Tetrahedron Vol. 41, No. 6, pp. 1057–1065, 1985
Printed in Great Britain.

0040–4020/85 $3.00 + .00
Pergamon Press Ltd.

ISOLATION OF THE MURICINS

EVIDENCE OF A CHEMICAL ADAPTATION AGAINST FOULING IN THE MARINE OCTOCORAL *MURICEA FRUTICOSA* (GORGONACEA)

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(*Received in USA* 26 *October* 1983)

Abstract—Chemical comparisons of the closely-related Pacific gorgonians *Muricea californica* and *M. fruticosa* have shown that the much less fouled *M. fruticosa* contains four new esterified aminogalactose saponins assigned as the muricins-1 through -4 (1–4). The structures of these new saponins were defined by chemical degradation yielding the aglycone pregna-5, 20-dien-3β-ol, and by extensive ^{1}H and ^{13}C NMR investigations. Muricins-2, -3 and -4 were found to possess 2-acetamidogalactopyranose components with unprecedented n-butyrate ester functionalities in the C-4′, C-6′ and the C-4′ and C-6′ positions, respectively. At approximately natural concentrations, the muricins were found to inhibit growth of the marine diatom *Phaeodactylum tricornutum*. The muricins did not, however, possess the ichthyotoxic, cytotoxic, or antimicrobial effects reported for numerous marine-derived saponins. The potential role of these compounds in contributing to the reduced fouling of *M. fruticosa* is discussed.

In tropical and subtropical marine habitats, the octocorals (Subclass Octocorallia, Phylum Cnidaria) are among the most abundant of the marine invertebrates. Unlike the hexacorals (Subclass Hexacorallia), which effectively sequester $CaCO_3$ into extensive and ornately-patterned external skeletons, the octocorals are soft-bodied, colonial cnidarians which generally lack the physical protection afforded by calcification. Of the six orders of octocorals, the encrusting true soft-corals (Order Alcyonacea) and the sea fans and sea whips or gorgonian corals (Order Gorgonacea) are by far the more widely distributed and abundant representatives. Although these latter two groups are ubiquitous in tropical marine habitats, their distributions are disjunct. In the western tropical Atlantic Ocean, in particular the Caribbean Sea, and also in the Eastern Pacific, the Gorgonians are the dominant octocorals. In the Indo–Pacific region and in the Indian Ocean, however, the true soft-corals or alcyonaceans represent the dominant octocoral fauna.

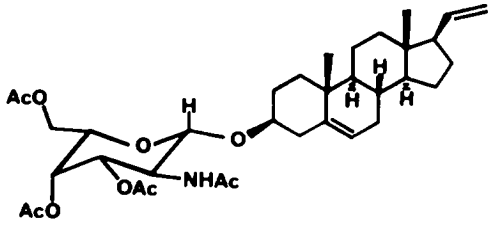
Over the last two decades, considerable chemical interest in these unique marine invertebrates has become evident. More that 200 papers have appeared describing the isolation of novel sesquiterpenoids, diterpenoids and products of fatty acid metabolism.[1] Since the majority of the octocorals contain significant concentrations of symbiotic dinoflagellates known as "zooxanthellae", controversy over the plant or animal origin of these latter metabolites has been considerable.[1] Several recent studies with selected gorgonians have, however, provided convincing evidence that terpenoids are products of gorgonian metabolism.[2–6]

Along with our continued interest in the novel natural products chemistry of marine octocorals, we and others have more recently begun to define the biological properties of these unique molecules. A large percentage of the metabolites from marine octocorals have subsequently been shown to possess antimicrobial, cytotoxic, algatoxic and ichthyotoxic properties.[1] Hence, considering the need for soft-bodied, sessile marine invertebrates to survive in predator-rich environments, these latter metabolites appear to be the foundation of a well-developed chemical defense adaptation. In this paper we wish to report on our recent investigations of California gorgonians of the genus *Muricea*. These studies provide additional evidence that biologically active secondary metabolites are functionally important in the adaptations of marine octocorals.

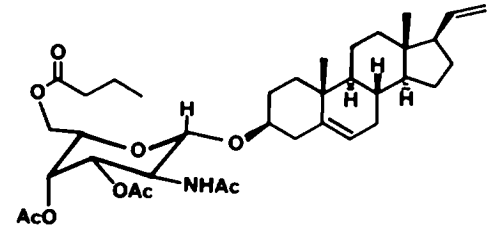
Muricea californica Aurivillius and *M. fruticosa* Verrill (Plexauridae) are the major and abundant *Muricea* species in California, and they are found growing together in habitats with high current flow. *M. californica* is described as red-brown in color with yellow polyps and reaches a maximum height of *ca* 40 cm. *M. fruticosa* is nearly identical in size and color, but possesses white polyps and may be slightly less abundant overall than *M. californica*. Other than the minor distinctions in morphology and polyp color, the similarities of these two gorgonians have made it difficult to distinguish them in and out of the water. The ecologies and life histories of these two species have been the subject of numerous biological investigations since 1960.[7–12] In each of these studies distinct differences in the fouling susceptibilities of these two gorgonians were noted. Although found within the same habitat, *M. californica* was consistently overgrown with typical encrusting plants and animals such as red algae, hydroids, ectoprocts, zoanthids, ascidians, molluscs and bryozoans. In contrast, *M. frutisoca* was found virtually free of surface fouling organisms in all studies.

The similarities of these two species in morphology and habitat suggested to us that chemical differences could be responsible for the observed lack of fouling in *M. fruticosa*. Indeed, collections of *M. californica* and *M. fruticosa* were found to be chemically dissimilar. While each contained almost identical mixtures

of triglycerides, sterols and fatty acids, extracts of *M. fruticosa* were also found to contain 4 new metabolites which are unique aminogalactose sponins containing pregnane-derived aglycones. We have assigned the trivial names muricin-1 through muricin-4 to these compounds and determined their structures as **1**–**4**.



