MARINE NATURAL PRODUCTS AS SOURCES OF ANTIVIRAL, ANTIMICROBIAL, AND ANTINEOPLASTIC AGENTS*

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Abstract. Extracts of marine species from Baja California and the
Caribbean have been examined on shipboard for a variety of bioacti-
vities and their constituents studied there by gas chromatography/
mass spectrometry. A very high proportion of the extracts has been
shown to be cytotoxic, a high proportion to be antibacterial or
antifungal and a surprisingly large number to be antiviral. Many
of these activities have been confirmed in more extensive assays
against tumor cells, pathogenic microorganisms and a battery of
viruses. A number of the compounds responsible for the activities
have been identified, including several new compounds. Of special
current interest are the didemnins, depsipeptides isolated from a
didemnid tunicate, which inhibit a number of RNA and DNA viruses
and exhibit potent cytotoxicity vs. tumor cell lines.

Systematic studies of marine natural products can be directed toward a particular phylum, such as sponges (Ref. 1), toward a particular class of compounds, such as sterols (Ref. 2), toward a class of compounds in a phylum, such as terpenes in gorgonians (Ref. 3), or toward a particular bioactivity, such as antineoplastic activity (Ref. 4). Our own interest in marine natural products arose initially out of our continuing studies on antibiotics (Ref. 5), and has been directed toward compounds possessing antimicrobial (antibacterial or anti-fungal) or, more recently, antiviral or antineoplastic activity. In the first instance, we sought to determine whether such activities are widely distributed among the marine phyla and, in the second instance, to ascertain whether the compounds responsible for the activities are of novel or useful structural types.

The primary collecting of marine species for examination was carried out during two expeditions (in 1974 and 1978) aboard the R/V Alpha Helix, a research vessel with laboratory space designed for marine biology and biochemistry studies and sponsored by the U.S. National Science Foundation. During each of these expeditions a comprehensive survey of specific bioactivities was carried out on shipboard, with assays designed to use small samples of the marine species (to avoid ecological disruption) on site (to allow recollection of species with significant activity). The first of these expeditions (AHBE 1974) examined the antimicrobial activities of some 831 species along the west coast of Baja California and in the

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*This paper is dedicated to the memory of Werner Bergmann, a pioneer in studies of marine natural products and an inspiring teacher of Yale students.

Gulf of California, which separates the west coast of the Mexican mainland from the Baja California peninsula, at sites selected to include a variety of climatic zones (Ref. 6,7). To speed the identification of antimicrobial compounds in the extracts, a gas chromatographic mass spectrometer was employed on the Alpha Helix (Ref. 8). These antimicrobial results, which have been reported in brief elsewhere (Ref. 7) and are repeated here in Table 1, showed that antimicrobial activity can be found in a number of phyla and is particularly prominent in the Porifera and the Echinodermata. The extracts from AHBE 1974

TABLE 1. Antimicrobial activity in phyla assayed during the Alpha Helix Baja Expedition 1974

Phylum	Number of Species Examined	% Species Active[a]				
		Overall	E.c.	B.s.	S.c.	P.a.
Porifera	71	37	18	32	13	17
Cnidaria	72	21	6	15	6	3
Ctenophora	3	0	0	0	0	0
Platyhelminthes	4	25	0	0	0	25
Nemertina	4	0	0	0	0	0
Entoprocta	1	0	0	0	0	0
Ectoprocta	13	23	8	23	0	8
Mollusca	199	16	4	14	5	9
Sipunculida	4	0	0	0	0	0
Annelida	37	16	3	16	3	5
Arthropoda	98	1	0	1	0	0
Chaetognatha	1	0	0	0	0	0
Echinodermata	83	43	0	17	27	16
Chordata	81	6	0	6	1	1
Cyanophyta	2	0	0	0	0	0
Chlorophyta	31	10	0	10	0	0
Chrysophyta	1	0	0	0	0	0
Phaeophyta	46	28	2	28	11	7
Rhodophyta	104	14	1	14	4	4
Tracheophyta	6	50	17	33	0	17

[a]E.c. = Escherichia coli, B.s. = Bacillus subtilis, S.c. = Saccharomyces cerevisiae, P.a. = Penicillium atrovenetum.

have been stored and a limited number of them have been examined subsequently for additional activities, including anti-Herpes activity and cytotoxicity against monkey kidney cells, L1210 (leukemia) cells and KB (nasopharyngeal cancer) cells (Ref. 9), as shown in Table 2. Although non-systematic, the latter assays did indicate occasional antiviral activity and widespread cytotoxicity in marine extracts and these indications led to our incorporating systematic antiviral and cytotoxicity assays into our second expedition, as described in the next paragraph. From time to time, additional samples of active species identified from the AHBE 1974 collection have been collected, as necessary, in Baja California (Ref. 10), employing a mobile laboratory consisting of a converted 26-foot GMC motor home equipped with chromatographic and other scientific equipment and collecting equipment (16-foot Boston Whaler, 85-horsepower outboard engine, scuba tanks, compressor, etc.).

The second expedition (AHCE 1978) explored Caribbean fauna and flora with similar goals. In this case, the ship's track commenced in Panama and the R/V Alpha Helix visited collecting sites in Panama, Colombia, Nicaragua, Honduras, Belize and Mexico (Figure 1). To the bioassays previously employed on the ship vs. the four microorganisms cited in Table 1 was added a screen for inhibition of a Herpes virus in monkey kidney cells (CV-1). The latter indicated not only antiviral activity but also cytotoxicity, which might predict antineoplastic activity. These results are summarized in Table 3. A shipboard assay was also carried out for stimulation of acetylcholine release by fish brain synaptosomes (Ref. 11). Table 3 shows again that the Porifera provide a rich source of antimicrobial activity. The larger number and proportion of sponge samples from AHCE 1978 relative to AHBE 1974 reflect in part the differing abundance of sponges in Caribbean vis-a-vis Baja California waters and in part a preferential collection of those phyla shown previously to provide likely sources of bioactive compounds.

Although the incidence of antimicrobial activity was quite high in the Porifera and Echinodermata, it was also above 25% in the Chordata, Cnidaria and the four major marine plant phyla. Moreover, antiviral activity was highest in the Cyanophyta, Phaeophyta and Chordata

TABLE 2. Antiviral activity and cytotoxicity in phyla collected during the Alpha Helix Baja Expedition 1974

Phylum[a]	% Species Active (Number of Species Examined)			
	HSV-1[b]	CV-1[c]	L1210[d]	KB[e]
Porifera	61 (13)	46 (13)	67 (6)	31 (52)
Cnidaria	0 (1)	100 (1)	54 (13)	28 (47)
Ectoprocta	(0)	(0)	(0)	10 (10)
Mollusca	(0)	(0)	(0)	5 (163)
Arthropoda	(0)	(0)	(0)	3 (68)
Echinodermata	67 (3)	50 (4)	0 (10)	27 (71)
Chordata	(0)	(0)	57 (7)	2 (47)
Chlorophyta	(0)	(0)	0 (1)	12 (26)
Phaeophyta	(0)	(0)	0 (1)	5 (43)
Rhodophyta	67 (3)	100 (3)	57 (7)	0 (93)

[a]No activity found in the following phyla (# of species examined): Ctenophora (3); Platyhelminthes (5); Nemertina (3); Entoprocta (2); Sipunculida (4); Annelida (31); Chaetognatha(1); and miscellaneous plants (6). [b]Inhibiting Herpes simplex virus, type I, at ≤ 200 μg/disc. [c]Cytotoxic to monkey kidney cells at ≤ 200 μg/disc. [d]Cytotoxic in L1210 tumor cell assay to the extent ID_{50} ≤ 10 μg/mL. [e]Cytotoxic in KB tumor cell assay to the extent ED_{50} ≤ 200 μg/mL.

TABLE 3. Antimicrobial and antiviral activities and cytotoxicity in phyla assayed during the Alpha Helix Caribbean Expedition 1978

Phylum	% Species Active[a] (Number of Species Examined)[b]						
	Overall Antimicrobial	E.c.	B.s.	S.c.	P.a.	HSV-1	CV-1
Porifera	82 (187)	14	41	19	11 (138)	14 (180)	62 (186)
Cnidaria	35 (70)	4	26	7	2 (66)	17 (69)	56 (70)
Ectoprocta	100 (1)	100	100	0	0	0 (1)	0 (1)
Mollusca	16 (20)	5	15	0	0 (17)	0 (21)	33 (21)
Annelida	33 (3)	33	0	0	0	0 (3)	0 (3)
Arthropoda	0 (6)	0	0	0	0	0 (6)	0 (6)
Echinodermata	58 (36)	0	3	50	26 (27)	16 (36)	72 (36)
Chordata	68 (27)	15	37	15	14 (22)	23 (26)	70 (27)
Cyanophyta	100 (5)	20	60	20	0 (4)	100 (5)	80 (5)
Chlorophyta	74 (42)	7	55	10	5 (41)	7 (42)	36 (42)
Phaeophyta	35 (19)	0	37	0	0 (18)	25 (19)	50 (19)
Rhodophyta	46 (43)	10	35	7	0	17 (42)	43 (42)
Tracheophyta	0 (3)	0	0	0	0	33 (3)	0 (3)

[a]Microbial, viral and cell line abbreviations same as for Tables 1 and 2.
[b]Number of species examined same as overall antimicrobial except as noted.

(Ascidiacea). We had earlier observed modest antiviral activity in other compound tunicates (Ref. 12,13); thus, the latter observation came as no surprise. Cytotoxicity was widely distributed among the phyla.

