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Marine natural products as leads to new pharmaceutical and agrochemical agents

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Abstract - A review of recent efforts to isolate and identify marine natural products with potential value as pharmaceutical or agrochemical agents is presented. Bioassays for and compounds with anti-neoplastic/antitumor, anti-microbial, insecticidal and plant growth regulatory activity are discussed.

INTRODUCTION

The study of natural products, their isolation and identification, has long been motivated by a quest for some benefit to man, the discoverer. The prolonged effort to determine the structure of the prototypical analgesic, morphine, is a classic example (1-3), as is the case of Tyrian purple, the first marine natural product to be characterized (4,5). Although marine organisms do not have a long history of medicinal applications like the terrestrial plants, some marine organisms have left an extensive record of hazard to mankind. The isolation and identification of saxitoxin (6), tetrodotoxin (7) and lyngbyatoxin (8) resulted from such reports.

Recent years have witnessed growing attention to an alternative view of natural products, a consideration of how these secondary metabolites serve the producing organism. Secondary metabolites might provide a number of functions -- protection against infection, overgrowth or predation; induction of metamorphosis; growth regulation or toxicity; and communication. The last dozen years have witnessed burgeoning interest and development in the chemical ecology of marine organisms. After early discoveries of algal sex attractants (9) and likely feeding deterrents from sponges (10) and molluscs (11), a number of research groups entered this arena. Clever bioassays were developed (12,13) and detailed studies of sponge-nudibranch (14), alga-mollusc and alga-fish (15) interactions followed. The recent identification of shark repellants from sole (16,17) is a marvelous example of the development and application of complex bioassay techniques to problems in chemical ecology.

A possible extension of discoveries in marine chemical ecology would be determination of applicability to agrochemical problems in insect control and plant growth regulation. One could, with an appropriate array of bioassays, study marine natural products simultaneously for pharmacological, ecological and agrochemical significance.

The approach of the author's group to marine natural products has been predicated on the employment of such a broad, multi-tiered bioassay program to make our studies of individual organisms as thorough as possible. The upper tier consists of *in vivo* anticancer assays at the National Cancer Institute, a variety of *in vitro* screens by collaborating pharmaceutical companies and insecticidal and herbicidal activity assays by collaborating agrochemical firms. The next level is comprised of assays performed by local collaborators; from this group, an important contribution to our work is provided by the differential DNA repair assay (18). This assay provides data, usually overnight, indicative of DNA intercalative anticancer activity (19). The key to our program, however, is the set of assays performed in house (antimicrobial, insecticidal, plant growth regulation) and in the field (antimicrobial, toxicity to brine shrimp) by research group personnel. Characterized by speed and simplicity, these assays serve, together with the differential DNA repair assay, as indicator prescreens to select organisms for chemical analysis and detailed study in more sophisticated assays, and as methods for bioassay guided fractionation.

This review comprises a description of some of our recent work, with emphasis on projects aimed at the development of leads to pharmaceutical or agrochemical agents.

ANTINEOPLASTIC AND ANTITUMOR ACTIVITY

The marine biosphere has long held great promise as a source of anticancer compounds. While a number of screening efforts have indicated a much higher percentage of antineoplastic/antitumor activity than terrestrial plants, only recently have marine natural products made their first appearance in clinical trials at the National Cancer Institute - first the didemnins (20), and then the bryostatins (21-24). The Ireland (25,26), Scheuer (27) and Suntory (28) groups have also discovered cytotoxic cyclic peptides related to the didemnins. These finds, together with the isolation of the unique cytotoxic sponge metabolites acanthifolicin (29), okadaic acid (30) and tedanolide(31), have reinvigorated interest and effort in anticancer agents from marine invertebrates.

Our work in this area is still in its early stages and the bulk of our effort has been expended on screening extracts. Table 1 provides a summary of the bioassay work conducted through May, 1985.