1



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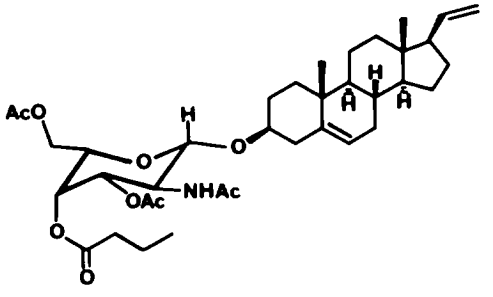

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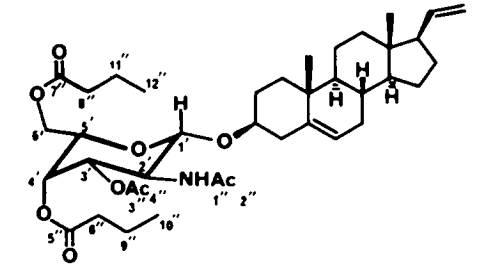
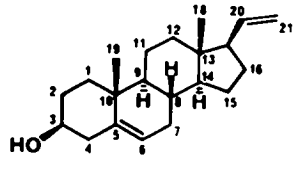


4



5

Muricin-1 (**1**) analyzed for $C_{35}H_{51}NO_9$ by field desorption mass spectrometry in combination with interpretation of ^{13}C NMR data (Table 1). The 11 degrees of unsaturation inherent in the molecular formula of **1** could be accounted for by only 2C double bonds and 4 ester or amide CO groups. Hence, muricin-1 possessed five rings. The combination of ^1H (Table 2) and ^{13}C NMR data gave considerable insight into the structure of **1**. Four Me resonances between δ 1.97 and 2.14 showed that the four CO groups were probably derived from acetyl residues. Further, a predominant group of methine resonances between δ 3.8 and 4.9 (7H), in conjunction with the presence of an obvious ^{13}C NMR acetal resonance (δ 99.4, d), suggested the presence of a cyclized, acetylated hexose unit. Comparison of the ^{13}C NMR bands from typical hexose acetate models showed that muricin-1 contained an acetylated aminohexose ring probably in the pyranose form. In particular, an off-resonance doublet carbon at 52.4 ppm with an abnormally large residual coupling constant (Table 1) was recognized as characteristic of the N-bearing carbon of aminosugars.[13–16]

Because of the complexity of the spectral data and the overall size of muricin-1, the structural details of the tetracyclic portion of the molecule were not readily determined. Hence, muricin-1 was hydrolyzed with 3N HCl at 40° for 3 hr and the aglycone **5** was obtained in high yield. Comparison of the ^1H NMR features of the isolated aglycone with those of muricin-1 showed that the aglycone was intact and had not undergone modification during the hydrolysis. The aglycone analyzed for $C_{21}H_{32}O$ by high resolution mass spectrometry and was confirmed as an alcohol by intense M^+-H_2O fragmentation and by IR absorption at 3401 cm^{-1}. The ^{13}C and ^1H NMR spectra of **5** (Tables 1 and 2) immediately suggested that the aglycone was a degraded sterol. Resonances for the Δ^5 double bond and the vinyl substituent at C-17 were readily apparent along with the typical bridgehead Me groups at C-18 and C-19, which appeared at characteristic chemical shifts in both the ^1H and ^{13}C NMR spectra. The presence of an alcohol methine band in the ^1H NMR spectrum (δ 3.53), with typical axial coupling constants, confirmed that **5** possessed an equitorial (β) alcohol substituent. Comparison of these data with several C_{21}-pregnane sterols suggested that **5** was pregna-5, 20-dien-3β-ol. This compound had, fortuitously, been previously isolated from a distantly related octocoral, the alcyonacean *Gersemia rubiformis*, or sea raspberry, found in the Atlantic Ocean near Newfoundland.[17,18] Indeed, comparison of the hydrolysis product **5** with the authentic natural product proved their identity. Aglycone **5** showed $[\alpha]_D - 43.6°$ (*c* 0.6, $CHCl_3$), which is in reasonable agreement with the optical properties of the previously defined natural product, $[\alpha]_D - 62.1°$ (*c* 0.7, $CHCl_3$), the absolute stereochemistry of which had been established by synthesis from natural progesterone.[19]

By subtraction of the molecular formula of **5** from the overall formula of muricin-1, the sugar component was shown to possess the composition $C_{14}H_{19}NO_8$. Earlier spectral data had shown that muricin-1 possessed four acetyl or acetate residues. Elimination of these substituents from the formula left $C_6H_{11}NO_4$ which is the formula of a typical aminohexose.

Table 1. ^{13}C NMR spectral assignments of muricins 1–4 and the aglycone 5[a]

