *Vesicular stomatitis* virus (VSV, an RNA virus). Plants, of course, have long provided man with antihypertensive, cardioregulatory, anti-inflammatory, analgesic, and hypnotic agents, deriving mainly from mankind's close interaction with them (15–18), and recent systematic studies of their antitumor (19) and antibacterial (20) properties have proved quite successful, but systematic assays for antiviral activity do not appear to have been carried out. In our search for antiviral agents from insects and plants we have been guided in part by bioautography involving overlaying a tlc plate on HSV-infected CV-1 (monkey kidney) cells and, after incubation, looking for areas of viral growth inhibition.[3]



		R	R′	R″	R‴
1	Eudistomin C	H	OH	Br	H
2	Eudistomin E	Br	OH	H	H
3	Eudistomin K	H	H	Br	H
4	Eudistomin L	H	Br	H	H



A=



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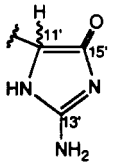
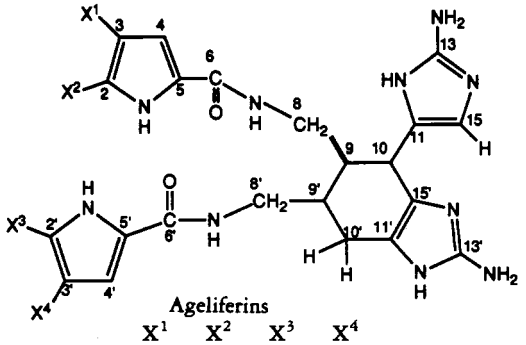


Sceptrins

	X^1	X^2	X^3	X^4	R^1
5	Br	H	H	H	A
6	Br	H	H	Br	A
7	Br	Br	Br	Br	A

Oxysceptrins

	X^1	X^2	X^3	X^4	R^1
8	Br*	H	H	H*	B
9	Br	H	H	Br	B



Ageliferins

	X^1	X^2	X^3	X^4
10	Br	H	H	Br
11	Br	H*	Br*	Br
12	Br	Br	Br	Br

*Substituents may be interchanged.

[3]G.R. Wilson and K.L. Rinehart, manuscript in preparation.

Initial antiviral testing (Table 1) of some 40 plant species (23 families, mainly from the western United States) suggests that terrestrial plants contain about the same proportion of antiviral compounds as do marine species. The HSV-1 assay has the fringe benefit of providing a guide to cytotoxicity by showing inhibition of monkey kidney

TABLE 1. Antiviral/Cytotoxicity and Antimicrobial Bioassays of Plant Extracts for 23 Families.[a]

Family	Species	In vitro Screens[b]			
		HSV-1	CV-1	*B.s.*	*M.l.*
Papaveraceae		—	—	—	—
Rhamnaceae		—	—	10	—
Sapindaceae		—	—	—	—
		—	—	—	—
		—	9 sh	—	—[c]
Passifloraceae		—	—	—	—
		—	—	—	—
Euphorbiaceae		—	—	—	—
		—	—	—	—
		—	—	—	—
		3 sp	—	—	—
Loranthaceae		—	—	—	—
Rutaceae		—	—	—	—
Garryaceae		—	—	—	—
Fabaceae		—	—	—	—
		—	—	—	—
		—	—	—	—
		—	—	—	—
		—	—	—	—
		—	—	—	—
	Amorpha fruticosa	?	35	9	7
Asclepiadaceae		—	—	—	—
		—	—	—	—
Asteraceae		—	—	—	—
		—	—	—	—
		—	—	—	—
Malesherbiaceae		—	—	—	—
Cupressaceae		25	—	—	—
Menispermaceae		—	—	—	—
Chenopodiaceae		—	—	—	—
Boraginaceae		—	—	—	—
Meliaceae		—	—	—	—
Elaeocarpaceae		—	—	7	7
Polypodiaceae		—	12 d	8	7
		—	—	—	—
		—	13 d	12	9
	Notholaena standleyi 2061	9	9 ls	10	7
	Notholaena standleyi 9235	—	9	9	7
	Notholaena standleyi 10910	—	9	14	9
Martyniaceae		—	7	7	—
Sterculiaceae		—	—	—	—
Saxifragaceae		—	—	—	—
Turneraceae		—	—	—	—

[a]20-μl extract on 6.35-mm disk; zones of inhibition in mm; sh = sharp zone; d = diffuse zone; ls = lightly stained; sp = small plaques; ? = unable to read due to cytotoxicity.

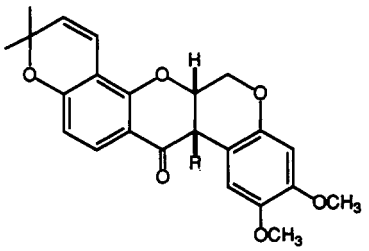
[b]HSV-1 = *Herpes simplex* virus, type 1; CV-1 = monkey kidney cells; *B.s.* = *Bacillus subtilis*; *M.l.* = *Micrococcus luteus*; no activity vs. *Escherichia coli* or *Penicillium atrovenetum*.

[c]9-mm zone vs. *Saccharomyces cerevisiae*.

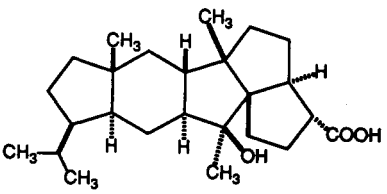
(CV-1) cell growth. Two species from Table 1 have been investigated, *Amorpha fruticosa* for its cytotoxicity and *Notholaena standleyi* 2061 for its antiviral activity.

In the case of *A. fruticosa*, the compounds responsible for the cytotoxicity were identified as the rotenoids deguelin [**13**] and tephrosin [**14**]. Tephrosin had been isolated earlier from *A. fruticosa* (21), but neither it nor any other compound from that species was reported to be cytotoxic. Deguelin, on the other hand, had not been found in *A. fruticosa* but was known to be cytotoxic (22).

More novel results were provided by *N. standleyi* 2061; it afforded notholaenic acid [**15**], a $C_{25}H_{40}O_3$ sesterterpene with anti-HSV-1 activity at a (relatively high) concentration of 25 μg/disk.



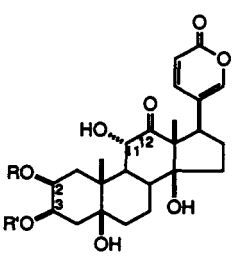
13 (−)-Deguelin: R=H
14 (−)-Tephrosin: R=OH



15 Notholaenic acid

Insects appear not to have been investigated for antiviral activity, but, as seen in Table 2, such activity can be found in a wide variety of insect orders. We have studied one of the species in Table 2 extensively—*Photinus pyralis*, a firefly. (Not all fireflies contain the antiviral compounds.) The compounds responsible are the known lucibufagins **16–20** (23), but their antiviral activity had not previously been noted (24), and its level is remarkable (Table 3). The most abundant, the diacetyl compound, inhibits viral shedding in the rabbit eye assay but does not appear to exert a therapeutic effect in that model.