TABLE 1. Bioassay Results, Antineoplastic/Antitumor Screening Program

Organism Subgroup	#Actives/#Tested		
	PS[a]	Toxic[b]	DNA Repair
Sponges	1/14	7/14	9/19
Tunicates	0/2	2/2	1/5
Coelenterates	0/2	0/2	1/2
Molluscs	0/1	1/1	1/1
Algae	0/5	2/5	1/7

[a]In vivo mouse P-388 leukemia
[b]At doses $\leq$ 400 mg/kg in PS assay

Some progress has been made in the fractionation of the in vivo active water soluble extracts of the sponge Cinachyra alloclada. This sponge (formerly Cinachyra cavernosa) was reported some time ago (32) to be active in the Walker M and Walker 256 assays, but no follow-up work had been conducted. We found antineoplastic activity concentrated in the water soluble extracts of Cinachyra alloclada (PS: T/C 133 @ 6.25 mg/kg); these extracts were also active against some solid tumor cell lines and in the differential DNA repair assay, exhibiting greatest inhibition against strain AB 1886 [uvr A6] (18).

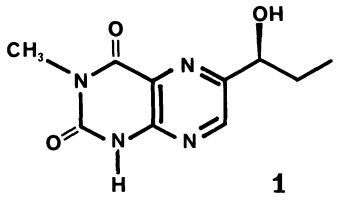
Fractionation of this material has proceeded through chromatography on Diaion HP-20 and gel permeation through Sephadex G-50, Bio-Gel P-2 and Sephadex G-15. The activity, surprisingly, was widely dispersed over a number of fractions, rather than concentrated in one or two. Ninhydrin and ammonium thiocyanate/cobaltous nitrate tests indicated the presence of nitrogenous compounds in these fractions; ^{13}C-NMR profiling of these fractions suggested the presence of polyamines, rather than peptides. Another unexpected observation was the immobility of a substantial amount of material in these fractions on both normal and reverse phase TLC plates. Ion exchange and centrifugal countercurrent chromatography are under investigation as final separation steps for this problem.

ANTIMICROBIAL ACTIVITY

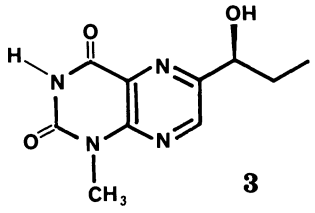
The very widely employed impregnated disk antimicrobial assay is utilized with six fungi (Candida albicans, Candida tropicalis, Aspergillus terreus, Phythium ultimum, Rhizoctonia solani, and Helminthosporium sativum) and three each of the Gram-positive (Staphylococcus aureus, Bacillus cereus and Corynebacterium michiganense) and Gram-negative (Escherichia coli, Pseudomonas syringae and Xanthomonas campestris) bacteria. With these organisms we can screen simultaneously for inhibition of human and plant pathogenic microorganisms.

Some time ago, an antimicrobial fraction from the cave dwelling calcareous sponge Leucetta microraphis yielded leucettidine, a novel pteridine which was assigned structure **1** (33). Since sufficient quantities of this minor metabolite were not available from the natural source for pharmacological assessment, a synthesis of this molecule was undertaken. An unusual route (Scheme I), assembly of the pyrimidine ring onto a preformed pyrazine, was selected to provide greater flexibility in the preparation of analogs. When the bicyclic ring system, **2**, was prepared (34), we discovered that the ^{1}H-NMR chemical shift for the N-methyl protons was at higher field (δ 3.50) than had been observed in leucettidine (δ 3.63). The realization that leucettidine was probably **3**, rather than **1**, was confirmed by Pfleiderer's synthesis of **3** in five steps from 1-methyl-7-hydroxylumazine (35). Our work on the synthesis of **1** and **3** continues.

The tunicate Eudistoma olivaceum, a member of the previously unstudied family Polycitoridae, can be found in very calm shallow water in Bermuda and in a number of locations in the Caribbean. The antimicrobial activity observed in field screens, inhibition of Staphylococcus aureus, was concentrated in the organic soluble extracts.