C	1 δ	1 J_R[b]	2 δ	2 J_R	3 δ	3 J_R	4 δ	4 J_R	5 δ	5 J_R
1	37.3 (t)	--	37.3 (t)	--	37.3 (t)	--	37.3 (t)	21.9	37.3 (t)	--
2	29.7 (t)[c]	--	29.7 (t)[c]	--	29.7 (t)[c]	--	30.1 (t)[c]	21.7	29.7 (t)[c]	--
3	70.5 (d)	39.2	70.5 (d)	39.6	70.5 (d)	38.1	71.8 (d)	36.5	71.8 (d)	--
4	38.8 (t)	--	38.8 (t)	--	38.9 (t)	--	38.9 (t)	27.7	37.3 (t)	--
5	140.3 (s)	--	140.3 (s)	--	140.3 (s)	--	140.8 (s)	--	140.8 (s)	--
6	122.0 (d)	48.0	122.0 (d)	47.4	122.0 (d)	44.6	121.6 (d)	47.3	121.6 (d)	--
7	32.0 (t)	--	32.0 (t)	--	32.0 (t)	--	32.0 (t)	--	31.7 (t)	--
8	37.2 (d)	--	37.2 (d)	--	37.3 (d)	--	37.2 (d)	--	32.0 (d)	--
9	50.4 (d)	19.4	50.4 (d)	18.6	50.4 (d)	--	50.4 (d)	18.8	50.4 (d)	--
10	36.8 (s)	--	36.8 (s)	--	36.8 (s)	--	36.8 (s)	--	36.6 (s)	--
11	20.7 (t)	--	20.7 (t)	--	20.7 (t)	--	20.7 (t)	--	20.7 (t)	--
12	29.5 (t)[c]	--	29.5 (t)[c]	--	29.5 (t)[c]	--	29.5 (t)[c]	22.0	32.0 (t)[c]	--
13	43.4 (s)	--	43.4 (s)	--	43.4 (s)	--	43.4 (s)	--	42.3 (s)	--
14	55.9 (d)	17.6	55.9 (d)	18.4	55.9 (d)	--	55.9 (d)	25.5	55.9 (d)	--
15	24.9 (t)	--	24.9 (t)	--	24.9 (t)	--	24.9 (t)	--	24.9 (t)	--
16	27.2 (t)	--	27.2 (t)	--	27.2 (t)	--	27.2 (t)	--	27.2 (t)	--
17	55.3 (d)	27.9	55.3 (d)	27.0	55.3 (d)	--	55.3 (d)	18.2	55.3 (d)	--
18	12.7 (q)	20.9	12.7 (q)	20.6	12.7 (q)	--	12.7 (q)	20.6	12.8 (q)	--
19	19.4 (q)	23.1	19.4 (q)	22.0	19.4 (q)	--	19.4 (q)	22.4	19.4 (q)	--
20	139.7 (d)	46.9	139.7 (d)	46.1	139.7 (d)	41.2	139.6 (d)	45.6	139.7 (d)	--
21	114.5 (t)	45.6	114.5 (t)	45.4	114.5 (t)	--	114.5 (t)	45.9	114.5 (t)	--
1'	99.4 (d)	48.2	99.4 (d)	47.2	99.4 (d)	44.8	99.4 (d)	47.7		
2'	52.4 (d)	38.0	52.4 (d)	37.7	52.4 (d)	38.1	52.3 (d)	37.3		
3'	69.6 (d)	46.5	69.7 (d)	45.9	69.7 (d)	42.0	69.8 (d)	44.5		
4'	66.8 (d)	47.9	66.8 (d)	47.5	66.5 (d)	45.2	66.5 (d)			
5'	79.8 (d)	36.5	79.8 (d)	36.2	79.8 (d)	--	79.7 (d)	35.9		
6'	61.5 (t)	--	61.3 (t)	--	61.5 (t)	--	61.4 (t)	40.4		
1"	170.3 (s)	--	170.3 (s)	--	170.3 (s)	--	170.3 (s)	--		
2"	23.5 (q)	--	23.5 (q)	26.5	23.5 (q)	--	23.5 (q)	26.7		
3"	170.3 (s)	--	170.3 (s)	--	170.3 (s)	--	170.3 (s)	--		
4"	20.7 (q)	--	20.7 (q)	27.4	20.7 (q)	--	20.7 (q)	--		
5"	170.3 (s)	--	170.3 (s)	--	172.9 (s)	--	172.9 (s)	--		
6"	20.7 (q)	--	20.7 (q)	27.4	36.0 (t)	--	36.0 (t)	27.3		
7"	170.3 (s)	--	173.0 (s)	--	170.3 (s)	--	172.9 (s)	--		
8"	20.7 (q)	--	35.9 (t)	26.6	20.7 (q)	--	36.0 (t)	27.3		
9"					18.6 (t)	--	18.6 (t)	23.4		
10"					13.6 (q)	--	13.6 (q)	21.7		
11"			18.3 (t)	23.0			18.3 (t)	--		
12"			13.6 (q)	21.1			13.6 (q)	21.7		

[a] ^{13}C NMR spectra were obtained at 50 MHz in 1% TMS/$CDCl_3$. Assignments were based on chemical shifts, multiplicities and J_R values and on comparison with model compounds

[b] J_R (residual coupling constant) values were measured in the off resonance spectra after offsetting the decoupler

[c] Assignments may be interchanged

The complete assignment of the aminohexose unit was accomplished by spin-decoupling analysis of the appropriate proton NMR signals in muricin-1. The anomeric proton at C-1′ was apparent as the lowest field sugar methine proton (δ 4.89). This proton was a doublet with $J = 8$ Hz, which confirmed it as an axial proton thus showing the hemiacetal linkage as equatorial or β.[20] By decoupling, all protons on the aminohexose ring were interrelated. Most conspicuous was the very small coupling constant between the C-3′ and C-4′ methine protons. Since C-3′ was clearly an axial proton, C-4′ must be equatorial and therefore, the C-4 hydroxyl must be axial. The acetylated amine functionality was readily established, as an equatorial substituent at C-2′ through these aforementioned decoupling experiments. Coupling of a D_2O-exchangeable N proton to the axial C-2′ proton ($J = 9$ Hz) was useful to establish the position of this group. Since all heteroatoms were equatorial except C-4′, and since all heteroatoms bear acetyl groups, the sugar component in muricin-1 was concluded to be 2′-acetamido-2′-deoxy-3′,4′,6′-tri-O-acetyl-β-galactopyranose. To confirm this conclusion the ^1H NMR spectral data of 1 were compared with those obtained from two synthetic aminogalactopyranosides produced by methylation and acetylation of

Table 2. 360 MHz ^{1}H NMR spectral assignments for muricin 1–4 and the aglycone 5[a]

H at C#	1		2		3		4		5	
	δ	m (J)	δ	m (J)	δ	m (J)	δ	m (J)	δ	m (J)
20	5.76	ddd (16,11,8)	5.76	ddd (16,11,8)	5.76	ddd (16,11,8)	5.76	ddd (16,11,8)	5.76	ddd (16,11,8)
-NH	5.64	d (9)[b]	5.67	d (8)[b]	5.51	d (8)[b]	5.61	d (9)[b]	--	
3'	5.41	dd (11,3)	5.41	dd (11,3)	5.41	dd (11,3)	5.40	dd (11,3.5)	--	
4'	5.36	bd (3)	5.36	bm (3)	5.38	bd (3)	5.38	bd (3.5)	--	
6	5.36	bd (3)	5.35	bm	5.36	bd (5)	5.38	bm	5.36	m (5.2)
21 (cis)	4.98	d (11)	4.98	d (11)	4.98	dd (11,2)	4.98	d (11)	4.98	d (11)
21 (trans)	4.97	d (16)	4.97	d (16)	4.97	dd (16,2)	4.97	d (16)	4.96	d (16)
1'	4.89	d (8)	4.89	d (9)	4.89	d (8)	4.89	d (8)	--	
6'a	4.16	dd (-11,6)	4.17	dd (-11,7)	4.17	dd (-11,7)	4.18	dd (-11,7)	--	
6'b	4.12	dd (-11,7)	4.10	dd (-11,7)	4.08	dd (-11,7)	4.08	dd (-11,7)	--	
5'	3.93	bt (7,3)	3.93	bt (7,6)	3.93	bt (7)	3.94	bt	--	
2'	3.81	ddd (11,9,8)	3.82	ddd (11,9,8)	3.79	ddd (11,9,8)	3.81	ddd (11,9,8)	--	
3	3.51	m	3.51	m (11,10,6,5)	3.51	m	3.51	m	3.53	m (11,11,4,3)
6"	2.14	s	2.14	s	3.38	t (7)	2.38	t (15)	--	
8"	2.04	s	2.27	t (7)	2.04	s	2.27	t (7)	--	
4"	2.00	s	2.00	s	1.99	s	1.99	s	--	
2"	1.97	s	1.97	s	1.97	s	1.97	s	--	
9"	--		--		--		1.68	m (8)	--	
11"	--		1.62	m (7)	--		1.62	m (7)	--	
19	0.99	s	0.99	s	1.00	s	1.00	s	1.02	s
10"	--		--		0.97	t (7)	0.98	t (8)	--	
12"	--		0.93	t (7)	--		0.94	t (7)	--	
18	0.60	s	0.60	s	0.60	s	0.60	s	0.61	s