INVERTEBRATE NEUROPEPTIDES.—Invertebrate species, terrestrial and marine, produce minute quantities of small (mol wt ca. 1000) neuropeptides with hormonal functions. In insects, these neuropeptides are found in a neurohaemal organ, the corpus cardiacum (corresponding to the mammalian hypophysis); they stimulate the mobilization of fats (adipokinetic hormones, AKHs) or sugars (hypertrehalosaemic factors, HrTHs) (25). Because insect corpora cardiaca are tiny, the amounts of the hormones are usually very limited (often ca. 1 μg). Nevertheless, a large number of insect neuropeptides have been identified (Table 4). In a collaboration between the laboratories of Illinois and Düsseldorf, we have assigned peptide sequences from daughter ion fast atom bombardment (fab) mass spectra, employing high-resolution tandem mass spectrometry (hrms/ms) when, as is usually the case, the ordinary fab spectra contain few, if



Lucibufagins

16 R=R′=Ac
17 R=Ac, R′=i-C_3H_7CO-
18 R=Ac, R′=C_2H_5CO-
19 R=H, R′=Ac
20 R=Ac, R′=H

TABLE 2. HSV-1 (CV-1) Antiviral (Cytotoxicity) Bioassay of Insect Extracts from 30 Species (7 Orders).

Order	Genus	Antiviral[a]	Cytotoxicity[b]
Orthoptera		−	0
		−	0
Hemiptera		+++	0
Homoptera		−	0
Coleoptera		−	0
		−	0
		−	0
		−	0
		+	0
		+	0
		−	0
		−	0
		+	0
		−	0
	Photinus pyralis	+++	0
		±	0
Hymenoptera		++	8
		−	0
		−	0
		+	0
		+++	0
Diptera		−	0
		−	0
Lepidoptera		−	0
		−	0
		−	0
		−	0
		−	0
		−	0
		+++	0

[a]−, No change in the number or diameter of viral plaques; ±, no change in the number of plaques, plaque diameter somewhat reduced; +, reduced number of plaques, plaque diameter markedly reduced, visible with unaided eye throughout the well; ++, plaques visible with the unaided eye only at the edge of the well, others detected by microscope; +++, no plaques detected by microscope.

[b]Diameter (mm) of cytotoxic zone centered on the application disk.

any, fragment ions. A case in point is the hypertrehalosaemic factor II from the stick insects *Carausius morosus, Sipyloidea sipylus*, and *Extatosoma tiaratum*, whose ordinary mass spectrum contains little sequence information but whose collision-induced decomposition tandem mass spectrum contains an adequate number of fragment ions to complete the sequence (Scheme 1) (30). Thus far, we (and others) have demonstrated that different insect families produce different neuropeptides, with the same genus containing the same peptides, and that all the peptides sequenced can be regarded as arising from sequential replacement of one base in the genetic code triad (Scheme 2) (33).

We have recently turned to marine invertebrates, which produce similar neuropeptides, though their functions are less well defined (42–44). These peptides are usually identified by radioimmunoassay for the *C*-terminal unit, for example, -RFamide (-Arg-Phe-NH_2). The peptide Pol-RFamide II isolated at Hamburg from the hydromedusa *Polyorchis penicillatus* contained the amino acids Glu, Leu, Gly, Arg, and Phe, identified by conventional amino-acid analysis. Hrfabms gave a molecular ion at *m/z* 916.5155,

TABLE 3. Antiviral Activities of the Lucibufagins.[a]

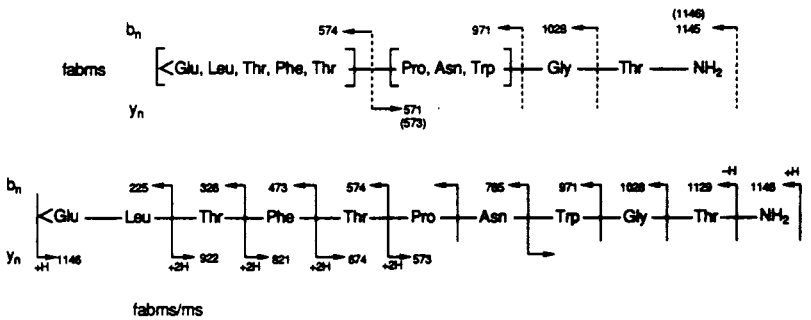
Compound	Dose per well	VSV[b]	HSV-1[c]
Lucibufagin[d]			
Ac_2	780 ng	++	+++
	20 ng	+	+
Ac-Isobutyryl	300 ng	++	++
	30 ng	+	+
Ac-Propionyl	100 ng	+	++
	30 ng	+	+
Mono-Ac	100 ng	+	++
Ribavirin	100 μg	+++	
	10 μg	++	
Ara A	100 μg		+++
	10 μg		+
Acyclovir	100 μg		+++
	10 μg		+++

[a]+, Reduced number of plaques, plaque diameter markedly reduced, visible with unaided eye throughout the well; ++, plaques visible with the unaided eye only at the edge of the well, others detected by microscope; +++, no plaques detected by microscope.

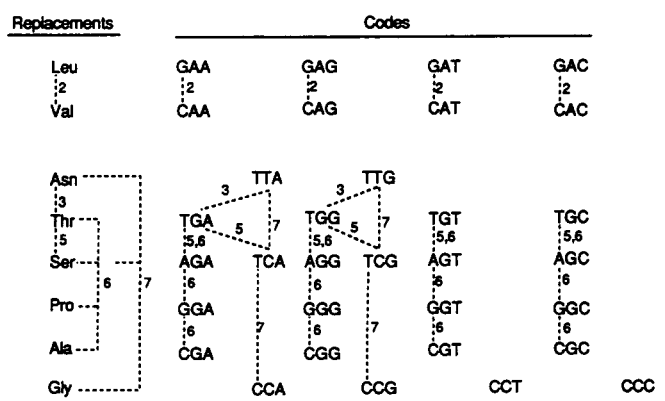
[b]*Vesicular stomatitis* virus.

[c]*Herpes simplex* virus, type 1.

[d]No cytotoxicity observed at these doses.



SCHEME 1



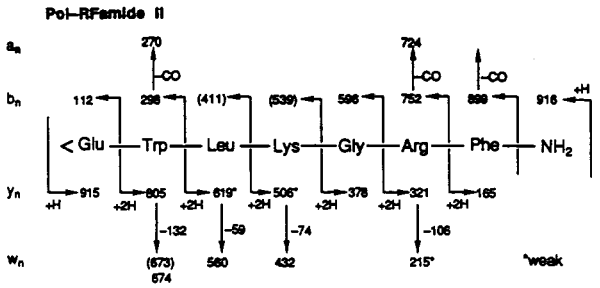
SCHEME 2

TABLE 4. Amino Acid Sequences of Insect Peptides.

Name[a]	Amino Acid										M	Species	References
	1	2	3	4	5	6	7	8	9	10			
Lom-AKH-I (AKH I)	<Glu	Leu	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Thr-NH_2	1158	*Locusta migratoria*,[b] *S. nitans*,[b] *Schistocerca gregaria*[b]	26, 27
Bld-HrTH (HTH-N, HTH-B)	<Glu	Val	Asn	Phe	Ser	Pro	Gly	Trp	Gly	Thr-NH_2	1073	*Nauphoeta cinerea*,[c] *Blaberus discoidalis*,[c] *Gromphadorhina portentosa*,[c] *Leucophaea maderae*,[c] *Blattella germanica*[c]	28–30
Cam-HrTH-II (HTF II)	<Glu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr-NH_2	1145	*Carausius morosus*,[d] *Sipyloidea sipylus*,[d] *Extatosoma tiaratum*[d]	30–32
Rom-CC-I (Ro I)	<Glu	Val	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Thr-NH_2	1144	*Romalea microptera*[e]	33
Mas-AKH (AKH-M, AKH-H)	<Glu	Leu	Thr	Phe	Thr	Ser	Ser	Trp	Gly-NH_2		1007	*Manduca sexta*,[f] *Heliothis zea*[g]	34, 35
Pea-CAH-I (M I)	<Glu	Val	Asn	Phe	Ser	Pro	Asn	Trp-NH_2			972	*Periplaneta americana*,[c] *Blatta orientalis*,[c] *Leptinotarsa decemlineata*[h]	30, 36–39
Pea-CAH-II (M II)	<Glu	Leu	Thr	Phe	Thr	Pro	Asn	Trp-NH_2			987	*P. americana*,[c] *B. orientalis*,[c] *Le. decemlineata*[h]	30, 36–39
Scg-AKH-II (AKH II-S)	<Glu	Leu	Asn	Phe	Ser	Thr	Gly	Trp-NH_2			933	*S. gregaria*,[b] *S. nitans*[b]	26, 27
Lom-AKH-II (AKH II-L)	<Glu	Leu	Asn	Phe	Ser	Ala	Gly	Trp-NH_2			903	*Lo. migratoria*[b]	26, 27
Grb-AKH (Ro II, AKH-G)	<Glu	Val	Asn	Phe	Ser	Thr	Gly	Trp-NH_2			919	*Gryllus bimaculatus*,[i] *R. microptera*[e]	33,40