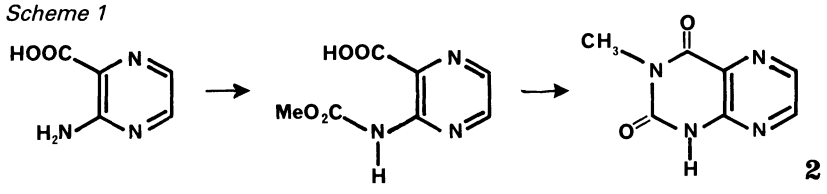


1



3

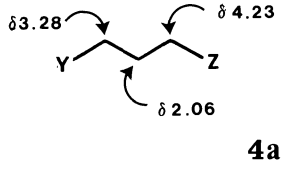
Scheme 1



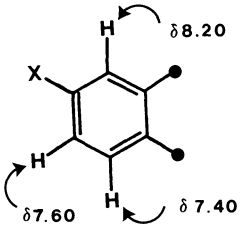
2

Step gradient gel permeation chromatography (36) of these extracts quickly led to a mixture of heteroaromatic compounds. Additional gel permeation chromatography proferred the major component of the mixture in nearly pure form.

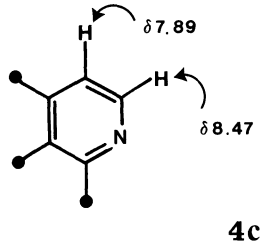
The molecular formula, $C_{15}H_{12}BrN_3$, determined by mass spectrometry, indicated a high degree of unsaturation. Examination of the ^{1}H-NMR spectral data led to development of three part structures - three contiguous methylenes with deshielding substituents at either end (**4a**), a 1,2,4-trisubstituted benzene (**4b**) and a 2,3,4-trisubstituted pyridine (**4c**). The lone remaining NMR signal, a broad singlet at δ 9.1, correlated well with a sharp IR absorption at 3370 cm^{-1} and, therefore, represented an N-H resonance. The proton chemical shifts in **4b** suggested that the substituent X was electronegative. If the bromine were placed there, the part structures could be assembled according to likely amino acid biosynthetic pathways to give **4**, derived from tryptamine and proline, or **5**, derived from tryptamine and glutamic acid. The IR, MS and NMR data all fit both structures quite well. Attempted acidic hydrolysis of the imine (6% hydrochloric acid, reflux) left unreacted starting material, ruling out **5**. This conclusion was supported by the reduction of **4** with sodium borohydride to the pyrrolidine. A minor component present with **4** differed only in the ^{1}H-NMR signals for the benzene ring protons, indicating that it possessed structure **6** (37).



4a

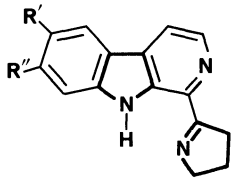


4b



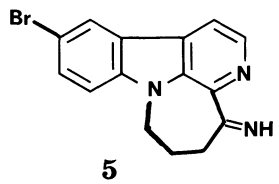
4c

During this same period, the Rinehart group completed a thorough examination of a substantial collection of E. olivaceum and reported the structures of a number of these β-carbolines, which they called eudistomins A-Q (38-40). As a consequence, **4** and **6** should be referred to as eudistomins H and G, respectively. Some of these compounds have been patented for antiviral activity (39).



4 R′ = Br, R″ = H

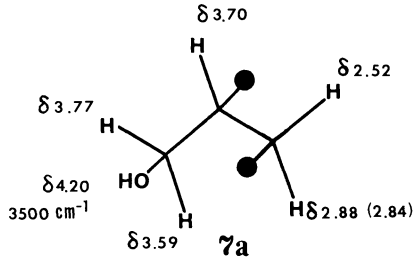
6 R′ = H, R″ = Br



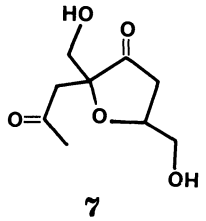
5

Our ongoing investigation of the soft coral Briareum polyanthes has resulted in the identification of five new briaran skeleton diterpenes which will be discussed later in this report. None of these compounds was responsible for the weak inhibition of Gram-negative bacteria exhibited by the organic soluble extracts. When these extracts were subjected to a modified Kupchan partition scheme (41), the activity was found in the ethyl acetate and, primarily, the water solubles. When these fractions were permeated through Sephadex LH-20, once with methanol-acetonitrile (4:1) and then with methanol-dichloromethane (1:1), two compounds were obtained; the more polar of the two was quite unstable and rapidly underwent transformation to the second. Characterization of the