[a] All ^{1}H NMR spectra were run in 1% TMS/$CDCl_3$. Assignments were based on ^{1}H NMR decoupling experiments and on comparison with model compounds.

[b] D_2O exchanged.

commercially available *N*-acetylgalactosamine (Table 3). A close correlation was observed between muricin-1 and the β anomer of the synthetic mixture.

To fully define the structure of muricin-1 the absolute stereochemistry of the aminogalactose component was required. Hydrolysis of **1**, followed by acetylation of the aqueous fraction, did yield mixtures of α- and β-tetra-O-acetyl-N-acetylgalactopyranose. However, the mixtures were difficult to separate and we considered rotation data unreliable. Therefore, the method of molecular rotation differences involving Hudson's Rules of isorotation was employed.[21–23] The method assumes that the molecular rotation of a glycoside is the sum of all rotational contributions from each chiral center in the molecule. Hence in **1**, the overall molecular rotation should simply be the combination of rotations of the pregnane and aminohexose components. Although this method is empirical, it has been routinely applied with good success to aminohexose pyranosides. The calculated negative molecular rotation between muricin-1 and aglycone **5** ($[M]_D$ − 32°) indicated that the sugar possesses the common D configuration. Therefore, by spectral interpretation and chemical degradation, muricin-1 (**1**) was fully defined as 3β-pregna-5,20-dienyl-2'-acetamido-2'-deoxy-3',4',6'-tri-O-acetyl-β-D-galactopyranoside.

Muricins-2 and -3 (**2**, **3**) both analyzed for $C_{37}H_{55}NO_9$ by field desorption mass spectrometry and by ^{13}C NMR analysis. Comparison of the ^{1}H and ^{13}C NMR spectra also showed **2** and **3** to be isomeric since each compound possessed identical resonances, albeit at slightly different chemical shifts. The molecular formulae for muricins-2 and -3 represented the addition of a C_2H_4 unit to muricin-1. Inspection of ^{13}C NMR data showed that this addition was in the form of two methylene groups. The ^{1}H NMR spectra for **2** and **3** showed only 3 acetyl Me resonances as compared to the 4 found in the spectrum of **1**. In **2** and **3**, however, additional bands were observed, which fully defined the presence of one n-butyrate ester group in each isomer. In muricin-2, for example, a 2H triplet was observed at δ 2.27, and the triplet was found to be coupled to a 2H multiplet at δ 1.62 (J = 7 Hz). This latter band was also coupled (J = 7 Hz) to a triplet methyl resonance found at δ 0.93. The same bands were also obvious in muricin-3, but at slightly different chemical shifts [δ 2.38 (2 H, t, J = 7 Hz), δ 1.68 (2 H, m, J = 7 Hz), δ 0.97 (3 H, t, J = 7 Hz)].

The ^{13}C and ^{1}H NMR spectra of **2** and **3** showed the presence of the same aglycone as in muricin-1. The high resolution mass spectrum also showed the expected fragmentation of $C_{21}H_{32}O$ (m/z 300.2433) assigned to the aglycone **5**. Consideration of these combined data led to the conclusion that muricins-2 and -3 were isomeric mono-n-butyrate esters or amides. With these data in hand, however, it was impossible to determine the locations of the butyrate esters/amides among the 4 possible sites of amide or ester formation on the aminogalactose ring.

Muricin-4 (**4**) analyzed for $C_{39}H_{59}NO_9$ by electron impact high resolution mass spectrometry and also by ^{13}C NMR analysis. This formula represented the addition of C_4H_8 to the formula of muricin-1, and of

Table 3. ^{1}H NMR spectral assignments for the acetylated aminosugar portion of muricins 1–4 compared with spectral data for model compounds, the α- and β-anomers of methyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-D-galactopyranoside

					methyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-D-galactopyranoside	
	1	2	3	4	β-anomer	α-anomer
-NH	5.64	5.67	5.56	5.76	5.95	5.89
C-1'	4.89	4.89	4.89	4.89	4.59	4.74
C-2'	3.81	3.82	3.82	3.82	4.05	4.52
C-3'	5.41	5.41	5.41	5.41	5.26	5.11
C-4'	5.36	5.35	5.35	5.35	5.33	5.33
C-5'	3.93	3.93	3.93	3.94	3.92	4.08
C-6'	4.12	4.10	4.08	4.08	4.12	4.08
	4.16	4.17	4.17	4.18	4.12	4.08
C-4'-OAc	2.14	2.14	--	--	2.15	2.14
C-6'-OAc	2.04	--	2.04	--	2.05	1.96
C-3'-OAc	2.00	2.00	1.99	1.99	2.00	2.03
C-2'-NHAc	1.97	1.97	1.96	1.96	1.97	1.96
C-4'-n-butyrate methyl	--	--	0.97	0.97	--	--
C-6'-n-butyrate methyl	--	0.93	--	0.93	--	--

C_2H_4 to those of muricins-2 and -3. Inspection of the NMR features of **4** yielded the predictable results. First, all resonances for the esterified aminosugar component and for the pregnadiene aglycone were intact. Decoupling experiments again resulted in complete assignments of numerous protons including all those of the aminogalactose component. The new C atoms in **4** were, not unexpectedly, found to be methylene groups. Based upon the presence of *two sets* of coupled bands in the ^{1}H NMR spectrum of **4** at *ca* δ 2.3, δ 1.6 and δ 0.9, muricin-4 was concluded to contain 2 butyrate ester/amide functionalities. As expected, only 2 acetyl Me resonances were observed in this compound. Hydrolysis of muricin-4 (10% H_2SO_4, 35°, 2 hr) gave the aglycone **5**, which was identical to that produced from **1** and also to the natural product from *G. rubiformis*.[17,18]