With the large number of active organisms shown in Tables 1-3 and a limited number of co-workers, it was necessary to arrange the active species in an order of priority according to some pre-considered quantitative measure of activity. This measure would then guide the studies toward the most interesting compounds, at least from a biological standpoint, the chemical standpoint being, in any event, unpredictable. For the AHBE organisms these prio-rity lists are shown in Tables 4-7 for activities against Escherichia coli, Bacillus subti-lis, Saccharomyces cerevisiae, Penicillium atrovenetum, Herpes simplex virus, type I, and L1210 or KB cells. Tables 4 and 5 derive, as noted above, from comprehensive on-site testing of essentially all the AHBE samples, Tables 6 and 7 from far more limited, and later, testing. The four tables indicate that while the highest incidence of activity may be in the Porifera and the Echinodermata (cf. Table 1), the most interesting activities are actually rather widely distributed among the phyla. Moreover, while there is some overlap with respect to
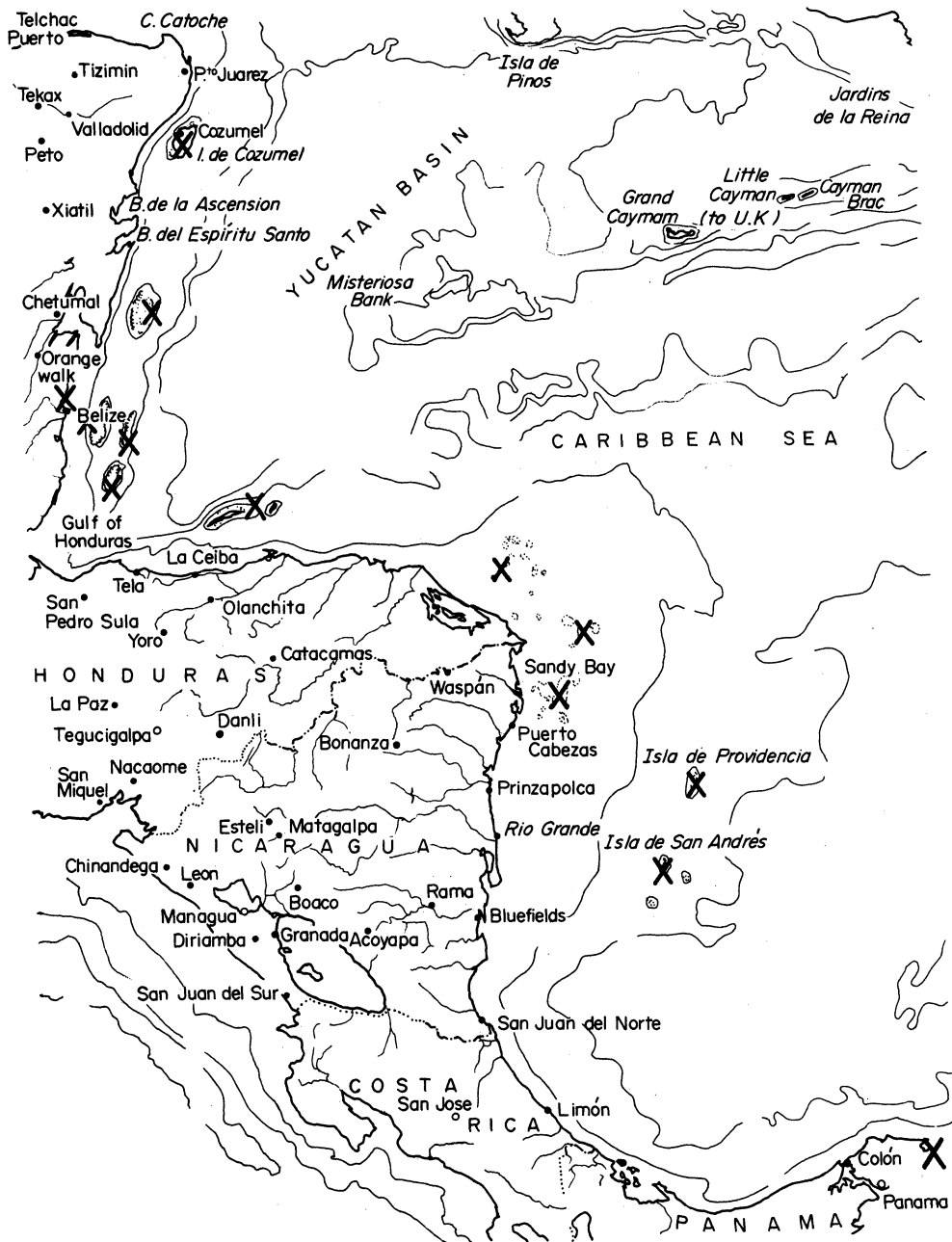
Fig. 1. Collecting sites (x) for the Alpha Helix Caribbean Expedition 1978.

antimicrobial activity (Tables 4, 5), the organisms most active against one microorganism are by no means those most active against others.

Among the species included in Tables 4-7, a number have yielded compounds reported previously by our group or by groups elsewhere (Fig. 2, 3). The antimicrobial compounds present in the sponge AHBE #1069 (Acarnus erithacus, Tables 4, 6, 7) were shown by Carter to be the acarnidines (1-3) (Ref. 14). The most active antimicrobial compounds present in Aplysina fistularis (formerly called Verongia aurea, sponges AHBE #852 and 585, Tables 4,5,7) were shown by Goo to include 4 (aeroplysinin-1), 5 and 6 (Ref. 15). An Aplidium species (presumably related to Chordata AHBE #554, Table 5) was shown by Carter to contain aplidiasphingosine (7) (Ref. 13). Laurinterol (8), identified by gas chromatography/mass spectrometry on shipboard (Ref. 8,16), was the principal component of Rhodophyta samples AHBE #513 and 943 (Laurencia decidua, Tables 4-7) (Ref. 17) and was shown in more detailed

TABLE 4. Priority list of species from AHBE 1974 active against bacteria

Escherichia coli			Bacillus subtilis		
Zone of Inhibition (mm)[a]	AHBE Sample	Phylum	Zone of Inhibition (mm)[a]	AHBE Sample	Phylum
30	#225	Mollusca	37	#454	Annelida
	454	Annelida	31	513	Rhodophyta
27	1102	Mollusca	30	871	Mollusca
23	451	Porifera	29	872	Mollusca
22	1131	Cnidaria	26	225	Mollusca
	852	Porifera		451	Porifera
21	1124	Porifera	25	556	Porifera
19	727a	Mollusca		57	Rhodophyta
	557	Mollusca		549	Rhodophyta
18	645	Porifera	24	727a	Mollusca
	729	Porifera	23	852	Porifera
	1130	Mollusca		888	Porifera
17	984	Rhodophyta		1124	Porifera
	1027	Porifera	22	729	Porifera
	1069	Porifera		1025	Mollusca
	1037	Cnidaria	21	288	Phaeophyta
	1105	Cnidaria		1051	Phaeophyta
	1301	Phaeophyta		1153	Phaeophyta
				645	Porifera
				149	Arthropoda

[a]From 100 µL of a 20-mL methanol-toluene (3:1) extract of 2 g of sample.

TABLE 5. Priority list of species from AHBE 1974 active against fungi

Saccharomyces cerevisiae			Penicillium atrovenetum		
Zone of Inhibition (mm)[a]	AHBE Sample	Phylum	Zone of Inhibition (mm)[a]	AHBE Sample	Phylum
31	#978b	Echinodermata	39	#1135	Mollusca
29	859	Porifera	30	513	Rhodophyta
26	451	Porifera	29	1122a	Mollusca
24	912b	Echinodermata		585	Porifera
	1009a	Echinodermata		451	Porifera
	238	Echinodermata	28	488	Mollusca
	556	Porifera	25	454	Annelida
	557	Mollusca	24	995a	Mollusca
23	554	Chordata		670	Mollusca
	483	Cnidaria		370	Mollusca
	229	Echinodermata	23	57	Rhodophyta
	871	Mollusca		727a	Mollusca
22	872	Mollusca	21	645	Porifera
21	589	Echinodermata		229	Echinodermata
	57	Rhodophyta			

[a]See footnote, Table 4.

tests to be highly antimicrobial (Ref. 18-20) as well as an inhibitor of L1210 cells and of reverse transcriptase (Ref. 18,19). Extracts of the red algae (Tables 4,6,7) Bonnemaisonia hamifera (AHBE #1013) and Asparagopsis taxiformis (AHBE #549 and 984) were shown to contain brominated ketones (e.g., 9-13) (Ref. 21-23). A recent systematic study of Dictyopteris undulata in our laboratory has identified the known compounds zonarol (14) (Ref. 24), yahazunol (15) (Ref. 25) and zonaroic acid (16) (Ref. 26) as the major compounds responsible for the antimicrobial activity of D. undulata (Phaeophyta, AHBE #288, Table 4). Other laboratories have identified the antimicrobial compounds dictyodial (17) and pachydictyol-A epoxide (18) in Dictyota flabellata (Phaeophyta, AHBE #1301, Table 4) (Ref. 27,28).
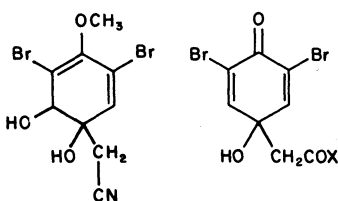
TABLE 6. Species from AHBE 1974 active against _Herpes simplex_ virus, type I

Activity	AHBE Sample	Phylum
+	#364	Porifera
	885	Porifera
	1033	Porifera
	1070	Porifera
	471	Porifera ·
±	785	Porifera
	969	Porifera
	1069	Porifera
	635	Echinodermata
	576	Echinodermata
	943	Rhodophyta
	1013	Rhodophyta

TABLE 7. Priority list of species from AHBE 1974 cytotoxic against L1210 or KB cells

Activity	AHBE Sample	Phylum
L1210 (ID_{50}, µg/mL)		
1.3	#984	Rhodophyta
1.8	943[a]	Rhodophyta
2.0	1127	Cnidaria
3.7	1035	Cnidaria
4.1	481	Cnidaria
	-	Arthropoda
4.3	869	Porifera
5.0	649	Cnidaria
	852	Porifera
5.6	785	Porifera
7.7	1043	Phaeophyta
KB (ED_{50}, µg/mL)		
<10	645	Porifera
	1069[a]	Porifera
	481	Cnidaria
	104	Echinodermata

[a]Also cytotoxic _vs._ CV-1 monkey kidney cells.