stable second compound proceeded in a typical fashion. Mass spectral analysis provided the molecular formula, $C_9H_{14}O_5$. Although all the ^{1}H-NMR resonances lay between 2 and 4.05 δ, decoupling experiments did provide part structure **7a** and indicated the presence of an isolated second hydroxymethyl group, along with an isolated methyl group and an isolated non-equivalent methylene. The ^{13}C-NMR spectrum revealed the presence of two saturated ketones and four sp^3 carbons bearing oxygen. The infrared spectrum exhibited a strong absorption at 1705 cm^{-1} and a shoulder at 1735 cm^{-1}. These data best fit the furanone **7** which was responsible for the inhibition of Pseudomonas aeruginosa and Xanthomonas campestris. This unique compound represents a new class of compounds heretofore unseen in the marine biosphere; **7** is currently undergoing broad pharmacological scrutiny, while efforts to define the sterochemistry of **7** and the structure of its companion compound continue (42).

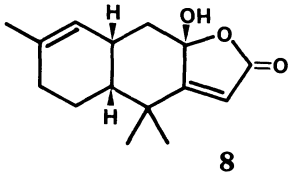


7a



7

Another organism whose extracts exhibited some antimicrobial activity is the sponge Dysidea etheria. Not long ago, we traced this activity to a mid-polar fraction from which we isolated the sesquiterpene lactone **8** (43), which inhibited the yeast Rhodoturula glutinus and the fungus Curvularia lunata, and a series of ceramides, **9** (44), which inhibited Corynebacterium michiganense.



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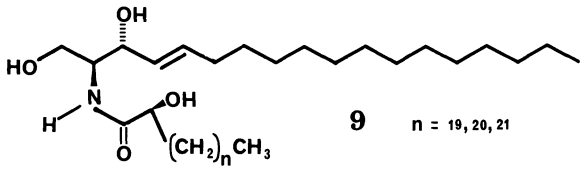


9 n = 19, 20, 21

PLANT GROWTH REGULATORY ACTIVITY

There have been a number of reports (45-50) on the discovery of growth regulators in marine algae, but most of those studies were comprised of qualitative analyses for known growth regulators. More recently, a number of studies have indicated the presence of growth promoting compounds of unknown structure in some algae (51-53). In contrast, little, if any, attention has been focused on growth regulators in sessile invertebrates. When one considers the intense competition for space on rocky and coralline substrates, the presence of growth regulators in invertebrates certainly seems plausible. Some organisms overgrow others, while some resist overgrowth. Chemical constituents are probably responsible for these competitive advantages.

Operating on the premise that novel growth regulators might be found in algae and competitively successful invertebrates, we initiated a program of screening for inhibition of germination and promotion or inhibition of seedling growth. The effects of ten different aqueous extracts on lettuce seed germination have been determined. Three algae, five sponges, one tunicate and one soft coral were examined; three of the extracts exhibited some inhibition of germination. Table 2 provides details of the assays on the three active extracts.

TABLE 2. Effects of Aqueous Marine Extracts on Seed Germination

Concentration mg/ml	# seeds germinated BA-17[a]	BT-9[b]	BT-11[c]
0.5	9/9	9/9	9/9
1	9/9	9/9	9/9
2	6/9	9/9	9/9
3	7/9	6/9	6/9
4	7/9	6/9	6/9
5	3/9	--[d]	--[d]
Control #1	9/9	9/9	9/9
Control #2	9/9	9/9	9/9

[a]Alga; [b]Soft Coral; [c]Tunicate; [d]Not tested

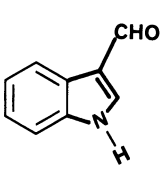
While none of the sponge extracts we tested inhibited germination, varying degrees of root growth inhibition were observed in those extracts. The tunicate (BT-11) extract was also a potent inhibitor of root growth in seedlings. Table 3 summarizes the results of these assays.