As with muricins-2 and -3, the unambiguous assignments of the locations of the 2 n-butyrate functionalities were needed for muricin-4. Fortunately, prior ^{1}H NMR investigations of the effects of lanthanide shift reagents on various methyl-2-acetamido-3, 4, 6-tri-O-acetyl-2-deoxy-D-pyranosides provided a foundation to make these assignments.[24] Prior studies showed that Europium shift reagents selectively complex with the amide CO group of acetylated aminosugars. This is as expected, since amide CO groups were previously shown to be stronger donors than esters toward lanthanide shift reagents.[25] With knowledge of the side of complexation, the rate of shift ($\Delta\delta$) of the acetate Me groups at C-3, C-4 and C-6 were reported for methyl-2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranoside. By use of these shift data, each acetate Me was located in space and proximity to the Europium atom. Extrapolation to zero concentration allowed the chemical shifts of all Me groups to be assigned (Table 4).

To confirm the earlier results and illustrate their compatibility with saponin aminopyranosides, the ^{1}H NMR behavior of muricin-1 (**1**) was measured with incremental additions of Eu(fod)$_3$ in $CDCl_3$. Virtually identical results were obtained (Table 4). The acetamido Me shifted to lower field at 3 times the rate of C-3′ acetoxyl Me. This allowed these latter Me singlets to be assigned in the original spectrum at δ 1.97 and δ 2.00, respectively. Similarly, the acetamido Me moved at 6 times the rate of the axial C-4′ acetoxyl Me and at 50 times the rate of the acetoxyl Me group at C-6′. These latter measurements were in very close accord with those from the model aminogalactopyranoside and allowed the C-4′ and C-6′ Me resonances to be established at δ 2.14 and δ 2.04, respectively, in muricin-1. Not unexpectedly, the axial ester Me group at C-4′ was the lowest field ester Me group.[26]

Once the shift behavior of muricin-1 (**1**) under these latter conditions, as well as the compatibility of Me group chemical shifts to model compounds were confirmed, the ^{1}H NMR shift behavior of muricin-2 (**2**) was measured next. Virtually identical results were obtained, which allowed the assignment of the three Me groups at C-2′, C-3′ and C-4′, based upon both their shift behavior ($\Delta\delta$) and their original chemical shifts. Since a Me group which showed the slow shifting behavior characteristic of C-6′ (1/50 rate of C-2′ methyl was clearly absent), the n-butyrate ester functionality in **2** was positioned at C-6′.

Shift studies with the isomeric monobutyrate muricin-3 (**3**) were not completed due to the limited quantities of this isomer. We can suggest, however, that the n-butyrate functionality should be placed at

Table 4. Results for the lanthanide-induced ($Eu(fod)_3$) shift study and acetyl methyl chemical shifts for muricins 1–4 compared with the model compounds, the α- and β-anomers of methyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-D-galactopyranoside

									methyl-2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-D-galactopyranoside			
	1[a]		2[b]		3[c]		4[b]		β-anomer[d]		α-anomer[d]	
C#	δ	Δδ	δ	Δδ	δ	Δδ	δ	Δδ	δ	Δδ	δ	Δδ
C-1'(-OMe)	--	--	--	--	--	--	--	--	3.51	0.8	3.37	1.7
C-2'(-NHAc)	1.97	1.9	1.97	4.2	1.97	--	1.96	8.3	2.00	7.2	2.03	14.7
C-3'(-OAc)	2.00	0.6	2.00	1.3	1.99	--	1.99	3.0	1.97	1.9	1.96	3.0
C-4'(-OAc)	2.14	0.3	2.13	0.7	--	--	--	--	2.15	0.2	2.14	0.6
C-6'(-OAc)	2.04	0.1	--	--	2.04	--	--	--	2.05	0.8	1.96	1.9

[a] Δδ values measured at 0.25 molar equivalents $Eu(fod)_3$

[b] Δδ values measured at 0.15 molar equivalents $Eu(fod)_3$

[c] Δδ values were not measured. Methyl group chemical shift assignments are evident based on comparison with muricins 1-2 and 4 and with the model compounds

[d] Values are quotations of literature reports[24]

the C-4′ axial position in this molecule, since the characteristic Me resonance at δ 2.14 confirmed that this position was lacking in the ^1H NMR spectrum of 3.

Lastly, the positions of the two n-butyrate esters in muricin-4 (4) were assigned in identical fashion. Two methyl groups, the C-2′ acetamido group and the C-3′ acetoxyl Me, were found to be intact by analysis of Europium shift data. Thus the n-butyrate esters were positioned at C-4′ and C-6′ in this metabolite.

The isolation of the muricins represents the first isolation of saponin derivatives from invertebrates of the Phylum Cnidaria. Saponins are, of course, common components of marine asteroids (starfish) and holothurians (sea cucumbers).[27,28] These organisms are the Phylum Echinodermata, where saponins are thought to provide a function in chemical defense. Echinoderm saponins are known to exhibit cytotoxic, hemolytic, ichthyotoxic, antiviral and antimicrobial activities, and apparently also induce escape reactions in certain molluscs.[29] While aminosugars are virtually ubiquitous components of nature, and found in the marine environment in structural polysaccharides such as chitin (polyglucosamine), aminosaponins have not been previously isolated from marine sources.[28] While this work was in progress we learned that several aminoglucose saponins had been described as the active components of the defensive secretion from the Japanese sole *Paradachirus pavoninus*.[30] Like the Red Sea sole *P. marmoratus*, the secretions from these fishes appear to be highly repugnant to predatory animals and their repellent effects on predatory sharks have been well documented.[31] Aminoglycosides from terrestrial sources are also known for their generally potent physiological effects.[32,33] The cytotoxic and antifungal metabolite septacidin, for example, contains the aminosugar 4-amino-4-deoxy-L-glucose.[34]

It is clear that there is ample precedent to predict biological activity from aminosaponins such as the muricins. In an attempt to establish a functional basis for these metabolites in reducing fouling on *M. fruticosa*, we measured the effects of 1–4 in several bioassays. Ichthyotoxicity against the marine pomacentrids *Dascyllus aruanus* and *Pomacentrus coeruleus* was measured, as previously described,[35] at concentrations of 10 ppm for 1 hr. None of the muricins were found to be toxic. Similarly, the muricins were found not to be effective when either their cytotoxic effects (fertilized sea urchin egg assay)[36] or antimicrobial properties (standard agar plate-assay disk method) were assessed. In contrast, the muricins, at 100 ppm concentrations, were found to effectively inhibit growth (70% inhibition) of the pennate diatom *Phaeodactylum tricornutum*.