4 AEROPLYSININ-I

5: X = NH$_2$
6: X = OH

$$H_2NCNH(CH_2)_5 \; N(CH_2)_3 \; NHCOCH = CCH_3$$

NH (double bond), R, CH$_3$
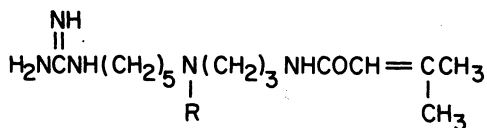
ACARNIDINES

1: R = -CO(CH$_2$)$_{10}$CH$_3$

2: R = -CO(CH$_2$)$_3$CH=CH(CH$_2$)$_5$CH$_3$ (**Z**)

3: R = -CO(CH$_2$)$_2$CH=CH(CH$_2$)$_2$(CH=CHCH$_2$)$_2$CH$_3$(all-**Z**)
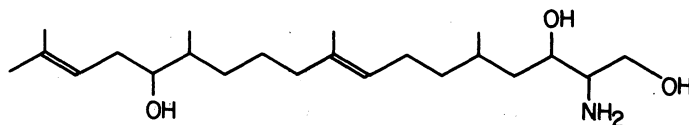
7 APLIDIASPHINGOSINE

Fig. 2. Some compounds found in extracts (of animal species) identified as bioactive during the Alpha Helix Baja Expedition 1974.

We have commented elsewhere (Ref. 29) regarding the rather vague use of the term "antibiotic" for compounds from marine sources having antibacterial properties and shall return to that theme later in this report. Suffice it here to state that, where the actual minimum inhibitory concentrations (MIC's) are known for compounds 1-18, they are generally large, too large to suggest clinical potential.

In the present report we shall concentrate our attention on the systematic studies carried out during and following our more recent Alpha Helix Expedition (AHCE 1978). Here again, the most active samples were initially arranged, as in Tables 8-13, in a priority order of their activities determined on shipboard. In this case first priority was assigned to activity against a _Herpes_ virus, followed by activity against _E. coli_, _P. atrovenetum_,
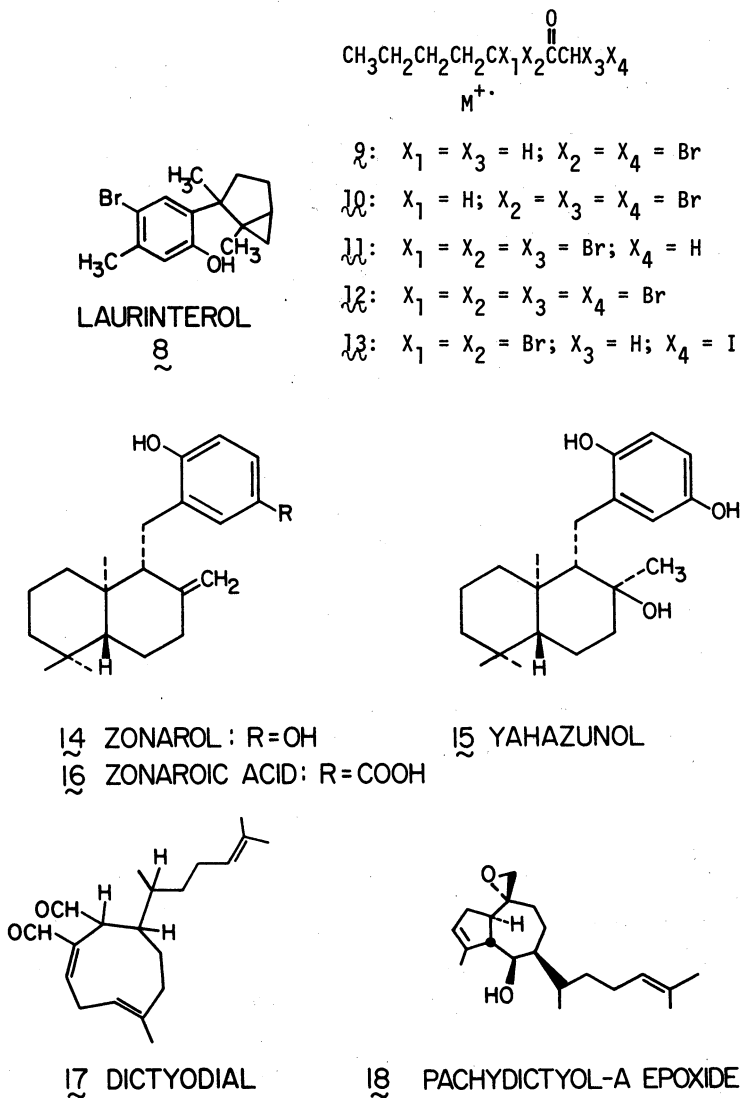
$$CH_3CH_2CH_2CH_2CX_1X_2\overset{\overset{\textstyle O}{\|}}{C}CHX_3X_4$$
$$M^{+ \cdot}$$

9: $X_1 = X_3 = H$; $X_2 = X_4 = Br$

10: $X_1 = H$; $X_2 = X_3 = X_4 = Br$

11: $X_1 = X_2 = X_3 = Br$; $X_4 = H$

12: $X_1 = X_2 = X_3 = X_4 = Br$

13: $X_1 = X_2 = Br$; $X_3 = H$; $X_4 = I$

LAURINTEROL
8

14 ZONAROL : R = OH

16 ZONAROIC ACID : R = COOH

15 YAHAZUNOL

17 DICTYODIAL

18 PACHYDICTYOL-A EPOXIDE

Fig. 3. Some compounds found in extracts (of algal species)
identified as bioactive during the Alpha Helix Baja Expedition 1974.

S. cerevisiae and B. subtilis, and finally cytotoxicity against monkey kidney cells. The
most active shipboard samples were then re-tested at The Upjohn Company--anti-Herpes samples
against a variety of viruses, antimicrobial samples against a broad spectrum of micro-
organisms including pathogens, cytotoxic samples against L1210 (leukemia) cells. This second
level of screening was designed to winnow the samples to those which might have some hope of
clinical candidacy. A striking feature of some of the Tables (8, 9) is the number of ex-
tracts active on shipboard which were found to be inactive in the secondary, pharmaceutical
screens. Although this may reflect, in part, the use of less sensitive microorganisms or
different cell lines, the principal reasons for the lack of activity are felt to be the
generally lower concentrations of the samples employed in the secondary screens and the
decomposition of many of the active compounds during storage (even, in some cases, in frozen
samples of marine species). Thus, the secondary screen tended to select for stable compounds
active at low concentrations. One heartening aspect of the secondary screening was a general
correlation between cytotoxicity toward monkey kidney cells and L1210 cells (Table 13).

The remaining sections of the paper will discuss results obtained with some of the more
active extracts passing the second level of examination in Tables 8-13.

TABLE 8. Priority list of species from AHCE 1978 active
against Herpes simplex virus, type I

Shipboard Activity[a]	AHCE Sample	Phylum	Secondary Testing[b]
++	#553	Chordata	-
+	100	Porifera	-
	175	Chordata	-
	55	Chordata	+
	371	Porifera	-
	319	Porifera	-
	112	Cnidaria	-
	126	Porifera	-
	1058	Rhodophyta	-
	1008	Tracheophyta	-
	755	Chordata	+
	441	Echinodermata	-
	177	Echinodermata	-
	84	Echinodermata	-
	155b	Cnidaria	NT[c]
	169	Cnidaria	-
	463	Porifera	-
	101	Porifera	+
	754	Porifera	+
	1019	Cyanophyta	+
	1173	Cyanophyta	+
	1072	Rhodophyta	-
	160	Chordata	-

[a]See footnote, Table 4. [b]Upjohn screen; + = active against
one or more viruses at 1 mg/mL. [c]NT = not tested.

TABLE 9. Priority list of species from AHCE 1978 active against E. coli

Shipboard Zone of Inhibition (mm)[a]	AHCE Sample	Phylum	Secondary Testing[b]
31	#137	Porifera	-
26	492	Cnidaria	-
25	522	Cnidaria	-
22	547	Chordata	-
21	141	Porifera	NT[c]
20	443	Chordata	-
19	61	Porifera	-
18	92	Porifera	-
	650	Porifera	+
	220	Cnidaria	NT[d]
	755	Chordata	-
	360	Porifera	-
17	64	Porifera	NT
	292	Porifera	-
	372	Porifera	-
	552	Porifera	-
	399	Porifera	-
	1237	Rhodophyta	-

[a,c]See footnotes, Table 4, 8. [b]Upjohn screen; + = active against one or more
microorganisms at 1000 µg/mL, dip-spotted. [d]Retesting at the Univ. of
Illinois indicated no activity vs. E. coli or B. subtilis.

An example of the shipboard extracts most active in inhibiting the growth of CV-1 (monkey
kidney) cells (Table 13) was that from the gorgonian AHCE #150 (Pseudoplexaura flagellosa).
Secondary screening of the extract against L1210 leukemia cells showed it to have ID_{50} 0.35
µg/mL, also placing it among the most L1210-inhibitory extracts. Field desorption mass
spectrometry (FDMS) of the extract indicated its major component to have the molecular
weight 376 and isolation of this major component by trituration with acetone, chromatography
over silica gel and crystallization yielded a compound with the molecular formula $C_{22}H_{32}O_5$
(mol wt 376.2239). The ^1H and ^{13}C NMR spectra of the isolated compound showed the

TABLE 10. Priority list of species from AHCE 1978 active against
P. atrovenetum

Shipboard Zone of Inhibition (mm)[a]	AHCE Sample	Phylum	Secondary Testing[b]
24	#547	Chordata	+
23	158	Echinodermata	+
21	552	Porifera	−
20	101	Porifera	+
	119	Echinodermata	+
19	378	Porifera	+
	441	Echinodermata	+
	443	Chordata	−
18	147	Porifera	−
	226	Porifera	+
	131	Echinodermata	+
	551	Echinodermata	−
17	143	Porifera	−
	159	Echinodermata	+
16	271	Porifera	−
	352	Porifera	+
15	166	Porifera	−
	137	Porifera	−
	372	Porifera	−
	650	Porifera	+
	544	Cnidaria	−

[a,b]See footnotes, Table 9.