TABLE 3. Effect of Aqueous Marine Extracts on Lettuce Seedling Root Growth

Concentration mg/ml	Root Length (mm) BT-11[a]	BS-2[b]	BS-9[b]	BS-15[b]	BS-24[b]	BS-32[b]
0.5	4.5	--[c]	6.7	16.5	18.4	17.0
1	2.7	5.0	3.9	7.9	12.2	15.6
2	2.3	3.4	3.2	7.1	14.5	9.3
3	1.6	3.4	2.8	6.2	14.4	8.8
4	1.4	3.1	1.8	2.5	--[c]	5.0
Control #1	17.1	16.8	7.4	21.1	26.8	26.0
Control #2	17.6	17.1	7.9	--[c]	20.1	25.0

[a]Tunicate; [b]Sponge; [c]Not tested

The extracts of Dysidea etheria (see BS-9, Table 3) have yielded three indoles, indole-3-carboxaldehyde (**10**), indole-3-acetamide (**11**), and the heretofore unknown hydroxy-diketone **12** (54). Indole-3-acetamide, a derivative of indole-3-acetic acid, is a known plant growth regulator or auxin. The novel **12** exhibited root growth promotion activity in the lettuce assay, somewhat weaker than indole acetic acid, but following the same profile. Maximum growth promotion occurred at 10^{-8} M.



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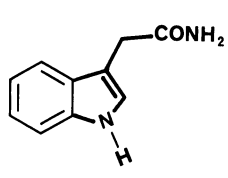

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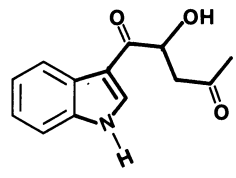
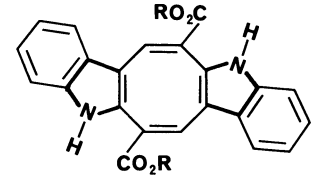


12



13 R = CH_3 **14** R = H

We had been involved for some time in a chemotaxonomic study (55) of the distribution of the unusual pigment caulerpin, **13** (56,57), in various species of green algae in the genus Caulerpa when it occurred to us that this seeming dimer of indole-3-acrylic acid might act as a plant growth regulator. Indeed, both caulerpin and the diacid **14**, obtained by mild alkaline hydrolysis of **13**, were active in promoting the growth of lettuce roots. Again, maximum activity was observed at 10^{-8} M and **13** and **14** were slightly less active than indole-3-acetic acid and more active than indole-3-pyruvic acid and indole-3-acrylic acid (58), two possible biogenetic precursors to caulerpin.

While the significance of caulerpin's plant growth regulatory activity to morphological distinctions in species of Caulerpa has yet to be fully elucidated, the identification of **12** and **13** as novel growth regulators should serve as a clear indication of the existence of a number of new marine natural products that act as plant growth regulators.

INSECT CONTROL

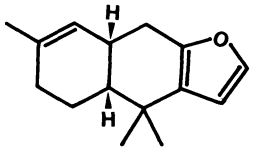
There are a number of examples of chemical defense against predation or herbivory in the literature on marine natural products (10-17) and similar patterns have been found in a number of terrestrial plant-insect relationships (59,60). If a compound were found to be toxic to or to inhibit feeding by marine invertebrates, might that compound not exert the same or similar effects on invertebrates in the terrestrial biosphere? To explore this possibility, an examination of the response of insects to marine natural products with suspected or demonstrated antifeedant behavior was initiated. Two insects have been used in these studies -- grasshoppers (Melanoplus sp.) and tobacco hornworms (Manduca sexta).

Grasshoppers were chosen as an assay organism because they are a major crop pest in the grain producing plains and plateau states, they are readily available year round from the Agricultural Experiment Station at Montana State, and they are relatively easy to maintain in the laboratory. Two species, Melanoplus bivitattus and Melanoplus sanguinipes, have been used in these assays.

Grasshoppers, however, wreak their damage as adults, while the great majority of agricultural damage is done by larval stage insects. The tobacco hornworm has served as a laboratory model for insecticidal screening (61). In one assay with Manduca sexta one can test for insecticidal activity, feeding deterrence, and juvenile hormone effects.

The sponge Dysidea etheria and the nudibranch Hypselodoris zebra have yielded a number of sesquiterpenes, some of which exhibit feeding deterrent activity against fish (43,62). The more abundant of these compounds, **15**, **16** and **17**, have been tested in the insect assays. Furodysinin (**15**) is toxic to grasshoppers at both doses and is the most potent and rapid acting of all the compounds studied thus far. To our surprise, furodysinin was not toxic to late larval stage tobacco hornworms at doses of 250 ppm in an artificial diet. Instead, all the test insects suffered some form of difficulty in molting. Most of the insects were unable to shed their molts completely and two hornworms developed double head capsules (63). Further tests of the tobacco hornworm's response to **15** are underway.