The absence of cytotoxicity, ichthyotoxicity and antimicrobial activity exhibited by the muricins is in strong contrast to other marine saponins. It appears that esterification greatly reduces the toxic effects of saponins. To determine if the unesterified "free saponins" were present in *M. fruticosa*, the aqueous fraction was examined. The aqueous phase was freeze-dried and acetylated and the organic soluble portion was fractionated as already described. No trace of the peracetyl saponin muricin-1 (1), nor any other aglycone-containing metabolite, could be detected.

The presence of diatom inhibition activity in the muricins may, however, play a significant role in reducing fouling on *M. fruticosa*. Numerous investigators have shown that marine fouling is a complex process involving the establishment of a preliminary microbiological film which is generally composed of diatoms, protozoa, bacteria and other microorganisms.[37,38] Macro invertebrate fouling is then thought to occur only after this primary process has become fully established. Hence, it may be that inhibition of photosynthetic (autotrophic) marine microorganisms is an important component in reducing overall fouling phenomena. Indeed, similar results and conclusions were recently reported in connection with investigations of the Atlantic gorgonians

Leptogorgia virgulata and *L. setacea*.[39] These gorgonians were found to produce significant quantities of homarine (N-methyl-2-carboxypyridine), which was also shown to inhibit diatom replication.

EXPERIMENTAL

General. IR and UV spectra were recorded on Perkin–Elmer model 137 and Perkin–Elmer model 124 double beam spectrophotometers. Optical rotations were recorded on a Perkin–Elmer model 141 polarimeter using a 10 cm microcell (1 ml). Proton NMR spectra were recorded at 360 MHz on an Oxford Magnetics-Nicolet computer instrument and ^{13}C spectra were recorded at 50 MHz on a Nicolet multinuclear, wide-bore instrument. All spectra were recorded with internal TMS as standard with $\delta = 0$. High-resolution electron impact and field desorption mass spectra were provided by the Bioorganic Biomedical Mass Spectrometry Resource Center, University of California, San Francisco. Melting points (uncorrected) were obtained using a Fisher-Johns apparatus and all solvents were purified by distillation from glass prior to use.

Collections, extractions: Muricea californica *and* M. fruticosa were collected simultaneously at four separate southern Californian locations between January 1979 and June 1980. After collection, samples were stored in either ethyl or isopropyl alcohol. The alcohol was decanted and the whole animal was repeatedly extracted with 70% CH_2Cl_2 in MeOH. The combined solvents were next removed under vacuum. The resulting aqueous residue was partitioned several times between CH_2Cl_2 and water. The organic layer was concentrated and dried over $MgSO_4$ to give a crude extract (usually 2–3% of the dry weight of the animal). Extracts of *Muricea californica* and *M. fruticosa* were fractionated by rapid filtration chromatography using TLC grade silica gel in a sintered glass funnel. Fractions were eluted with mixtures of isooctane, CH_2Cl_2 and EtOAc.

Comparison of the organic extracts of Muricea californica *and* M. fruticosa

Muricea californica was collected in La Jolla, Catalina and Los Coronados Islands (southern California). A total of 5.7 g crude dichloromethane extract was obtained from 200 g dry weight of *M. californica* (collected in Catalina in June, 1980) (2.9% dry wt). The aqueous residue was set aside for further study. Separation of the dichloromethane extract, as described above, resulted in the isolation of 50 mg of ergosterol peroxide from a fraction eluted in 25% EtOAc–CH_2Cl_2 (0.9% extract, 0.03% dry wt). Ergosterol peroxide, a sterol peroxide previously isolated from marine[40,41] and terrestrial organisms,[42] was the only secondary metabolite found in the extract of *M. californica*.

Muricea fruticosa was collected simultaneously with *M. californica*. A total of 3.0 g crude extract was obtained from 200 g dry wt of *M. fruticosa* (collected in Catalina in June, 1980) (1.5% dry wt). The aqueous residue was set aside for further study. Separation of this extract also gave 30 mg ergosterol peroxide (eluted with 25% EtOAc–CH_2Cl_2). ^1H NMR analysis of the fraction eluted with 50% EtOAc–CH_2Cl_2 showed a mixture containing both fatty acids and compounds **1**–**4** (0.5% extract, 0.01% dry wt for each compound). Collections of *M. fruticosa* from other locations yielded similar results with varying mixtures of the muricins (**1**–**4**). These compounds were rechromatographed by HPLC on silica with 60–75% EtOAc–isooctane. Reverse phase (C_{18}) HPLC using 90–95% MeOH–water was also employed to finally separate **1**–**4** from mixtures containing fatty acids. The compounds were eluted from silica HPLC in the following order of increasing polarity: **4**–**3**–**2**–**1**.

Muricin-1, *3β-pregna-5,20-dienyl-2'-acetamido-2'-deoxy-3',4',6'-tri-O-acetyl-β-D-galactopyranoside* (**1**). Compound **1** was purified by silica HPLC with 75% EtOAc isooctane. Compound **1** showed $[\alpha]_D^{25} = 26.3°$ ($c = 1.6$, $CHCl_3$); IR ($CHCl_3$): 3367–3546 (w), 2967, 1754 (s), 1686 (s), 1462, 1374, 1247, 1134, 1079, 1045, 1018 cm^{-1}. Field desorption mass spectrometry (FDMS) gave: M + 1 630 (100) (M^+ 629 for $C_{35}H_{51}NO_9$), 571 (15) (M^+–OAc), 338 (15), 332 (10) ($M^+-C_{21}H_{30}O$), 315 (10), 301 (10) ($C_{21}H_{33}O$), 259 (13). The absolute stereochemistry of the aminosugar moiety in **1** was obtained using Hudson's rules of isorotation:[20–22] $[M]_D$ (sugar) ~ $\Delta[M]_D = [M]_D$ saponin—$[M]_D$(aglycone) where $[M]_D = [\alpha]_D \times MW/100$. For muricin-1, $[M]_D$ (**1**) = $-26 \times 629/100 = -164$. For the aglycone **5**, $[M]_D$ (**5**) = $-44 \times 300/100 = -132$. Therefore, $\Delta[M]_D = (-164) - (-132) = -32$. Since the $[M]_D$ of the β-D-anomeric sugar is $-61(-17 \times 361/100)$ and the $[M]_D$ of the α-D-anomer is $+325$ ($+90 \times 361/100$), the aminosugar in **1** was assigned as the β-D-anomer. The corresponding $[M]_D$ for the β-L-aminosugars would have been $+61$ and -325, respectively.