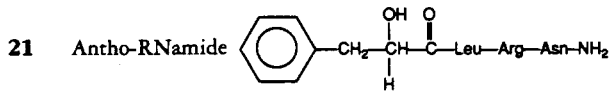
[a]The new nomenclature used is according to Raina and Gäde (41). Former names are in parentheses.
[b]Locust.
[c]Cockroach.
[d]Stick insect.
[e]Lubber grasshopper.
[f]Tobacco hornworm.
[g]Corn ear worm moth.
[h]Potato beetle.
[i]Cricket.

which argued a *C*-terminal amide and Trp unit. The tandem mass spectrum suggested the sequence <Glu-Trp-Lys-Leu-Gly-Arg-Phe-NH_2, and that peptide was synthesized commercially by solid phase techniques. However, the chromatographic and fab mass spectrometric behaviors were very slightly different for the synthetic and natural peptides. In particular, a fabms/ms peak at *m/z* 560 in the natural compound was replaced by a peak at *m/z* 545 in the synthetic material. We interpret the fragmentation for the *Polyorchis* peptide in terms of the w ions (45), involving loss of a side chain portion from the Lys and Leu units (Scheme 3).



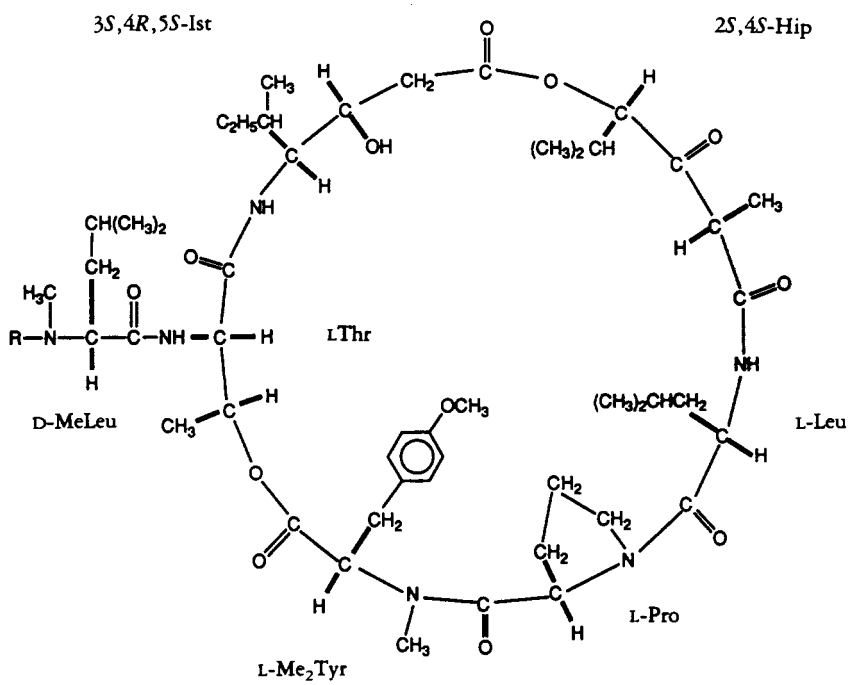
SCHEME 3

Another very intriguing invertebrate neuropeptide, Antho-RNamide [**21**], is produced along with Antho-RWamides **22** and **23** and Antho-RFamide [**24**] (42,43), by the sea anemone *Anthopleura elegantissima*. In this case, amino-acid analysis and fabms/ms defined the peptide sequence but left 149 mu unidentified, assigned as $C_9H_9O_2$ by hrfab/ms. Acetylation indicated a hydroxyl group and loss of CO in the fab tandem mass spectrum indicated a carbonyl group, and the ^1H nmr spectra of phenyllactic and hydrocinnamic acid derivatives suggested the former unit; this was confirmed by synthesizing the antho-RNamide structure shown. It differs greatly from those of other marine invertebrate neuropeptides.



22 Antho-RWamide I <Glu-Ser-Leu-Arg-Trp-NH_2
23 Antho-RWamide II <Glu-Gly-Leu-Arg-Trp-NH_2
24 Antho-RFamide <Glu-Gly-Arg-Phe-NH_2

ANTITUMOR MARINE NATURAL PRODUCTS.—*Didemnins* **25–40**.—The didemnins were originally reported in 1981 (46,47), but they continue to occupy center stage, with didemnin B being the only marine natural product currently in clinical trials as an anti-cancer agent (6,48). [At least one other, bryostatin 1 (49), is scheduled for clinical trials.] Not only do the didemnins have antitumor properties, but also they possess potent antiviral (50,51) and immunosuppressive (52) activities. Our continued examination of the large *Trididemnum solidum* extracts on hand has provided a number of new didemnins, including didemnins X and Y (53,54), and additional derivatives and analogues of didemnins A and B are regularly prepared (Table 5). In general, acylation of the *N*-methylleucine unit of didemnin A gives a much more active cytotoxic agent, while reduction of the keto group of Hip nearly eliminates activity. Some derivatives,



25 Didemnin A: R=H

26 Didemnin B: R=CH$_3$CHOHC-N(L-Lac)-C(=O)-(L-Pro)-

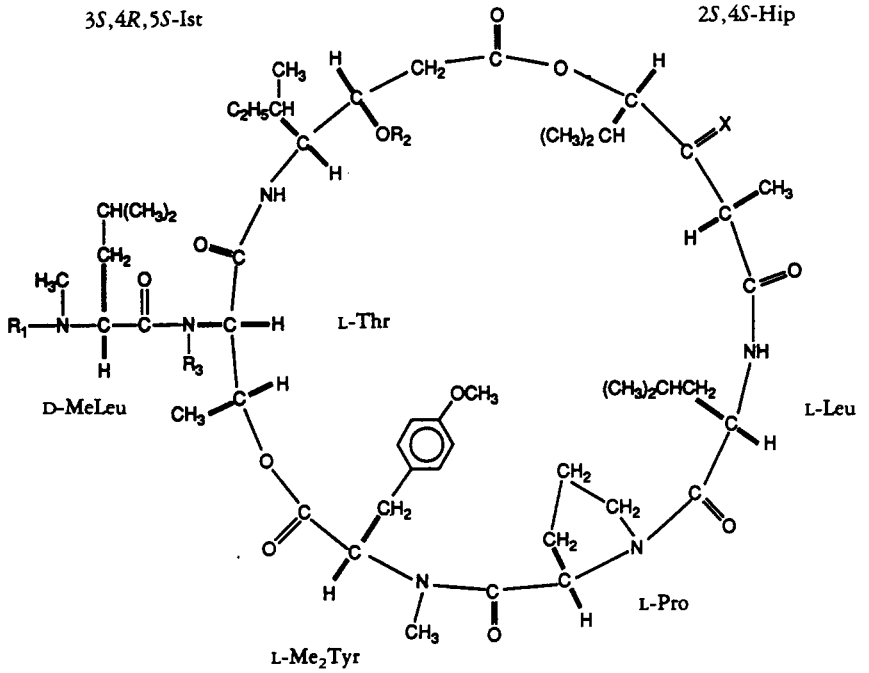
27 Didemnin C: R=L-Lac
28 Didemnin D: R=L-pGlu-(L-Gln)$_3$-L-Lac-L-Pro-
29 Didemnin E: R=L-pGlu-(L-Gln)$_2$-L-Lac-L-Pro-
30 Didemnin X: R=Hydec-(L-Gln)$_3$-L-Lac-L-Pro-
31 Didemnin Y: R=Hydec-(L-Gln)$_4$-L-Lac-L-Pro-