The other two Dysidea metabolites tested, 5-acetoxy- and 5-hydroxy-nakafuran-8 (**16** and **17**), exhibited a different activity profile against the grasshopper. The acetate **16** was toxic at the high dose and antifeedant at the lesser dose, while the alcohol **17** was antifeedant at both doses. Both compounds are being tested in the tobacco hornworm assay.



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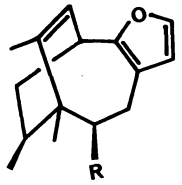
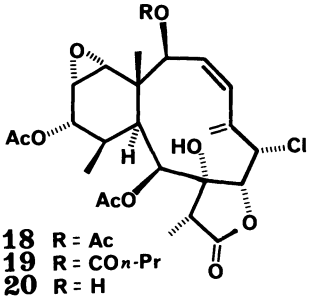
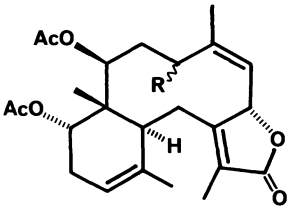


16 R = OAc
17 R = OH



18 R = Ac
19 R = CO*n*-Pr
20 R = H



21 R = H
22 R = OH

Some time ago, we initiated a study of the soft coral Briareum polyanthes to ascertain whether there was a chemotaxonomic basis for the species distinction between the Briareum found in Bermuda and the more common B. asbestinum found throughout the Caribbean. We have found a series of new diterpenes (**18–22**) with the briaran skeleton (41,64,65). It has been implied (66,67) that this class of compounds and the asbestinins (66,68) serve as a chemical defense against predation in the soft corals and sea pens which produce them, but this has not yet been demonstrated by bioassay.

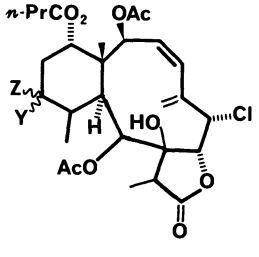
Two of these compounds, brianthein Y (**19**) and brianthein W (**21**) have been tested in the grasshopper assay. Brianthein Y proved toxic at the high dose, but inactive at the lower dose; brianthein W produced no deleterious effect at either dose. Results of all the grasshopper assays are summarized in Table 4.

TABLE 4. Insecticidal Activity of Selected Marine Isolates[a]

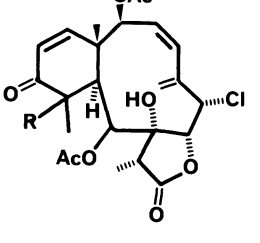
Compound	3 mg/insect	1 mg/insect
15	toxic	toxic
16	toxic	antifeedant
17	antifeedant	antifeedant
19	toxic[b]	inactive
21	inactive	inactive

[a]tested against the grasshopper Melanoplus sanguinipes
[b]also toxic at 3 mg/insect against M. bivitattus

We have recently isolated four additional briaran diterpenes from the sea pen Ptilosarcus gurneyi - the previously known ptilosarcone, **23**, and ptilosarcenone, **24** (69), together with two new compounds, **25**,**26**. Not only do we anticipate resolving the lingering questions about the stereochemistry of **23** and **24** (41,69), but testing of this entire group of diterpenes (**18–26**) in the insect assays should provide some indication of which of the numerous functional groups present in this class of compounds are required for the activity observed.