TABLE 11. Priority list of species from AHCE 1978 active against
S. cerevisiae

Shipboard Zone of Inhibition (mm)[a]	AHCE Sample	Phylum	Secondary Testing[b]
35	#492	Cnidaria	−
24	628	Porifera	+
23	650	Porifera	+
	754	Porifera	−
	352	Porifera	+
	158	Echinodermata	+
22	89	Porifera	+
	163	Porifera	+
	119	Echinodermata	+
	159	Echinodermata	+
	470	Echinodermata	NT[c]
	473	Echinodermata	NT
21	137	Porifera	−
	639	Porifera	+
	244	Echinodermata	+
20	485	Porifera	+
	474	Echinodermata	NT
	241	Chordata	−

[a,b]See footnotes, Table 9.

appropriate number of quaternary (one), olefinic (two) and acetate (one) methyl groups, and the melting point (+144 °C) and rotation ($[\alpha]_D$ +72°) matched those for the known antitumor compound (Ref. 30) crassin acetate (19), isolated previously by Ciereszko and Weinheimer from a number of Caribbean gorgonians, including P. flagellosa. Assay of the pure crassin acetate confirmed its moderate activity against L1210 cells (ID_{50} 0.20 μg/mL). Although the compound is not new, it was reported to have marginal antitumor activity (T/C 130 at 50 mg/Kg vs. P-388 lymphocytic leukemia) (Ref. 30b); its isolation from an extract sug- gested by the CV-1 assay to have potential antitumor activity argues the promise of the shipboard tissue culture assay.
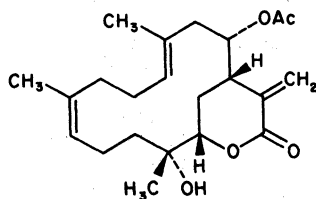
19 CRASSIN ACETATE

TABLE 12. Priority list of species from AHCE 1978 active against B. subtilis

Shipboard Zone of Inhibition (mm)[a]	AHCE Sample	Phylum	Secondary Testing[b]
34	#1060	Rhodophyta	+
33	1058	Rhodophyta	-
31	1028	Rhodophyta	-
30	252	Porifera	-
29	1027	Rhodophyta	-
28	650	Porifera	+
	747	Porifera	+
26	137	Porifera	-
	59	Porifera	+
25	148	Cnidaria	+
	1215	Chlorophyta	-
24	141	Porifera	NT[c]
	485	Porifera	+
23	658	Porifera	-
	522	Cnidaria	-
	1211	Rhodophyta	-

[a-c]See footnotes, Table 9.

The Caribbean red alga Laurencia intricata provided some of the most active extracts assayed on shipboard against Bacillus subtilis (Table 12, AHCE #1060; cf. also Table 14). Gas chromatography/mass spectrometry (GC/MS) of this and other extracts of L. intricata (AHCE #1060, 1165, 1227 and 1244, Table 14) showed the presence of a single major component, together with a number of minor components, whether the sample was collected in Colombian, Honduran, Belizean or Panamanian waters. The similarity of the GC traces confirmed the utility of GC (even on shipboard) as a promising tool for chemotaxonomy of Laurencia species, as indicated in our earlier papers (Ref. 17,18).

The major component of L. intricata satisfied all the criteria advanced earlier (Ref. 17,18) for its identity with isolaurinterol (20, Scheme 1): the molecular ion appeared at m/z 294 and contained one bromine atom, the compound formed an acetate, hence contained a hydroxyl group, and it did not give the ion due to loss of C_5H_8 (M - 68 at m/z 226) characteristic of laurinterol (8). We have recently investigated the use of methylene unit values (Ref. 31), comparing retention times to those of a series of alkanes, as an aid to characterizing marine metabolites in the field (Ref. 32). This procedure has the advantage of being more reproducible than the simple retention times previously employed by us (Ref. 8,17) and does not require such elaborate instrumentation as GC/MS. In any event, isolaurinterol has MU = 19.0 (Table 15). The major component of L. intricata, however, has MU = 19.9 and, thus, cannot be isolaurinterol. Accordingly, the major component of L. intricata sample AHCE #1227 was isolated by chromatography over a silica gel column followed by preparative thin-layer chromatography on a silica gel plate. The ¹H NMR spectrum of the isolated compound's acetate was compared with that reported for isolaurinterol acetate (21), which confirmed that indeed a different material was present in L. intricata. On the other hand, the general overall similarity of the spectra suggested a closely related compound and a comparison with literature properties revealed the identity of the ¹H NMR spectrum of the acetate of the compound from L. intricata with that reported for allolaurinterol acetate (22), the derivative of allolaurinterol (23), previously isolated from Laurencia filiformis forma heteroclada (Ref. 33). Allolaurinterol was reported to undergo prototropic rearrangement to a cyclic ether, filiformin (24) (Ref. 33), in the same way that isolaurinterol (20) undergoes rearrangement to aplysin (25, Scheme 1) (Ref. 34). The material from L. intricata

TABLE 13. Priority list of species from AHCE 1978 cytotoxic against CV-1 cells

Cytotoxicity Index, Shipboard[a]	AHCE Sample	Phylum	Secondary Cytotoxicity $(ID_{50}, \mu g/mL)$[b]
318	#418	Mollusca	0.30
283	650	Porifera	0.17
136	639	Porifera	0.094
116	265	Chordata	NT[c]
103	721	Porifera	NT
98	485	Porifera	3.7
95	59	Porifera	0.16
89	754	Porifera	NT
89	150	Cnidaria	0.35
85	303	Cnidaria	NT
80	751	Porifera	NT
80	755	Chordata	0.90
79	421	Echinodermata	NT
76	642	Porifera	NT
72	761	Porifera	NT
72	30	Cnidaria	3.0
72	1020	Rhodophyta	19
67	40	Porifera	3.2
67	82	Cnidaria	3.4
63	32	Cnidaria	3.8
63	27	Cnidaria	0.70
63	1028	Rhodophyta	7.6
63	641	Porifera	>1
62	156	Cnidaria	0.83
62	179	Porifera	>2.5
62	181	Porifera	0.28

[a]Zone of inhibition of CV-1 cells, extrapolated to 100 μL of a 20-mL methanol-toluene (3:1) extract of 2 g of sample. [b]Upjohn screen; L1210 cells. [c]NT = not tested.
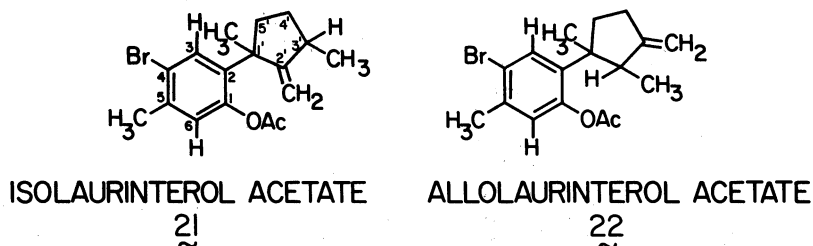

ISOLAURINTEROL
20

APLYSIN
25

LAURINTEROL
8

ALLOLAURINTEROL
23

FILIFORMIN
24
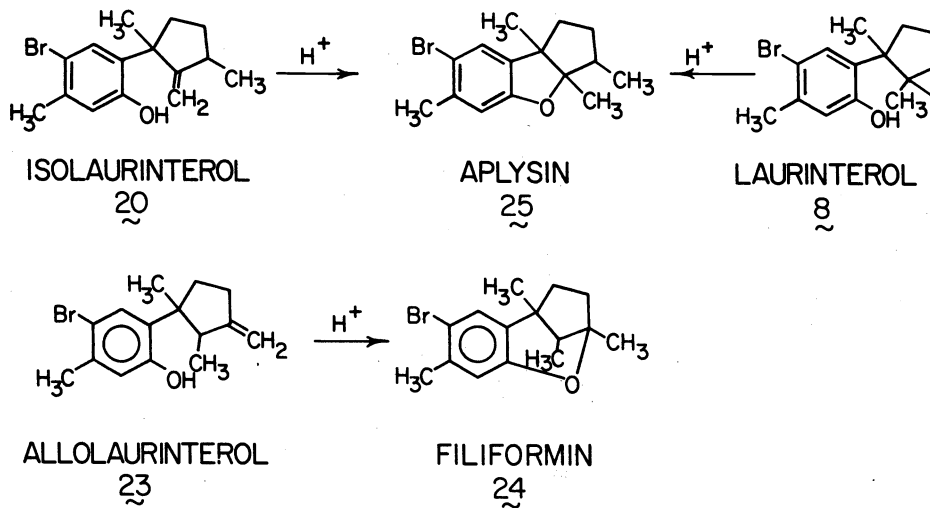
Scheme 1. Conversions of Laurencia-derived phenols to cyclic ethers.

ISOLAURINTEROL ACETATE
21

ALLOLAURINTEROL ACETATE
22

TABLE 14. Bioactivities of <u>Laurencia</u> extracts

AHCE Sample	Collecting Site[a]	Activity[b]						
		E.c.	B.s.	S.c.	P.a.	CV-1	L1210	MU[c]
L. intricata								
#1060	C	–	34	15	–	28	8.3	19.9
1165	B	15(–)	28(16)	15	NT	13	10	19.9
1227	H	15	26	14	NT	20	NT	19.9
1244	P	–(–)	21(15)	–	NT	NT	19	19.9
L. obtusa								
Form I								
#1020	C	–	14	–	–	72	19	15.2
1026	C	–	28	–	–	67	NT	21.2
1027	C	–	29	–	–	67	NT	21.2
1068	C	–	28	–	–	44	NT	21.2
1120	H	–	26	–	–	25	NT	21.2
1128	H	NT(–)	20(–)	NT	NT	NT	7.0	21.2
1164	B	–(–)	–(–)	–	–	14	11	20.0
1175	B	–	15	–	–	21	NT	15.0
Form II								
#1028	C	–	31	13	–	63	NT	21.2
1100	H	–	23	–	–	58	NT	21.2
1121	H	–	28	–	–	38	NT	21.2
1170	B	–(–)	30(–)	–	–	50	7.6	21.2
1228	H	NT(–)	16(–)	NT	NT	NT	28	21.2
Form V								
#1093	H	–	14	–	–	0	NT	20.8
Form VII								
#1083	H	–	–	–	–	60	NT	15.0
1141	B	–(–)	16(–)	–	–	50	25	21.3
1149	B	NT(–)	14(–)	NT	NT	NT	25	21.2
1211	B	–	23	–	–	35	25	20.0
1216	B	NT(–)	–(–)	NT	NT	NT	5.1	17.7
1225	H	NT(–)	–(–)	NT	NT	NT	2.1	20.0
Form VIII								
#1058	C	–	33	–	–	35	8.8	21.2
1065	C	–	24	–	–	34	NT	15.2

[a]C = Colombia, H = Honduras, B = Belize, P = Panama. [b]Abbreviations as in Tables 1 & 2; zone of inhibition (mm) of 100 μL of standard extract [supernatant from 2 g of sample homogenized in 20 mL of methanol-toluene (3:1)]. Parenthetical values refer to zones of inhibition in the secondary screen (<u>cf</u>. footnote b, Table 9). [c]MU = methylene unit value of most abundant component in GC trace.