Hydec=*n*-C$_7$H$_{15}$-CH(OH)-CH$_2$-C(=O)-

such as methylene didemnin A and *N,O*-diacetyldidemnin A, have improved antiviral/cytotoxicity ratios; others have even greater (tenfold) immunosuppressive potency than didemnin B.

ECTEINASCIDINS.—Another group of compounds from tunicates has occupied our attention for the past few years (55,56), but the history of the material is considerably longer. Beginning in 1969 (57) and continuing through 1983 (58), a number of reports appeared regarding the antitumor activity of extracts of the colonial tunicate *Ecteinascidia turbinata*, with exceptional activity in vivo against P-388 mouse leukemia (T/C to 275, 4 of 6 cures in one experiment). During that period Lichter and co-workers (59,60) also described the remarkable activity of extracts of this tunicate as immunomodulators; the extracts inhibit mitogenesis in vitro and both splenomegaly (graft-versus-host reaction) and skin allograft rejection in vivo. However, the identification of bioactive compounds from *E. turbinata* did not follow.

Although we extracted *E. turbinata* during the Alpha Helix Caribbean Expedition



32 *N*-Acetyldidemnin A: $R_1=CH_3CO$-; $R_2=R_3=H$; X=O

33 *N*-Propionyldidemnin A: $R_1=CH_3CH_2\overset{\overset{O}{\|}}{C}$-; $R_2=R_3=H$; X=O

34 *N*-Leucyldidemnin A: $R_1=(CH_3)_2CHCH_2CH(NH_2)\overset{\overset{O}{\|}}{C}$-; $R_2=R_3=H$; X=O

35 *N*-Prolyldidemnin A: R_1=L-Pro-; $R_2=R_3=H$; X=O

36 *N,O*-Diacetyldidemnin A: $R_1=R_2=CH_3CO$-; $R_3=H$; X=O

37 *O,O'*-Diacetyldidemnin B: $R_1=CH_3CH(OCOCH_3)\overset{\overset{O}{\|}}{C}$-L-Pro-; $R_2=CH_3CO$-; $R_3=H$; X=O

38 Methylene didemnin A: $R_1+R_3=$-CH_2-; $R_2=H$; X=O

39 Dihydrodidemnin A: $R_1=R_2=R_3=H$; X=H, OH

40 Nordidemnin B: $R_1=CH_3\underset{\underset{OCOCH_3}{|}}{CH}\overset{\overset{O}{\|}}{C}$-L-Pro-; $R_2=R_3=H$; X=O

1978 and found it to be modestly cytotoxic, our efforts really began with larger collections in Belize in 1981. The initial extracts were only modestly active [ID_{50} 0.15 μg/ml vs. L1210 leukemia cells in vitro, T/C 126 (200 mg/kg) vs. P-388 murine leukemia in vivo] due to the minute amounts present of ecteinascidins, the compounds ultimately isolated from *Ecteinascidia*. A number of bioassay techniques were developed to assist in isolating the active compounds (55). First, we demonstrated activity against CV-1 monkey kidney cells, allowing us to follow the isolation of the compounds by assay against CV-1 cells. Second, we developed a cytoautography assay employing those CV-1 cells to locate active compounds by tlc as the separation proceeded (55).[4] Presum-

[4]T.G. Holt and K.L. Rinehart, manuscript in preparation.

TABLE 5. Cytotoxicity of Didemnins and Derivatives.

Didemnin	L1210 IC_{50}, μg/ml
A	0.014
B	0.0025
C	0.011
D	0.0050
E	0.0030
G	0.006
X	0.0044
Y	0.0056
nor-B	0.0078
$-CH_2$-A	0.0065
N-Ac-A	0.0024
N,O-Ac_2-A	0.015
Z-A	0.015
Pro-A	0.014
H_2-A	0.52
O,O'-Ac_2-B	0.0016

ably a difficulty in earlier attempts to isolate the active components from *E. turbinata* was reliance on in vivo assays, which were time-consuming and involved long turnaround times. Third, we surveyed a number of bacteria and found that *E. turbinata* concentrates inhibited the growth of *Micrococcus luteus* (formerly *Sarcina lutea*). The antibacterial assay then allowed us to follow the separation of the compounds much more easily. However, it must be noted that the activity of the ecteinascidins (Et's) against bacteria is far less than that against mammalian cells (55). In the early stages of isolation, activity in tissue culture was the guiding assay.

Our standard isolation procedure [extraction with MeOH-toluene (3:1) and successive partitions with toluene, $CHCl_3$, EtOAc, and *n*-BuOH] indicated the majority of the activity to reside in the $CHCl_3$ extract (Table 6); thus, the compounds appeared to be slightly polar. Numerous attempts at purification, employing normal or reversed-phase chromatography, usually led to extensive loss of activity, and in addition, the compounds appeared to be sensitive to light, acid, and enzymatic degradation. A most effective method for purification ultimately proved to be centrifugal countercurrent chromatography (ccc, Ito coil) (61,62). During this small-scale equivalent of counter-

TABLE 6. Ranges of Potencies for Biological Activities of Partition Fractions.

Fraction	*Bacillus subtilis*[a] 100 μg/disk (mm)	HSV-1[b] 100 μg/disk	CV-1[a,c] 100 μg/disk (mm)	L1210 ID_{50} (μg/ml)	P-388	
					Dose (mg/kg)	T/C (%)
$C_6H_5CH_3$	0	− to ±	0–18	0.84–0.24	200	110
$CHCl_3$	0–19	− to +	22–36	0.07–0.0075	25	154
EtOAc	0	− to +	0–21	4.4–3.8	400	117
n-BuOH	0	− to ±	0–15	13–1.4	400	129

[a]12.5-mm disk.

[b]−, No change in the number or diameter of viral plaques; ±, no change in the number of plaques, plaque diameter somewhat reduced; +, reduced number of plaques, plaque diameter markedly reduced, visible with unaided eye throughout the well.

[c]36-mm well.

current distribution, the compounds are not exposed to chromatographic adsorbent. Activity emerging from the Ito coil was monitored by tissue culture assay against CV-1 cells, as described above. The activities of some fractions from the Ito coil are shown in Table 7. Interestingly, although fraction 2 shows the greatest cytotoxicity, fraction 6 is the most active in vivo.