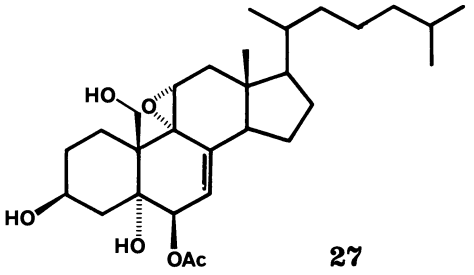
23 Y,Z = O
25 Y=OH, Z=H



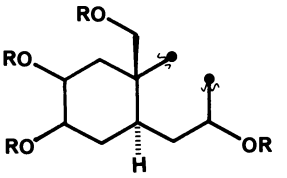
24 R = H
26 = OH

Since the first discovery of this class of diterpenes by the Oklahoma (67,70) and Brussels (71) groups, additional members have been identified by the Faulkner (69,72,73), Roche (74), Ahond and Poupat (75,76), Fenical (77) and Cardellina (41,64,65,78) groups. While a great variety of functional groupings has been observed in the cyclohexane ring throughout the series, there are, in general, two subgroups in this class of compounds that reflect differences in the cyclodecane ring. In one, a diene system and allylic chloride are present; in the other group, that combination of functionality is absent and, typically, those compounds are far less functionalized than the former group. Enough representatives of this class of compounds are now available to probe the structure-activity relationships in the series.

Schmitz (79) has isolated the most oxidized sterol yet found in a sponge from an unidentified species of Dysidea from Guam and assigned structure **27** to this interesting find. Our collections of Bermudian Dysidea etheria have yielded substantial quantities of highly functionalized sterols. Preliminary analysis of these fractions suggested some differences from the Schmitz sterol. Purification has just been achieved by gel permeation chromatography and reverse phase HPLC; preliminary ^{1}H-NMR chemical shift and decoupling data led to proposal of part structure **28** (80) for the major compound in the sterol complex. While the oxygen functionality at C-19 is reminiscent of **27**, the C-2 and C-7 hydroxyls and their absence at C-5 and C-6 would indicate a clearly distinct molecule, perhaps related to the ecdysones.



27



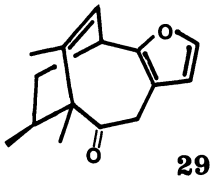
28

In the tobacco hornworm assay, the polar sterol fraction from D. etheria was not toxic at 250 ppm, but the larvae reared on sterol impregnated diet grew to only 35% of the weight of controls, even though both groups of hornworms consumed the same amount of diet. Further testing of these compounds will follow their structural characterization.

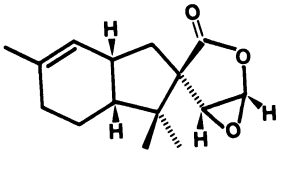
SECONDARY METABOLITES OF *DYSIDEA ETHERIA*

In terms of natural products, Dysidea is probably the most studied sponge genus. A number of species from every major collection area has been studied and a tremendous diversity of secondary metabolites has been revealed, including halogenated diphenyl ethers (81-84), sesquiterpenes (43,85-91) sesquiterpene-quinones (92-96), a sesterterpene (97), a sesterterpene-quinone (98), oxidized sterols (79,80) and a variety of halogenated nitrogenous compounds (94-104). Similarities in carbon skeleton and functionality patterns between this latter group and blue-green algal metabolites like malyngamide A (105) and the pukeleimides (106,107) support the concept (91) that these compounds might well be produced by endosymbiotic cyanophytes, but there has been no verification of this hypothesis.

As described earlier in this report, our broad-based investigation of D. etheria has yielded a number of secondary metabolites. We have found a total of seven sesquiterpenes thus far, including the known furodysinin and furodysin (88) and five new compounds - furodysinin lactone, **8** (43), three nakafuran-8 derivatives with oxygen functionality at C-5, **16**, **17** and **29** (43,62), and dysetherin, **30** (108), with the very unusual β, γ-epoxy- γ - lactone array. Our initial examination of the water soluble extracts has revealed the presence of the nucleosides adenosine, 2'-deoxyadenosine, 2'-deoxyguanosine, thymidine and 2'-deoxyuridine (109). We have now isolated a total of twenty-one interesting metabolites from Bermudian Dysidea etheria. The indoles, ceramides and nucleosides all represent classes of compounds isolated for the first time from Dysidea.



29



30

SUMMARY

Many of the compounds reviewed herein might have gone unnoticed if a multiple screen bioassay program were not in use. The furanone **7** would almost certainly have escaped our attention if the polar extracts had not been assayed. The nucleosides from Dysidea were found during our search for plant growth regulatory compounds. Even at this program's early stage, these results are evidence that compounds representing a variety of structural types will continue to be isolated from marine organisms and many of these will exhibit striking pharmacological or agrochemical activity.

ACKNOWLEDGEMENTS

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