TABLE 15. Methylene unit values (MU) for <u>Laurencia</u>-derived compounds

Compound	MU
Laurene	14.9
Debromoisolaurinterol	16.3
Debromoallolaurinterol	17.0
Debromolaurinterol	17.3
Filiformin (24)	18.0
Aplysin (25)	18.1
Isolaurinterol (20)	19.0
Allolaurinterol (23)	19.9
Laurinterol (8)	20.0
Filiforminol	20.3
Elatol (26)	21.2
Bromoallolaurinterol	22.3
Bromolaurinterol	22.5

was, accordingly, converted with acid to a cyclic ether, whose properties identified it as filiformin (24), confirming the identification of allolaurinterol. The bioactivity of the allolaurinterol sample was interesting in that it, like laurinterol (Ref. 19), is active in inhibiting reverse transcriptase (RDDP).

Another <u>Laurencia</u> species, <u>Laurencia obtusa</u>, also provided several of the most active extracts in inhibiting <u>Bacillus subtilis</u> on shipboard (Table 12, AHCE #1058, 1028, 1027, 1211; <u>cf</u>. also Table 14). In addition, several of the samples showed strong cytotoxicity toward CV-1 cells (Table 13, AHCE #1020, 1028; <u>cf</u>. also Table 14). In the case of <u>L. obtusa</u>, several forms were identified (Table 14), perhaps corresponding to subspecies. Lack of homogeneity of the species was also indicated by the

variability in bioactivities of the samples (Table 14) and by the differing gas chromato-
graphic traces of L. obtusa extracts. The correspondence between forms judged morphologi-
cally identical and those judged the same by their GC traces was by no means perfect, but in
general the gas chromatographic trace for AHCE #1027 was typical of L. obtusa Forms I (AHCE
#1026, 1027, 1068, 1120, 1128), II (AHCE #1028, 1100, 1121, 1170) and VIII (AHCE #1058).
Forms V and VII gave different traces (as did some of the samples originally assigned to
Forms I, II and VIII, Table 14). The characteristic GC pattern for Forms I, II and VIII was
one containing a single major constituent (MU 21.2) whose molecular weight was 332 and which
was easily demonstrated to contain one bromine atom and one chlorine atom by the isotope
peak pattern for the molecular ion. Thus, the typical gas chromatogram of L. obtusa is
dominated by a compound quite different from allolaurinterol. The compound of molecular
weight 332 was accordingly isolated by chromatography over silica gel. The ^{1}H NMR spectrum
indicated clearly a compound containing two quaternary·methyl groups (1.07s, 1.08s) and one
vinyl methyl group (1.70m).

In this case, comparison with known compounds revealed the previous isolation from L. elata
(Ref. 35) and later from L. obtusa from the Atlantic zone (Ref. 36) of a compound with simi-
lar properties--elatol (26). Closer comparison of the ^{1}H NMR spectrum of the material iso-
lated from the Caribbean L. obtusa with that reported for elatol confirmed the identity of
the two. However, our sample of elatol has the opposite rotation and, thus, the opposite
absolute configuration from the previously described elatol (Ref. 35,36).

There was a generally close correspondence between the degree of inhibition of gram-positive
bacteria (B. subtilis, Table 14) and the amount of elatol in the alga as judged by GC traces.
On the other hand, there was little correlation of extracts of L. obtusa between activity
against B. subtilis and cytotoxicity toward CV-1 cells. Thus, cytotoxicity did not corre-
late well with the presence of elatol (Table 14), although elatol has been reported to inhi-
bit sea urchin egg cell division (Ref. 37). More importantly, extracts of L. obtusa were
only modestly active toward L1210 leukemia cells and thus not especially promising as a
source of antitumor compounds.

It is of some interest that different specimens of L. obtusa produce quite different types
of secondary metabolites. As shown above, the characteristic material in a number of Carib-
bean Laurencia obtusa samples is elatol, which has also been reported (as the enantiomorph)
from Atlantic L. obtusa by González, et al. (Ref. 36). However, as also shown above, other
Caribbean samples contain different major metabolites, and the characteristic compounds in
several different Mediterranean L. obtusa samples have recently been shown (Ref. 38) to
include obtusenyne (Ref. 39), obtusadiol (Ref. 40) and obtusin (Ref. 41), as well as the
previously unreported laurencienyne (27) (Ref. 42).

26 ELATOL

27 LAURENCIENYNE
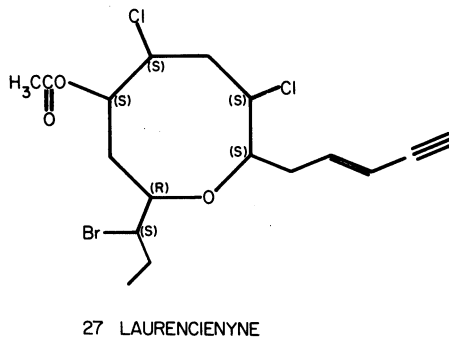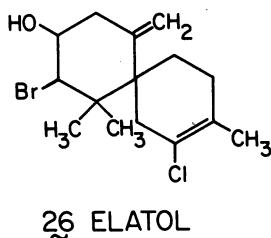
We turn now from red algae to sponges collected during AHCE 1978. A rich source of marine
antibacterial compounds in the past has been the genus Aplysina. In Tables 9 and 12 several
of the most active extracts vs. E. coli and B. subtilis are from Aplysina samples or related
genera and some of these have been investigated. For example, a sample of A. fistularis
(AHCE #527), represented in Table 9 by AHCE #64, gave 5, previously isolated in our labora-
tory from A. fistularis from the Gulf of California (Ref. 15). Similarly, extraction of
Verongula gigantea, sometimes classified as Aplysina gigantea (Ref. 43), gave aeroplysinin-1
(4), also isolated from Baja California A. fistularis (Ref. 15). Although these are some of
the most active antibacterial compounds produced by Aplysina species, the sponges owe their
high places on the priority lists more to the abundance of the compounds rather than to
their specific activity, since their MIC's are rather high. Compound 5, for example, has
MIC = 250 µg/mL vs. K. pneumoniae, 500 µg/mL vs. S. aureus, S. pyogenes, D. pneumoniae,

E. coli, S. schottmuelleri and Ps. aeruginosa and 1000 µg/mL vs. S. faecalis. Aeroplysinin-1
(4) has been reported (Ref. 15) to have lower antimicrobial activity than 5.

Extracts of sponges identified as Aplysina fistularis insularis and Aplysina lacunosa (AHCE
#141 and 360, respectively) were among those most active against B. subtilis and E. coli
(Tables 12 and 9) and the pattern of activities (B. subtilis > E. coli, no antifungal acti-
vity, modest cytotoxicity, Table 16) of these and a number of other samples classified as
these two Aplysina species or as Smenospongia aurea was similar. Although three sponge
species were ostensibly represented, GC/MS of the sponge extracts showed most of the samples
to contain one or more of three components--two closely related compounds containing bromine
and a third compound lacking halogen (Table 16).

TABLE 16.   Properties and constituents of extracts of related sponges (AHCE samples)

	Aplysina fistularis insularis (Duchassaing and Michelotti, 1864)		Aplysina lacunosa (Pallas, 1766)			Smenospongia aurea (Hyatt, 1875)			Aplysina sp.
	#141	286	180	360	259[a]	295	462	583	92
Activity[b]									
E.c.	21	13	17	18	15	-	-	NT	18
B.s.	24	16	20	21	17	16	14	NT	17
S.c.	-	-	-	-	15	-	-	NT	-
P.a.	-	-	-	-	-	-	NT	NT	-
Antimi-crobial	NT	NT	-	NT	NT	NT	NT	NT	-
CV-1	18	0	25	20	12	32	28	NT	15
L1210 ID$_{50}$	NT	NT	8.2	NT	NT	NT	NT	NT	12
Constituents									
Monobromo	+	-	-	+	-	+	-	+	+
Dibromo	+	-	-	+	-	+	-	+	+
Non-brominated	-	-	+	+	+	+	+	+	+

[a]Originally identified as A. spongelii (= A. lacunosa), not reconfirmed.   [b]See footnotes,
Tables 1, 2 & 9.

The extract from sponge #583 was separated by acid extraction into basic and neutral compo-
nents. On the basis of GC/HREIMS of the basic material the two bromine containing compounds
were identified as mono- and dibromo-N,N-dimethyltryptamines (28 and 29, respectively).
From our earlier work with brominated indoles (30-33) from Laurencia brongniartii (Ref. 44)
we anticipated the bromines in the dibromoindole derivative would be at C-5 and C-6 (29)
and that the single bromine in the monobromoindole should be at C-5 (as in 28) or C-6. The
^{1}H NMR spectrum (CD$_3$COCD$_3$) of a 2:1 mixture of the mono- and dibromoindoles agreed with the
aromatic regions of 30 and 32, and the assignment can be made as 28 and 29.