For the initial characterization of the ecteinascidins we turned to combined liquid chromatography (lc, moving belt)/fabms (63,64) in which the reconstructed ion current trace paralleled closely the uv chromatographic trace (Figure 1). Moreover, lc/fabms located Et 743 in a fraction near 9 min and Et 745 in a fraction near 13 min, and showed two separate fractions (5 and 8 min) for Et's 759 A and B. The mass spectra obtained for these traces were even better than mass spectra obtained on isolated samples; the latter showed matrix ions, but the lc/fab mass spectra were obtained without matrix (Figure 2), because the transport of the belt provides a renewable sample. Note the absence in Figure 2 of matrix peaks in the low mass region where a number of intense Et ions (204, 218, 224, 250, etc.) are found. Other regions of intense ions are near *m/z* 500 (495, 477, 463, etc.) and, of course, the "molecular ion" region (M + H at *m/z* 744).

Accurate mass values were obtained on the [M + H] ions of the six ecteinascidins (designated Et's 729, 743, 745, 759A, 759B, and 770), but assignment of molecular formulas awaited a ^{13}C-nmr spectrum, which was initially obtained on 600 μg of Et 743. This showed that compound to have 39 carbon atoms, and from the accurate mass value the molecular formula $C_{39}H_{41}N_3O_{10}S$ was assigned. The presence and amount of sulfur were confirmed by an Electron Spectroscopy for Chemical Analysis (ESCA) study carried out at The Upjohn Company. The molecular formulas (from hrfabms) show that the ecteinascidins are closely related, Et 729 and Et 743 being homologues, Et 745 the reduced form of Et 743, Et 759A and Et 759B being oxidized versions of Et 743, and Et 770 having an additional carbon, hydrogen, and nitrogen (vs. Et 743).

The biological activities differ (Table 8), with Et 729 being the most active in vivo

TABLE 7. Bioactivities of CCC Fractions of *Ecteinascidia turbinata*.

Fraction	*Micrococcus luteus*	L1210	P-388	
	(1 μg/disk[a]) (mm)	ID_{50} (ng/ml)	Dose (mg/kg)	T/C (%)
SM[b]	9	2.6	0.5	188
1	8	1.6		
2	12	0.37	0.063	129
3	15	0.92		
4	12	1.6		
5	13	0.88	0.19	200
6	12	0.78	0.13	233
7	12	0.88		
8	7	3.4		
9		12		
10		16		
11		19		
12		19		
13		14		
14		15		
15		23		
16		84		

[a]6.35-mm disks.
[b]SM = Starting Material.

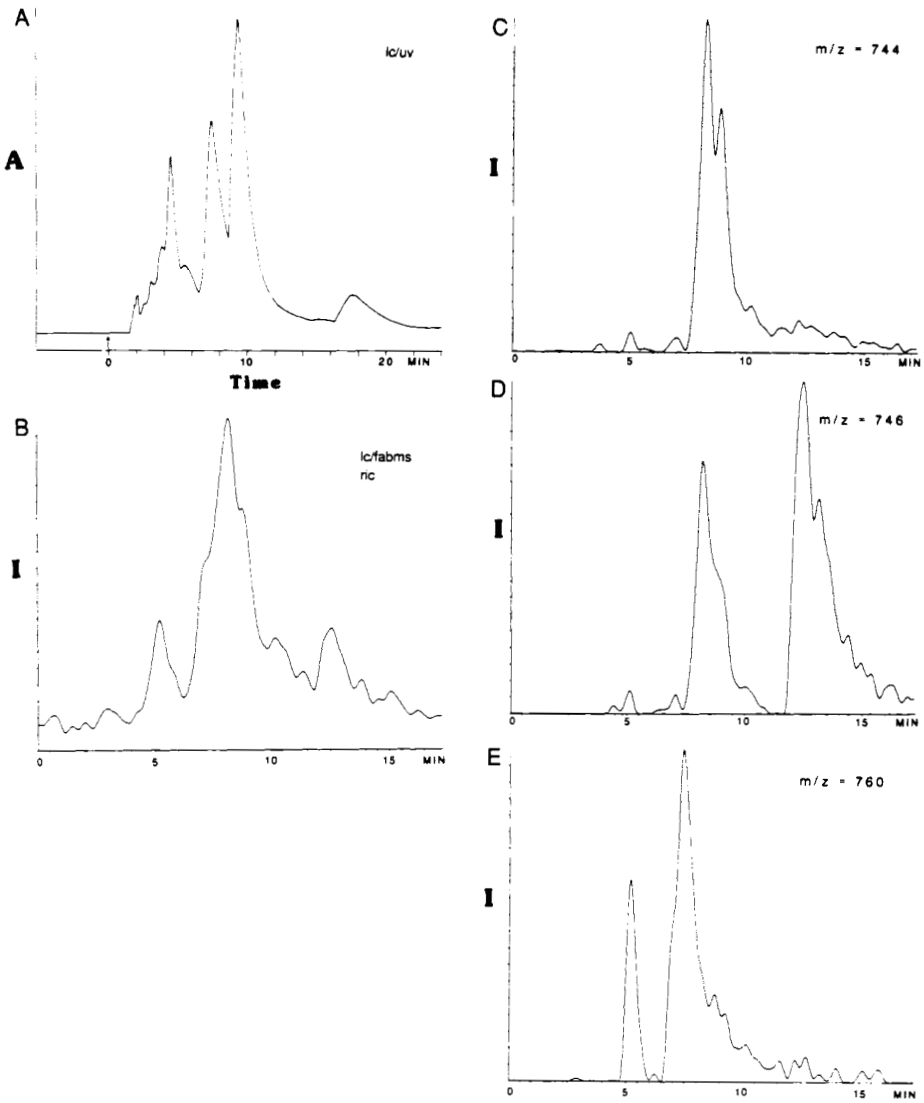


FIGURE 1. A: Uv chromatogram for a mixture of ecteinascidins 743, 745, 759A, and 759B [Whatman Partisil 5 ODS column (5 μm, 4.6 × 250 mm), MeOH-H_2O-NH_4HCO_2 (70:30:0.1), 1.0 ml/min]. B: Reconstructed ion chromatogram (ric) for the same mixture [Alltech C18 microbore column (10 μm, 1 × 250 mm), MeOH-H_2O-NH_4HCO_2 (70:30:0.1), 100 μl/min]. C: Ion chromatogram for *m/z* 744. D: Ion chromatogram for *m/z* 746. E: Ion chromatogram for *m/z* 760.

against P-388 and even more potent against B16 melanoma, while Et 743 is more cytotoxic and Et 745 is generally much less active.

Structural characterization of the ecteinascidins began with the uv spectrum (Figure 3), which was in accord with the presence of one or more phenolic units, with low extinction coefficients in the 280-nm region, high extinction coefficients in the 200-nm region, and a bathochromic shift in base. The ir spectrum indicated two carbonyls, a phenolic ester at 1760 cm^{-1}, and an aliphatic ester at 1740 cm^{-1}.