Muricin-2, *3-β-pregna-5,20-dienyl-2'-acetamido-2'-deoxy-3',4'-di-O-acetyl-6'-O-n-butyryl-β-D-galactopyranoside* (**2**). Compound **2** was purified from extracts of *Muricea fruticosa* by silica HPLC with 70% EtOAc–isooctane. Compound **2** showed $[\alpha]_D^{27} = -29.7°$ ($c = 1.4$, $CHCl_3$); IR ($CHCl_3$): 3340–3520 (w), 2960, 1745, 1680, 1370, 1240, 1070 cm^{-1}. Field desorption mass spectral measurement (FDMS): M + 1 658 (100) ($C_{37}H_{56}NO_9$), M^+ 657 (23) ($C_{37}H_{55}NO_9$), 367 (10) ($M^+-C_{21}H_{29}$), 283 (45) ($C_{21}H_{31}$), 205 (42), 171 (12), 153 (20). Hudson's rules of isorotation were used as described above for **1** to determine the absolute stereochemistry of the aminosugar. For muricin-2, $[M]_D = -30 \times 657/100 = -197$ and $\Delta[M]_D = (-197) - (-132) = -65$, close to the $[M]_D$ value of -61 for the β-D-anomeric sugar.

Muricin-3, *3-β-pregna-5,20-dienyl-2'-acetamido-2'-deoxy-3',6'-di-O-acetyl-4'-O-n-butyryl-β-D-galactopyranoside* (**3**). Compound **3** was purified by silica HPLC with 65% EtOAc–isooctane. Compound **3** showed $[\alpha]_D^{27} = -30.3°$ ($c = 1.2$, $CHCl_3$); IR ($CHCl_3$): 3350, 2960, 1750, 1680, 1520, 1490, 1370, 1270, 1170, 1080, 1040 cm^{-1}. FDMS: M + 1 658 (20) ($C_{37}H_{56}NO_9$), M^+ 657 (24) ($C_{37}H_{55}NO_9$), 377 (3), 376 (42) ($M^+-C_{21}H_{29}$), 283 (37), 282 (100) ($C_{21}H_{30}$); HRMS: *m/z* 300.2433 (0.4) (Calc 300.2453 for $C_{21}H_{32}O$), 282.2344 (61.4) (Calc 282.2348 for $C_{12}H_{30}$), 267.2108 (26.4) (calc 267.2113 for $C_{21}H_{27}$). For muricin-3, $[M]_D = -30 \times 657/100 = -197$ and $\Delta[M]_D = (-197) - (-132) = -65$, close to the $[M]_D$ value of -61 for the β-D-anomeric sugar.

Muricin-4, *3-β-pregna-5,20-dienyl-2'-acetamido-2'-deoxy-3'-O-acetyl-4',6'-di-O-n-butyryl-β-D-galactopyranoside* (**4**). Compound **4** was purified by silica HPLC with 70% EtOAc–isooctane. Compound **4** showed m.p. 119–121°, $[\alpha]_D^{27} = 35.8°$ ($c = 0.8$, $CHCl_3$); IR ($CHCl_3$): 3500 (w), 2980, 1740, 1680, 1460, 1370, 1300, 1160, 1090, 1080 cm^{-1}. ^1H NMR (Me_2CO–d_6): δ 7.10 [1 H, d(9) (D_2O exchanged)], 5.80 [1 H, ddd (16, 11, 8)], 5.35 (1 H, bd (3.5)], 5.35 (1 H, m), 5.19 [1 H, dd (11, 3.5)], 4.96 [1 H, d (11)], 4.95 [1 H, d (16)], 4.89 [1 H, d (8)], 4.18 (1 H, m), 4.08 (1 H, m), 4.06 (1 H, m), 4.02 [1 H, ddd (11, 9, 8)], 3.55 (1 H, m), 2.40 (2 H, t (9)], 2.26 [2 H, 5 (7)], 1.90 (3 H, s), 1.85 (3 H, s), 1.59 [2 H, m (7)], 1.48 (1 H, m), 1.08 (3 H, s), 0.98 [3 H, t (8)], 0.91 [3 H, 5 (7)], 0.64 (3 H, s).^{13}C NMR (50 MHz, Me_2CO-d_6); 173.1 (s), 172.9 (s), 170.2 (s) × 2, 141.5 (s), 140.3 (d, J_R = 44.7), 122.2 (d, 46.6), 115.0 (t), 100.7 (d, 45.8), 79.8 (d, 35.0), 71.4 (d, 44.1), 71.2 (d, 37.7), 67.5 (d, 46.9), 62.1 (t), 56.6 (d, 27), 56.1 (d, 17.6), 51.6 (d, 38.0), 51.4 (d), 44.0 (s), 39.6 (t), 38.1 (t), 37.5 (s), 36.3 (t), 32.7 (t), 27.8 (t), 25.4 (t), 32.1 (q, 26.0), 21.3 (t), 20.6 (q), 19.7 (q), 19.2 (t), 18.9 (t), 13.8 (q, 20.8), 13.0 (q, 20.9) ppm. HRMS: M^+ 685.4152 (0.1) (Calc 685.4190 for $C_{39}H_{59}NO_9$), 300.2455 (2.1) (Calc 300.2453 for $C_{21}H_{32}O$), 285.2204 (1.0) (Calc 285.2218 for $C_{20}H_{29}O$) 282.2350 (48.7) (Calc 282.2345 for $C_{21}H_{30}$), 267.2105 (19.3) (Calc 267.2113 for $C_{20}H_{27}$), 231.1742 (1.8) (Calc 231.1479 for $C_{16}H_{32}O$), 229.1939 (3.5) (Calc 229.1956 for $C_{17}H_{25}$). Hudson's rules of isorotation were employed for muricin-4: $[M]_D$ (**4**) = $-36 \times 685/100 = -247$, $\Delta[M]_D = (-247) - (-132) = -115$, closer to the $[M]_D$ value for the β-D-anomer (-61) than for the α-D-anomer ($+325$).