28 (M=266.0416)   29 (M=343.9513)
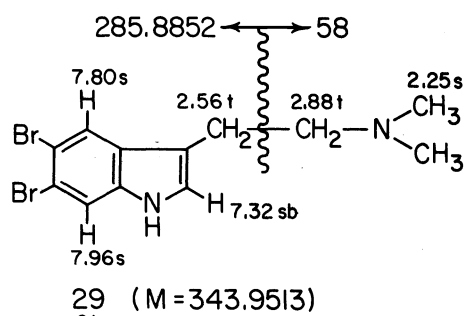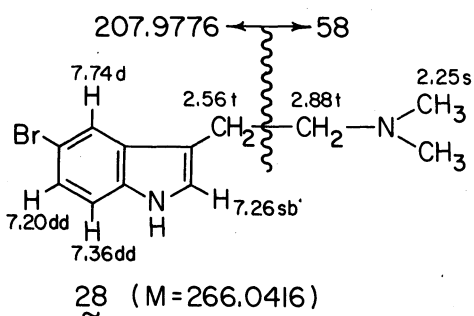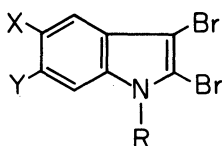
30: X = Br, Y = H, R = CH$_3$

31: X = H, Y = Br, R = CH$_3$

32: X = Y = Br, R = H

33: X = Y = Br, R = CH$_3$

The molecular formula of the halogen-free compound was indicated by gas chromatography/high resolution electron impact mass spectrometry (GC/HREIMS) to be C$_{21}$H$_{30}$O$_3$ (314.2243) and the characteristic peaks at m/z 191.1791 (C$_{14}$H$_{23}$) and m/z 123.0455 (C$_7$H$_7$O$_2$) indicated it to have the structure of a sesquiterpene-substituted hydroquinone (Ref. 26). The ^{1}H NMR spectrum of the isolated C$_{21}$ compound [0.78s (3H), 0.92s (3H), 1.06s (3H), 1.11d (3H)] argued for a structure closely related to that of zonarol, though the skeleton appeared to be slightly rearranged, judging from the number of methyl groups present. While we were in the process of isolating an adequate sample of the sesquiterpenic hydroquinone for structural elucidation, the same compound and also the brominated dimethyltryptamines were reported from Smenospongia aurea and S. echina by the Faulkner group (Ref. 45) and the structures assigned as 28, 29 and 34 (aureol), with aureol containing a rearranged zonarol structure as determined by X-ray crystallography.[a] Identity of melting point, rotation and spectral properties (especially ^{13}C NMR signals) assures the identity of our sesquiterpene-substituted hydroquinone with aureol.
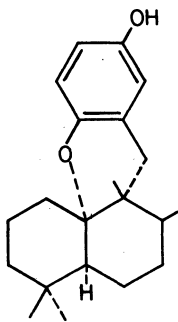
34 AUREOL

Before leaving the subject of sponges we should note the utility of the antimicrobial spectrum of an extract in characterizing the compounds present. As an example, from the AHCE collection a number of sponges were identified as having interesting antimicrobial activity (Table 17). The sponges were initially identified on shipboard as belonging to several species or not identified at all. However, when they were subjected to the secondary screen the broad antimicrobial spectrum shown for the extracts (Table 17) was so comparable (B. subtilis > B. fragilis > K. pneumoniae > M. avium > S. lutea > S. aurea, etc.) that it seemed likely that the compounds present might well be similar or the same. Indeed, preliminary chemical examination of the sponge extracts by FDMS indicated the occurrence of a number of the same molecular ions in all three groups of sponge samples. Although the sponge species are still not certain, the compounds present appear for the most part to be the same. The structures of the compounds will be discussed elsewhere, but the utility of a broad antimicrobial screen in relating compounds is evident. However, even these compounds, derived from one of our most potent extracts, were not sufficiently active against pathogens to warrant further investigation as antibacterial agents. One of them, for example, had a MIC of 25 µg/mL vs. Streptococcus pyogenes, an MIC of 100 µg/mL vs. S. pneumoniae and Staphylococcus aureus, and MIC's > 100 µg/mL vs. 29 other bacteria. Most marine antibacterial agents simply do not compare favorably with classical antibiotics derived from terrestrial microorganisms.

The final compounds for discussion are a group of peptides derived from a Caribbean tunicate, an as yet unidentified member of the family Didemnidae, probably a Didemnum species. Extracts of this tunicate were already observed on shipboard to inhibit Herpes virus (Table 8, AHCE #55) and to exhibit potent toxicity to monkey kidney cells (Table 13, AHCE #755). The antiviral activity was subsequently confirmed in the secondary screens against not only Herpes simplex virus (types I and II) but against another DNA virus (vaccinia virus), as well as against a number of RNA viruses--influenza (PR8), parainfluenza-3 (HA-1), Coxsackie A-21 (COE) and equine rhinovirus (E.R.)--as seen in Table 18. Antiviral activity in the

[a]It is somewhat amusing to note that the time employed for identification of the compounds in both laboratories appears to have been approximately two years, the Faulkner group having visited the Caribbean on the Alpha Helix in September 1977 and our own group in February 1978, and more amusing to note that our Caribbean expedition on the Alpha Helix was originally scheduled for September 1977, but was delayed by the grant review process.

TABLE 17. Bioactivities of sponge samples (AHCE)

	Antimicrobial Activity, Zone of Inhibition (mm)								
	#59	89	101	215	163	170	650	639	747
Shipboard Assays[a]									
E.c.	-	-	14	14	14	-	18	-	-
B.s.	26	21	23	24	21	23	28	21	28
S.c.	20	22	22	21	22	17	23	21	18
P.a.	15	16	20	14	15	13	15	NT	NT
Upjohn Testing[b]									
S.a.	15		17		14		17	16	16
B.s.	19		24		18		25	24	22
S.l.	15		26		tr		20	17	15
K.p.	17		20		16		21	21	19
E.c.	0		0		0		0	0	0
P.v.	0		15		0		0	0	0
Ps.a.	0		0		0		0	0	0
M.a.	16		19		0		20	19	17
S.p.	0		17		0		0	0	0
P.o.	0		0		0		0	0	0
C.a.	0		NT		0		14	14	tr
B.f.	18		24		15		24	22	19
C.p.	0		0		0		15	tr	tr

[a]See footnotes, Table 1. [b]S.a. = Staphylococcus aureus, S.l. = Sarcina lutea, K.p. = Klebsiella pneumoniae, P.v. = Proteus vulgaris, Ps.a. = Pseudomonas aeruginosa, M.a. = Mycobacterium avium, S.p. = Saccharomyces pastorianus, P.o. = Penicillium oxalicum, C.a. = Candida albicans, B.f. = Bacteroides fragilis, C.p. = Clostridium perfringens.

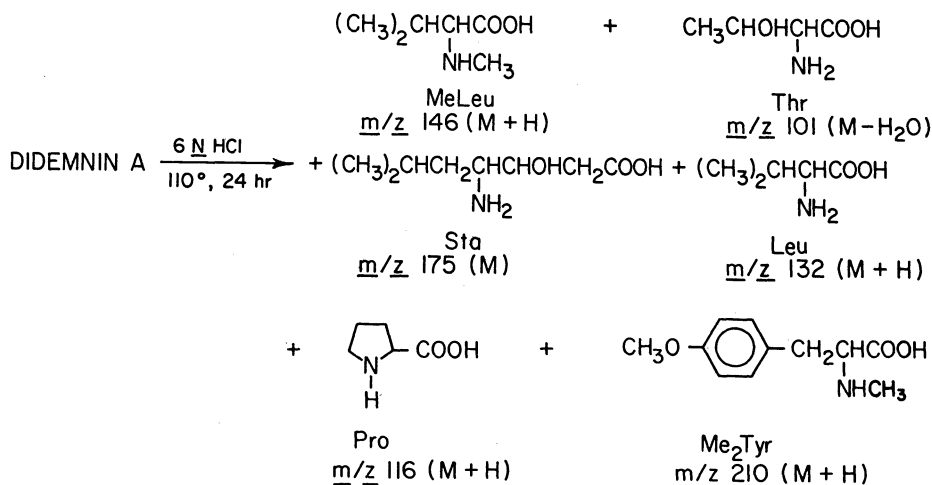TABLE 18. Bioactivities of tunicate (family Didemnidae) samples

	AHCE Sample								
Antiviral Assays[a]	#55	241	580	614	634	676	738	484	755
Shipboard HSV-1	+3	±2	NT	NT	NT	NT	NT	±	+
Secondary testing[b]									
HSV-1	2/4	1/2	2/4	0/4	4/4	0/4	4/4	4/4	0/4
HSV-2	2/4	1/3	2/4	0/4	4/4	0/4	4/4	4/4	0/4
Vacc	1/4	0/3	2/4	0/4	4/4	0/4	4/4	4/4	0/4
PR8	2/0	2/3	NT	4/0	4/4	4/0	4/4	4/4	4/4
HA-1	1/3	3/4	2/4	2/4	4/4	2/4	4/4	4/4	0/4
COE	2/3	3/4	2/4	2/4	NT	2/4	NT	NT	0/4
E.R.	2/4	2/4	2/4	2/4	4/4	2/4	4/4	4/4	0/4
Cytotoxicity[c]									
CV-1	35	49	NT	NT	NT	NT	NT	0	70
L1210, ID_{50} (μg/mL)	0.015	0.16	0.052	0.26	NT	0.030	NT	0.20	0.90

[a]HSV-1, HSV-2 = Herpes simplex virus, types I and II; Vacc = vaccinia virus; PR8 = influenza virus; HA-1 = parainfluenza-3 virus; COE = Coxsackie A-21 virus; E.R. = equine rhinovirus. All expressed as cytotoxicity/virus inhibition (1 = 1-10, 2 = 10-20, 3 = 20-30, 4 = 30-40 mm zone of inhibition). [b]Upjohn Company screen at 1 mg/mL. [c]See footnotes, Table 9.

tunicate extract was followed through the purification shown in Scheme 2. The compounds isolated, didemnins A, B and C, have the activity profiles shown in Table 19, which shows all three compounds to have antiviral activity and cytotoxicity. Didemnin A appears to be especially promising as an antiviral agent and didemnin B to be highly cytotoxic to L1210 cells, thus a potentially useful antitumor agent. Didemnin C, obtained in small quantities, is also antiviral. Each of the three didemnins is a mixture of homologs. The molecular

Didemnum sp.
500 g + 500 mL IPA

1) MeOH:C_7H_8 : 3:1
2) aq. $NaNO_3$

H_2O Toluene

$CHCl_3$

H_2O $CHCl_3$

evapn.