The three nitrogens were found in separate, roughly equivalent units with one nitrogen each, as demonstrated by hrfabms and fabms/ms measurements on the major ions found in the fab mass spectrum (Scheme 4). Thus, although the ion at *m/z* 204

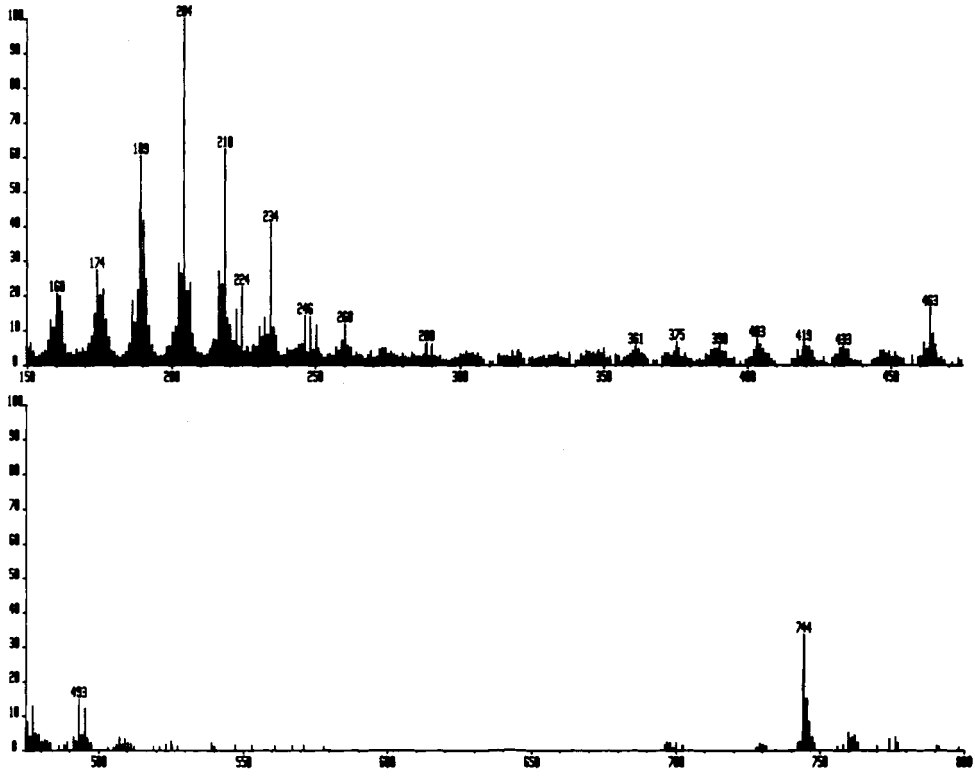


FIGURE 2. Lc/fab mass spectrum of ecteinascidin 743.

could in principle have resulted from cleavage of the [M − CH_2SH] ion to give the *m/z* 204 and the *m/z* 495 ions, tandem mass spectrometry (ms/ms) demonstrates that the *m/z* 204 ion itself arises from the ion at *m/z* 495 (and, thus, is part of the latter unit), as shown in Scheme 4.

The ^{1}H-nmr spectrum showed a number of isolated recognizable groupings (Scheme 5) including three singlet aromatic hydrogens, two aromatic methyl groups, two aromatic methoxyls, an *N*-methyl, and an aromatic methylenedioxy unit. The two esters, two methoxyls, and methylenedioxy unit account for eight of the ten oxygens; the formation of a di-*O*-methyl derivative (aromatic methoxyls) confirmed the remaining two as phenolic hydroxyls. In addition to the isolated groupings, a number of relatively small spin systems could be identified involving no more than three carbons per system, as shown in Scheme 6. The spin systems were established by extensive spin decoupling but more definitively by COSY spectroscopy, as shown in Table 9.

TABLE 8. Biological Activities of the Ecteinascidins.

	Mol. formula	L1210	P-388		B16	
		IC_{50}[a]	Dose[b]	T/C	Dose[b]	T/C
Et 729	$C_{38}H_{39}N_3O_{10}S$		3.8	214	10	246
Et 743	$C_{39}H_{41}N_3O_{10}S$	0.5	15	167		
Et 745	$C_{39}H_{43}N_3O_{10}S$	88	250	111		

[a]Concentration for 50% inhibition in ng/ml.
[b]Dose in μg/kg.

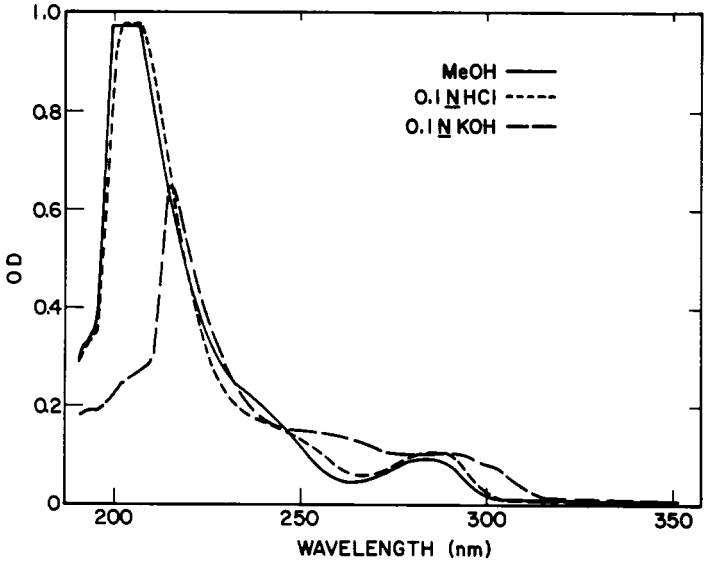
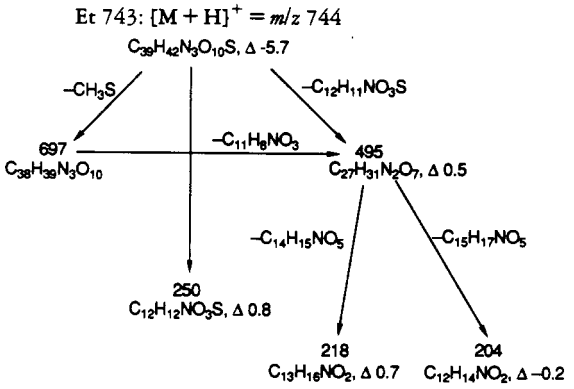
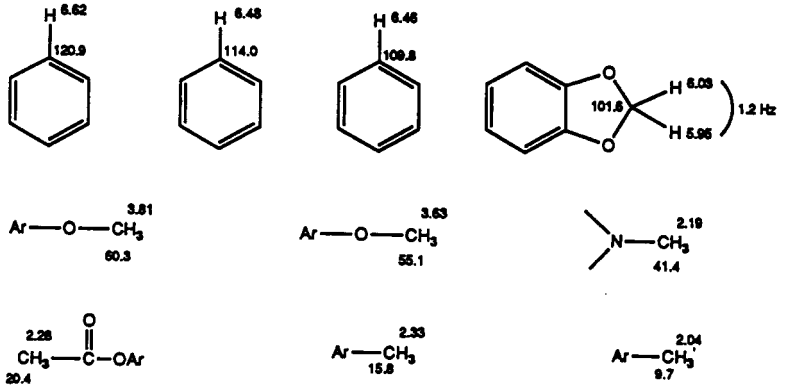


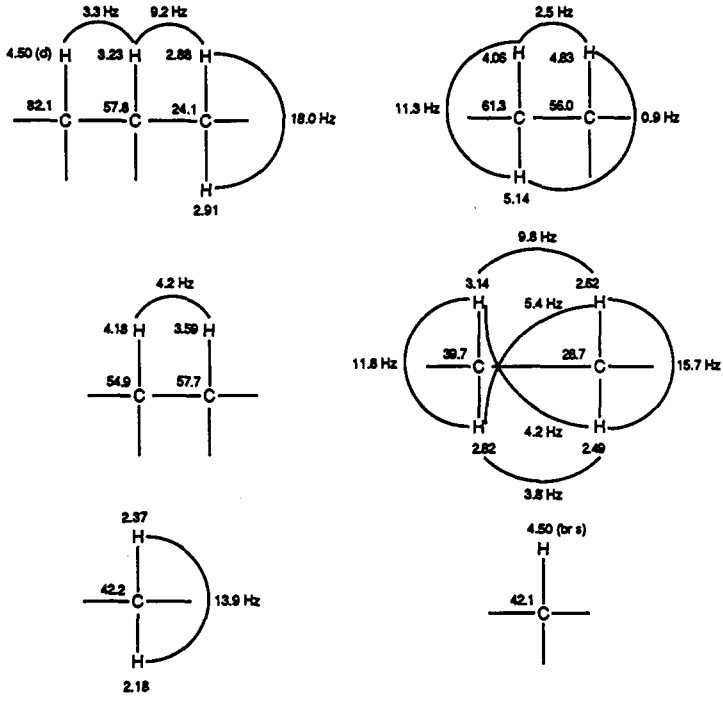
FIGURE 3. Uv spectrum (MeOH) of ecteinascidin 743.