Hydrolysis of muricin-1. Compound **1**, 24.5 mg (3.9×10^{-5} mole), in 2 ml EtOH was treated with 1 ml of 1.0 N HCl and heated for 3 hr at 40°. The product was partitioned several times between CH_2Cl_2 and water and the

organic layer was concentrated to give 11.8 mg (3.9×10^{-5} moles) of a single product, deduced to be relatively pure by ^{1}H NMR analysis. The product was identified as **5**, pregna-5,20-dien-3β-ol, by comparison with an authentic sample previously isolated from *Gersemia rubiformis*. The aglycone **5** showed $[\alpha]_D^{25} = 43.6°$ (c = 0.6, $CHCl_3$), IR ($CHCl_3$): 3401, 2950, 2865, 1450 cm^{-1}, HRMS: M^+ 300.2443 (7.3) (M^+ Calc 300.2453 for $C_{21}H_{32}O$), 282.2372 (16.2) (Calc 282.2348 for $M^+-H_2O-CH_3$), 246.1994 (3.1) (Calc 246.1984 for $M^+-H_2O-CH_3$), 246.1994 (3.1) (Calc. 246.1984 for $C_{17}H_{26}O$), 242.2050 (5.4) (Calc 242.2035 for $C_{18}H_{26}$), 213.1632 (21.8) (Calc 213.1643 for $C_{16}H_{21}$). The aqueous fraction from this reaction was placed in a desiccator containing potassium hydride and placed under a high vacuum for 16 hr. A total of 5.5 mg of a white powder was recovered. Attempts to dissolve this powder in a suitable ^{1}H NMR solvent ($CDCl_3$, Me_2CO-d_6, MeOH-d_4, D_2O) were unsuccessful. The powder could be dissolved in 6 ml pyridine, however, and was acetylated with 3 ml Ac_2O for 20 hr at room temp. The reaction was quenched by adding ice and water and the resulting soln was extracted several times with EtOAc. The organic layer was washed successively with 5% HCl and 5% bicarbonate soln and dried over $MgSO_4$. The recovered product, 4.0 mg of a yellow oil, was determined to be a mixture of 1,3,4,6-tetra-O-acetyl-N-acetyl-α-D(+)-galactosamine (10%) and 1,3,4,6-tetra-O-acetyl-N-acetyl-β-D(+)-galactosamine (1%) by ^{1}H NMR analysis. Comparison of the ^{1}H NMR spectra with those of the synthetic products produced from acetylation of N-acetyl-D-galactosamine (Aldrich Chemical Company) showed the products were identical and to have been produced in the same relative proportion.

Hydrolysis of muricin-4. Compound **4**, 15 mg (2.2×10^{-5} mole) in 3 ml MeOH, was treated with 3 ml of a 10% H_2SO_4 soln. The reaction flask was warmed to 35° for 2 hr and then stirred overnight at room temp. The resulting mixture of products was partitioned several times between EtOAc and water. The organic layer was washed with a sat $NaHCO_3$aq, concentrated and dried over $MgSO_4$ to yield 6.2 mg (2.1×10^{-5} mole) of **5**. The product obtained was identical in all regards to the product obtained from the hydrolysis of muricin-1.

Investigation of the aqueous extracts of M. californica *and* M. fruticosa. The aqueous residues from both gorgonians (isolation described earlier) were lyopholized for 20 hr to yield approximately 1 g quantities of yellow powders (0.9% dry wt *M. fruticosa*, 1.7% dry wt *M. californica*). Both yellow solids were treated with 10 ml Ac_2O in 20 ml pyridine for 24 hr at room temp. The resulting mixtures were partitioned several times between EtOAc and water. The organic layers were washed sequentially with 5% HCl and 5% $NaHCO_3$ solns, dried over $MgSO_4$ and concentrated. Yields of 0.1 and 0.2 g of organic soluble ($CHCl_3$) residues were obtained from *M. fruticosa* and *M. californica*, respectively. Proton NMR analysis of each of these mixtures showed them to contain acetylated simple sugars. The mixtures did not contain the low-field NMR bands characteristic of **5** nor could any evidence be gathered for the presence of the expected compound, muricin-1.

Lanthanide induced shift (LIS) experiments with the muricins. Consecutive 0.05 molar aliquots of Eu(fod)$_3$, in $CDCl_3$, were added to an NMR tube containing **1–4** until final concentration of 0.15–0.25 molar equivs were reached. The ^{1}H NMR spectra were recorded after each addition of Eu(fod)$_3$ and double resonance (decoupling) experiments were performed in some cases to detect the downfield shift of each proton. The induced shifts ($\Delta\delta$) were determined by plotting the chemical shift of each proton signal against the molar quantity of Eu(fod)$_3$ added. The results of the shift study are reported in Table 4.

Algal growth bioassay. An aliquot of 5 μl from an axenic culture of the marine pennate diatom *Phaeodactylum tricornutum* Bohlin was used to inoculate Pyrex culture tubes containing 1 mg GPM seawater (a nutrient medium) and 100 μg of each compound. Compounds **1–4** were introduced into the media in 2.5 μl EtOH. The diatom cultures were grown under continuous light for four days at 24° along with EtOH and GPM seawater controls. Cell densities were counted using a haemocytometer, under a microscope. At 100 ppm, muricin **1–4** inhibited the growth of the marine diatom from 60–80% compared with the controls. At higher concentrations (200 ppm) complete inhibition was observed.

Acknowledgements—This research is a result of financial support from NOAA, Office of Sea Grant, Department of Commerce, under grant NA80AA-D-00120, and in part, by the California State Resources Agency, project R/MP-22. The U.S. Government is authorized to reproduce and distribute for governmental purposes. We wish to acknowledge the mass spectral support provided by the Bioorganic Biomedical Mass Spectrometry Resource Center at the University of California, San Francisco (A.L. Burlingame, Director) which is supported by the NIH under grant RR00719 from the Division of Research Resources. MMB wishes to acknowledge the California and National Sea Grant Program for additional support in the form of a graduate fellowship, and to thank Mr. James Lance for assistance in performing antialgal bioassays. We are indebted to Dr. John Wright, UC-San Diego NMR Center, for his kind assistance with NMR methodology.

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