878 mg
solid
108 mg

SiO_2 column
$CHCl_3$ → $CHCl_3$:MeOH : 95:5

trace   6.1 mg   34.7 mg
Didemnin   Didemnin   Didemnin
C   B   A
R_f 0.69 R_f 0.62 R_f 0.49

Prep TLC
SiO_2
$CHCl_3$:MeOH : 9:1

Didemnin
A

Scheme 2.   Isolation and purification of didemnins.

TABLE 19. Bioactivities of didemnins

	ID_{50} (µg/mL)		
	A	B	C
RNA viruses[a]			
ML cells	25	<1.5	6
COE virus	1.5	<1.5	<1.5
ratio	17	∿1	>4
ML cells	12	<1.5	6
E.R. virus	1.5	<1.5	<1.5
	17	∿1	>4
DNA viruses[a]			
Vero cells	50	12	50
HSV-1 virus	3	1.5	<1.5
	17	8	>33
Vero cells	50	12	50
HSV-2 virus	1.5	1.5	<1.5
	33	8	>33
L1210 cells	0.019	0.0011	

[a]Virus abbreviations same as in Table 18.

weights of the major components of didemnins A, B and C were established by FDMS as 942, 1111, and 1014, respectively. Didemnin A was chosen for initial study due to its lower molecular weight.

Didemnin A was first suspected to be a peptide from the three NH protons near 8 ppm in its ^{1}H NMR spectrum. Recognition of its peptide nature allowed application of a procedure for structure assignment of peptides recently developed in our laboratory and highly dependent on advanced mass spectrometric techniques (Ref. 5c). Hydrolysis of the peptide gave a mixture of amino acids (Scheme 3), which was analyzed by FDMS, identifying molecular ions. The amino acids were also derivatized with trifluoroacetic anhydride and n-butyl alcohol and analyzed by GC/MS. The latter technique identified leucine (Leu), threonine (Thr) and proline (Pro), identifications confirmed by co-injection of derivatized authentic samples of those three amino acids and by identification in an amino acid analyzer.

$$(CH_3)_2CHCHCOOH \quad + \quad CH_3CHOHCHCOOH$$

(under first) NHCH$_3$ (under second) NH$_2$

MeLeu
$\underline{m/z}$ 146 (M + H)

Thr
$\underline{m/z}$ 101 (M − H$_2$O)

DIDEMNIN A $\xrightarrow[110°, 24 \text{ hr}]{6 \text{ N HCl}}$ + $(CH_3)_2CHCH_2CHCHOHCH_2COOH$ + $(CH_3)_2CHCHCOOH$

(NH$_2$) (NH$_2$)

Sta
$\underline{m/z}$ 175 (M)

Leu
$\underline{m/z}$ 132 (M + H)

+   (pyrrolidine ring) N—COOH   +   CH$_3$O—(benzene ring)—CH$_2$CHCOOH

H   NHCH$_3$

Pro
$\underline{m/z}$ 116 (M + H)

Me$_2$Tyr
$\underline{m/z}$ 210 (M + H)

Scheme 3. Hydrolysis of didemnin A to its component amino acids.

Three other amino acids were also identified by GC/MS in the hydrolyzate. N-Methylleucine (MeLeu) was suspected from the similar fragmentation of its derivative to that of Leu and the presence in the ^{1}H NMR spectrum of didemnin A of two (non-acylated) N-methyl singlets (at 2.4 and 2.6 ppm). Co-injection of an authentic sample of derivatized MeLeu confirmed the identification of the amino acid. The second N-methyl peak in the ^{1}H NMR spectrum and an aromatic O-methyl peak (3.8 ppm) were believed to be associated with a compound giving a molecular ion at $\underline{m/z}$ 361 in its derivative's GC/chemical ionization (CI) mass spectrum (Scheme 4) as well as an intense peak at $\underline{m/z}$ 121 (C$_8$H$_9$O). The amino acid was assigned from these observations as N,O-dimethyltyrosine (Me$_2$Tyr) and the assignment was confirmed by comparison with an authentic sample (Ref. 46), including co-injection of its derivative on GC.

The two remaining peaks in the gas chromatogram could both be ascribed to the same C$_8$H$_{17}$NO$_3$ amino acid, one GC peak representing the N,O-bis(trifluoroacetyl) derivative of the butyl ester, the other GC peak representing the compound resulting from loss of trifluoroacetic acid from the bis(trifluoroacetyl) derivative. In its mass spectrum the N,O-bis(trifluoroacetyl) derivative loses trifluoroacetic acid and also shows an intense fragment ion for C$_5$H$_{12}$NCOCF$_3$ (Scheme 4). Among compounds with the formula C$_8$H$_{17}$NO$_3$, the hydroxy amino acid statine, 4-amino-3-hydroxy-6-methylheptanoic acid (Sta), isolated from pepstatin (Ref. 47), has recently been intensively investigated (Ref. 48). Comparison of synthetic samples of the R,S (threo) and S,S (erythro) isomers of statine (Ref. 49) with the amino acid from didemnin A by GC/MS of the trifluoroacetyl butyl ester confirmed the identity of the didemnin hydroxy amino acid with a stereoisomer different from that found in pepstatin.

If one adds together the molecular formulas assignable to the six aminoacyl units--Leu, Pro, Thr, MeLeu, Me$_2$Tyr, and Sta--the partial formula C$_{41}$H$_{66}$N$_6$O$_9$ is obtained. HRFDMS and HREIMS carried out on didemnin A gave the value 942.5678 for the molecular ion, and if the accurate mass (786.4891) corresponding to the partial formula C$_{41}$H$_{66}$N$_6$O$_9$ is subtracted from that, the residue 156.0787 corresponds best to the formula C$_8$H$_{12}$O$_3$ (156.0786). Indeed, the FD mass spectra of basic and partial acid hydrolyzates of didemnin A (see below) both contained ions for C$_8$H$_{12}$O$_3$ (e.g., 156.0794).
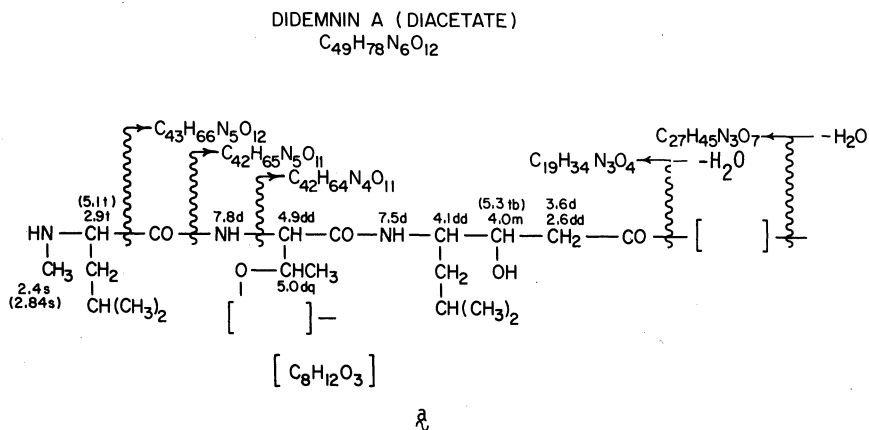
Treatment of didemnin A with acetic anhydride gave a diacetyl derivative (M = 1026; COCH$_3$, 1.9 and 2.1 ppm), whose ^{1}H NMR spectrum included a shifted N-methyl group (2.8 ppm) and CH-N proton (5.1 ppm) for the MeLeu unit and a shifted carbinol hydrogen (5.3 ppm) for the Sta unit, indicating the methylamino group of MeLeu and the hydroxyl group of Sta to be the only two acylable groups. The N-terminal amino acid of didemnin A was required by this
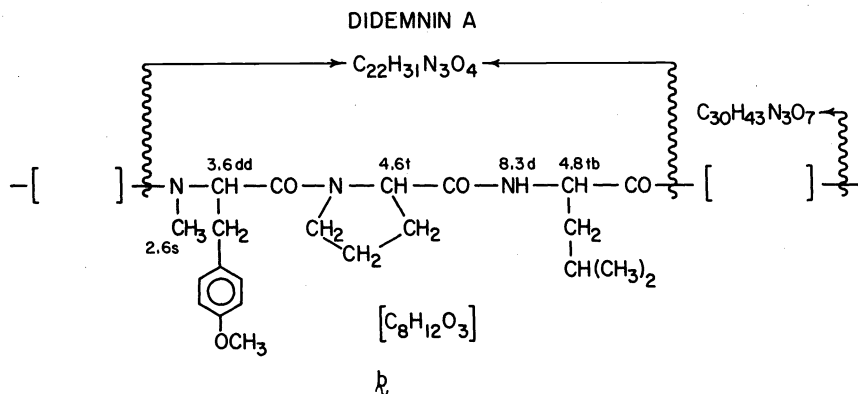
$$M = 361 \ (GC/CI)$$

$$M = 423 \ (GC/CI)$$
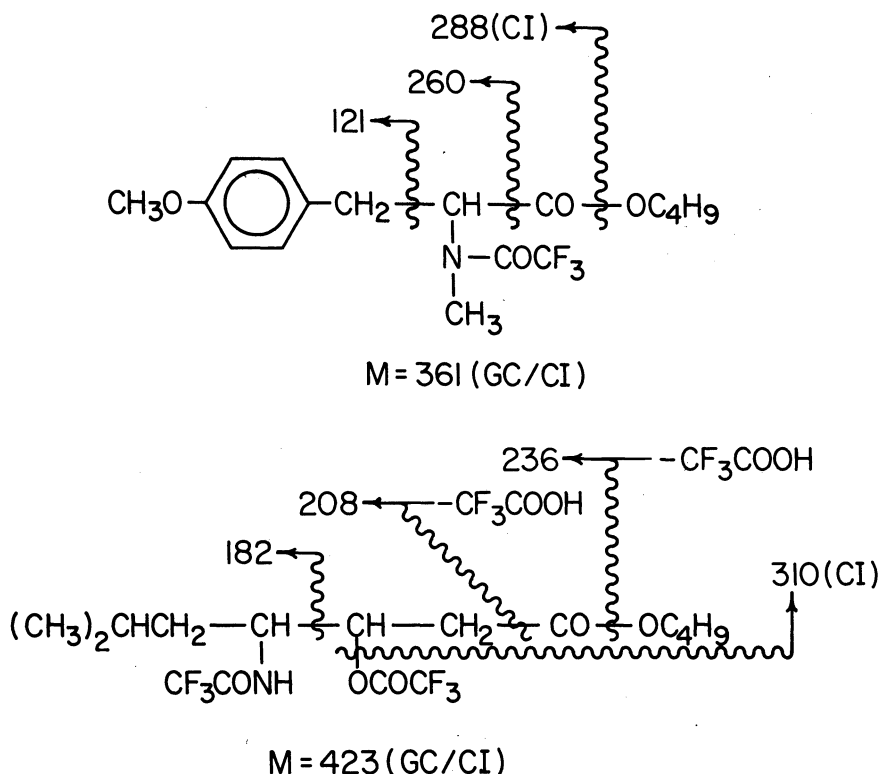
Scheme 4. Mass spectral fragmentations of Sta and Me_2Tyr derivatives.