SCHEME 4



SCHEME 5



SCHEME 6

The hydrogens are further linked by HETCOSY spectra to the carbons to which they are attached, as shown in Table 10. The chemical shifts of a number of the protons and carbons argue for their attachment to nitrogen. The most effective procedure for combining the isolated spin systems is long-range coupling, both heteronuclear and homonuclear. Thus, one tetrahydroisoquinoline unit (A) is defined by the long-range coupling shown in Scheme 7 and the nOe results shown in Scheme 8. Similarly, tetrahydroisoquinoline units B and C are defined by long-range coupling and nOe spectroscopy. The two other tetrahydroisoquinoline units (B, which overlaps A, and C) could be only partially defined by long-range coupling and nOe spectroscopy at the time of this presentation, as shown in Schemes 7 and 8, respectively.

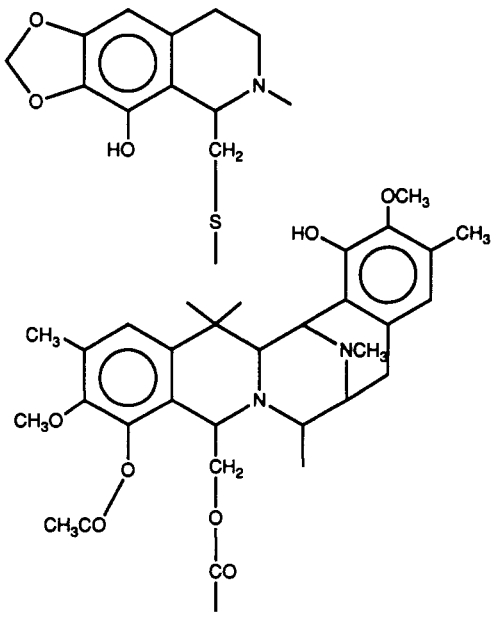
At the time of this material's presentation, we favored the nearly complete arrangement **41**. Subsequently, however, we have assigned the complete formula **42**, based on a reexamination of HMBC data and new negative ion hrfabms measurements, which indicate the addition of H_2O to our original molecular formula (65). Similar structures, but lacking the C-21 hydroxyl and N-2′ proton (*i.e.*, C-21, N-2′ joined, a suggestion we originally made at the time of this presentation), have been assigned to Et 729 and Et 743 by Wright *et al.* (66) but have been revised to **42** (with minor stereochemical differences) to accord with our new negative ion fabms data.

The possible biogenetic pathway shown in Scheme 9 involves initial condensation of two tyrosine units to form a diketopiperazine, which could then be reduced to a bis(carbinolamine), which cyclizes to form unit A. Ring closure to form unit B would then be effected by an aldehyde derived from serine. Oxidation of the phenol in unit B to a quinone methide followed by nucleophilic addition could then attach unit C. Introduction of additional ring oxygens and methylation by methionine could be carried out at various stages. The overall scheme at this stage is highly speculative, but the outline appears reasonable.

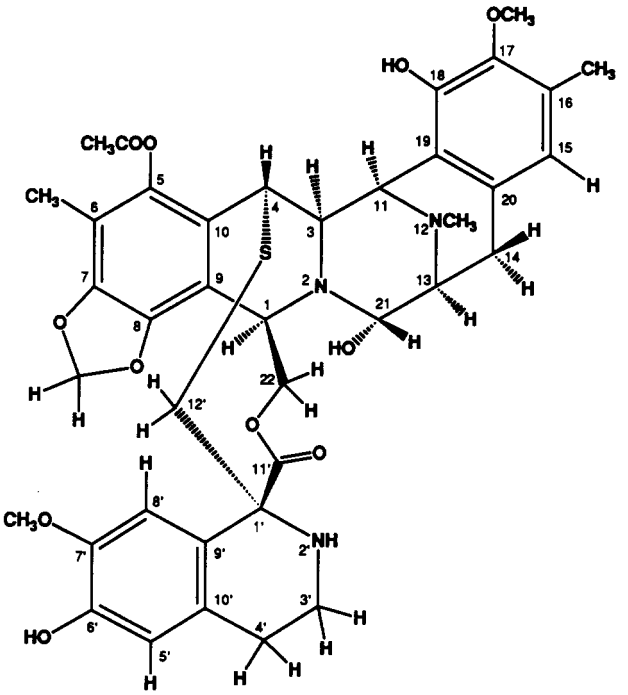
TABLE 9. ^1H-^1H COSY Data, Including Long-Range Coupling, for Ecteinascidin 743.

δ ($CDCl_3$)	Coupled Protons, δ ($CDCl_3$)	
	Short-Range	Long-Range
6.62		2.33, 2.91, 2.88
6.48		2.62, 2.49
6.46		3.63
6.03	5.95	
5.95	6.03	
5.71		
5.14	4.83, 4.06	
4.83	5.14, 4.06	3.59, 2.04
4.50 (d)[a]	3.23	
4.50 (br s)[a]	3.59	
4.18	3.59	3.23
4.06	5.14, 4.83	
3.81		
3.63		6.46
3.59	4.18, 4.50 (br s)	4.83
3.23	4.50 (d), 2.91, 2.88	4.18
3.14	2.82, 2.62, 2.49	
2.91	3.23, 2.88	6.62, 2.33
2.88	3.23, 2.91	6.62, 2.33
2.82	3.14, 2.62, 2.49	
2.62	3.14, 2.82, 2.49	6.48
2.49	3.14, 2.82, 2.61	6.48
2.37	2.18	
2.33		6.62, 2.91, 2.88
2.28		
2.19		
2.18	2.37	
2.04		4.83

[a]The two protons at δ 4.50 are distinguished by d = doublet, br s = broad singlet.

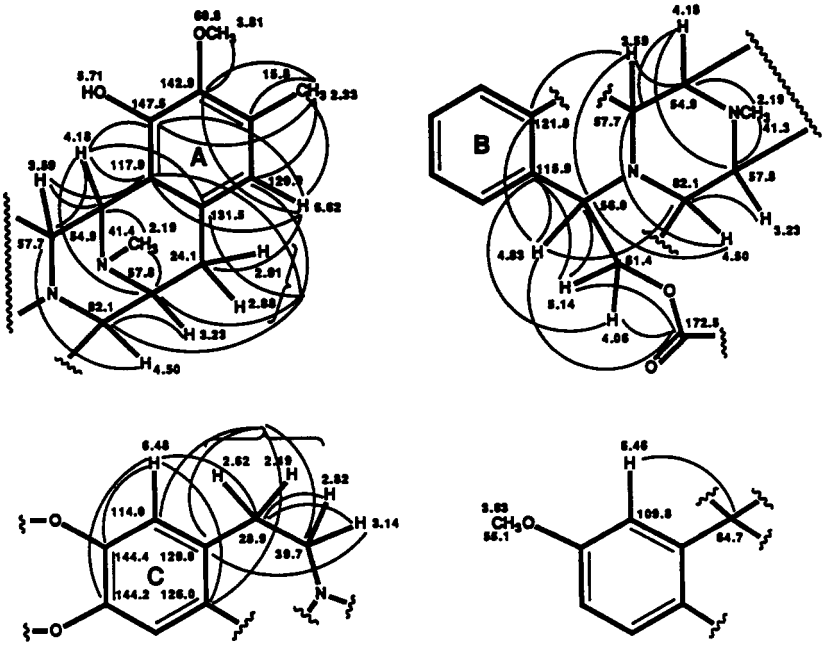


41

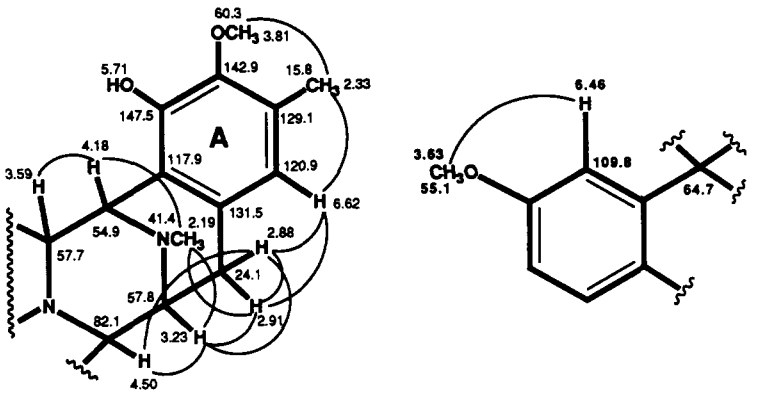


4:2

The structures of the other ecteinascidins follow directly from that of Et 743; that is, they are *N*-demethyl (Et 729), reduced at C-21 (Et 745), and cyano at C-21 (Et 770) analogues and *N*-oxides (Et 759 A, Et 759B) of Et 743 (55). The complete assignment of the ecteinascidins proved remarkably difficult due to the minute amount of the mate-



SCHEME 7



SCHEME 8

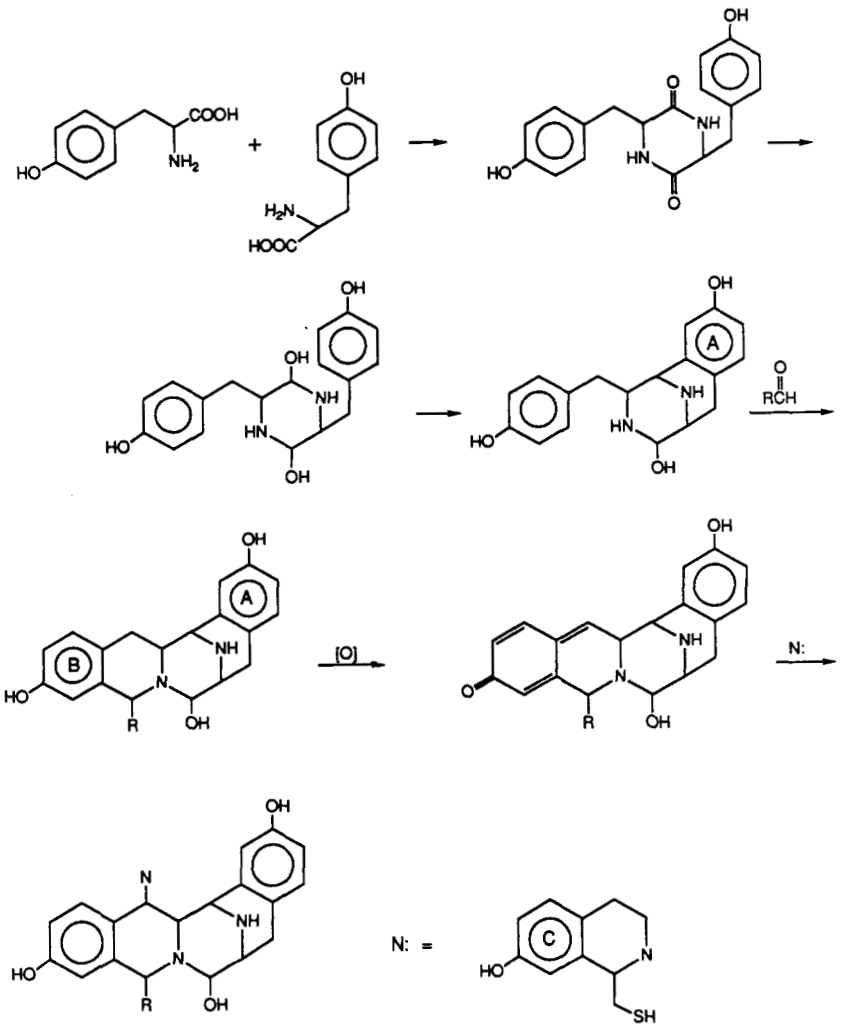
rial available, its tendency to decompose under a variety of conditions, and lack of correlation between the isolated structural units. Nevertheless, the remarkable activity of the compounds appears to fully warrant their introduction to the pharmaceutical scene at this juncture.

The current scene is propitious for the organic chemistry of natural products. Newer isolation techniques involving chromatographic methods, sensitive bioassays, bioautography, and radioimmunoassay have been developed, and newer methods of structure elucidation, especially hrfabms, lc/fabms, and fabms/ms, allow study at the microgram level. With more material (10–100 μg), newer nmr pulse sequences pro-

TABLE 10. ^{13}C-nmr Absorptions for Ecteinascidin 743, Including DEPT and HETCOSY Results.

^{13}C nmr[a] δ, multiplicity[b]	^{1}H nmr δ, multiplicity[b]	^{13}C nmr[a] δ, multiplicity[b]	^{1}H nmr δ, multiplicity[b]
172.5, s		64.7, s	
168.3, s		61.3, t	5.14 (1H, dd)
147.5, s			4.06 (1H, dd)
145.1, s		60.3, q	3.81 (3H, s)
144.4, s		57.8, d	3.23 (1H, br d)
144.2, s		57.7, d	3.59 (1H, br d)
142.9, s		56.0, d	4.83 (1H, br s)
141.3, s		55.1, q	3.63 (3H, s)
140.4, s		54.9, d	4.18 (1H, br d)
131.5, s		42.2, t	2.37 (1H, br d)
129.1, s			2.18 (1H, d)
129.0, s		42.1, d	4.50 (1H, br s)
126.0, s		41.4, q	2.19 (3H, s)
121.8, s		39.7, t	3.14 (1H, ddd)
120.9, d	6.62 (1H, s)		2.82 (1H, ddd)
117.9, s		28.9, t	2.62 (1H, ddd)
115.9, s			2.49 (1H, ddd)
114.0, d	6.48 (1H, s)	24.1, t	2.91 (1H, br d)
112.5, s			2.88 (1H, dd)
109.8, d	6.46 (1H, s)	20.4, q	2.28 (3H, s)
101.6, t	6.03 (1H, d)	15.8, q	2.33 (3H, s)
	5.95 (1H, d)	9.7, q	2.04 (3H, s)
82.1, d	4.50 (1H, d)		

[a]Taken in $CDCl_3$.
[b]s = singlet, d = doublet, t = triplet, q = quartet, br = broad.



SCHEME 9

vide an inordinate amount of information, limited only by sample size, on previously intractable compounds.

With the tools at hand, organic chemists can attack ever more challenging problems with smaller amounts of material. It is truly an exciting time for natural products research.

ACKNOWLEDGMENTS

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