observation to be MeLeu and this was confirmed by the very facile loss of $C_6H_{14}N$ [$(CH_3)_2CHCH_2CHNHCH_3$] from the molecular ion of didemnin A.

Extensive studies employing HRMS, combined with extensive decoupling of the ^{1}H NMR spectrum, assigned the partial structures a and b. For example, proton decoupling located protons related to the amide bonds involving the amino groups of Thr and Sta, while the sequence of a was established by ions in the HREI mass spectrum of didemnin A. Another important ion was that at m/z 210.1358 ($C_{11}H_{18}N_2O_2$, MeLeu → Thr - H_2O). In addition, the partial acidic hydrolyzates of didemnin A gave similar FDMS ions, e.g. 386.2646 ($C_{19}H_{36}N_3O_5$, MeLeu → Thr → Sta + H). Similarly, decoupling showed the relation of the amide-bonded amino group of Leu and the other groups in b, while the sequence of b was established by the HREIMS ions indicated, as well as by ions in the mild basic hydrolyzate of didemnin A at 288.1467 ($C_{16}H_{20}N_2O_3$, Me_2Tyr → Pro), 420.2492 ($C_{22}H_{34}N_3O_5$, H-Me_2Tyr → Pro → Leu → OH + H), and 576.3279 ($C_{30}H_{46}N_3O_8$, H-[$C_8H_{12}O_3$]→Me_2Tyr → Pro → Leu → OH + H). Another important ion

DIDEMNIN A (DIACETATE)
$C_{49}H_{78}N_6O_{12}$

a

DIDEMNIN A

$$C_{22}H_{31}N_3O_4$$

$$C_{30}H_{43}N_3O_7$$

$$-[\quad]-N-CH-CO-N-CH-CO-NH-CH-CO-[\quad]$$

3.6dd (CH): CH$_3$, CH$_2$; 2.6s; 4.6t (CH): CH$_2$, CH$_2$, CH$_2$; 8.3d, 4.8 tb (CH): CH$_2$, CH(CH$_3$)$_2$

OCH$_3$

$$[C_8H_{12}O_3]$$

ḁ

in the mild basic hydrolyzate spectrum is that found at m/z 601.3359 ($C_{32}H_{47}N_3O_8$, Sta → [$C_8H_{12}O_3$] → Me$_2$Tyr → Pro), which links units ḁ and ḅ as

MeLeu → Thr → Sta → [$C_8H_{12}O_3$] → Me$_2$Tyr → Pro → Leu →,
              |
              O—
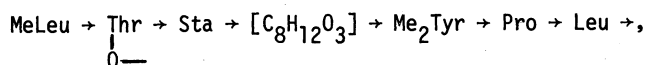
requiring closure of Leu on the hydroxyl of Thr, giving the depsipeptide structure ç for didemnin A.

MeLeu → Thr → Sta → [$C_8H_{12}O_3$] → Me$_2$Tyr → Pro → Leu ⌐
              | |
              O——┘

ç
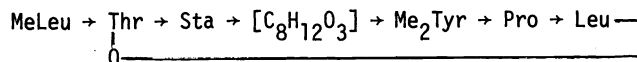
To provide insight into the nature of the $C_8H_{12}O_3$ unit, the ^{13}C and ^1H NMR spectra of didemnin A were examined. The ^{13}C spectrum contained 49 carbon signals, including those for a keto carbon (205.1 ppm) and one carboxy or carboxamido carbon (one of seven near 175 ppm) not accounted for by the 6 aminoacyl residues. The ^1H NMR spectrum contained signals for the groups d and e, identified by extensive homonuclear decoupling. Groups d and e can be reasonably combined with keto and carboxyl carbons in only one way to form a $C_8H_{12}O_3$ unit, as f. This unit is then a hydroxyisovalerylpropionyl (Hip) group, derived from 4-hydroxy-3-oxo-2,5-dimethylhexanoic acid. Incorporating unit f into unit ç then gives 35 as the structure for didemnin A.

4.2q 1.35d
−CH−CH$_3$
  |

d

5.2d   2.4m 0.9d
−CH−CH(CH$_3$)$_2$
  |
  O−

e

$$\rightarrow O-CH-\overset{O}{\overset{\|}{C}}-CH-\overset{O}{\overset{\|}{C}}\rightarrow$$
(CH$_3$)$_2$CH   CH$_3$

f

R → MeLeu → Thr → Sta → Hip → Me$_2$Tyr → Pro → Leu ⌐
                 | |
                 O————————————————————————————————————┘

35: R = H
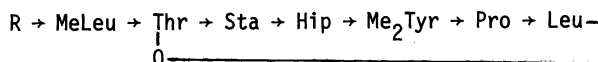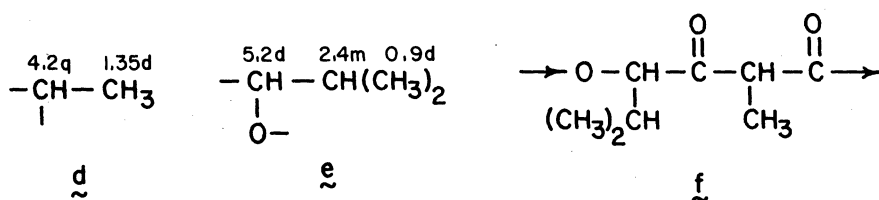
36: R = CH$_3$CHOHCO → Pro →

37: R = CH$_3$CHOHCO →

The structure for didemnin B follows directly from that for A, since the HRFDMS molecular ion for didemnin B (1112.6442, $C_{57}H_{90}N_7O_{15}$, M + H) indicates an additional $C_8H_{11}NO_3$ not found in didemnin A. Acidic hydrolysis of didemnin B gives one extra mole of Pro, accounting for C_4H_7NO. The MeLeu and the extra Pro unit can be shown to be acylated from the ^1H NMR spectrum, which also identifies the remaining $C_3H_4O_2$ as a lactyl, $CH_3CH-CO\rightarrow$, group.

$$OH$$

Thus, the structure of didemnin B is 36. Didemnin C (1014.5873, $C_{52}H_{82}N_6O_{14}$, HRFDMS) contains an acylated MeLeu unit and is assigned structure 37. With their potent antiviral and cytotoxic activities and their easily modifiable structures, the didemnins offer considerable therapeutic promise.[a]

All three didemnins contain as minor components homologs, with norstatine (4-amino-3-hydroxy-6-methylhexanoic acid) replacing statine. The occurrence of this amino acid is of special interest, since it is presumably derived biosynthetically from valine and acetate. Its biosynthesis may, thus, be related to that of Hip.

One of the hopes for success of marine natural products research is that the compounds' structures identified will be sufficiently simple that they can be synthesized for testing in vivo, either in their own right or in an analog program. Such an analog program has already been initiated for study of the earlier reported acarnidines (Ref. 50) and polyandrocarpidines (Ref. 51). The didemnins, relatively simple peptides with antiviral and cytotoxic properties, are, as noted above, exceedingly tempting targets for synthesis and modification by synthesis. Whether or not any of the compounds described in the present report prove ultimately useful in clinical studies, we feel that some of the materials have sufficient promise to establish the validity of our systematic approach toward the study of biologically active compounds from marine sources.

During the course of the research a surprising number of biological and chemical techniques were shown to be systematically feasible on shipboard, including antimicrobial, antiviral, and cytotoxicity assays, gas chromatography and gas chromatography/mass spectrometry (by our group), haloperoxidase and organic halogen assays (by Dr. L. P. Hager's group), and acetylcholine release assays (by Dr. W. O. McClure's group). Of course, such a systematic approach would not have been possible without the availability of the R/V Alpha Helix.[b] In that context, it is regrettable that this type of highly fruitful study of biologically active compounds in marine species can apparently never be repeated. The National Science Foundation's Ships Board, in early 1980, removed the R/V Alpha Helix from service as the only NSF vessel directed primarily toward studies of marine biology, and transferred it to the pool of NSF vessels engaged primarily in studying oceanography.

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[a]Footnote added in proof. We have recently revised the sequence shown for didemnins A, B and C to →Leu→Pro→Me$_2$Tyr→ rather than the reverse. Thus, 35-37 are

R→MeLeu→Thr→Sta→Hip→Leu→Pro→Me$_2$Tyr ⌐
⌊────O────────────────────────────────┘
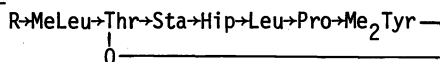
Also, the tunicate has recently been tentatively identified as a Trididemnum species.

[b]One prominent reviewer of our AHBE 1974 proposal argued that everything we proposed to do on that expedition could be better done from a shore station.

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