Amaroxocanes A and B: Sulfated Dimeric Sterols Defend the Caribbean Coral Reef Sponge *Phorbas amaranthus* from Fish Predators

Brandon I. Morinaka,[†] Joseph R. Pawlik,[‡] and Tadeusz F. Molinski*[,†,§]

Department of Chemistry and Biochemistry and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, California 92093-0358, and Department of Biology and Marine Biology, Center for Marine Science, University of North Carolina Wilmington, 5600 Marvin K. Moss Lane, Wilmington, North Carolina 28403-3297

Received October 15, 2008

Two new dimeric steroids, amaroxocanes A (**1**) and B (**2**), were isolated from *Phorbas amaranthus* collected on shallow coral reefs off Key Largo, Florida. The two compounds are comprised of two sulfated sterol cores bridged by an oxocane formed by different oxidative side-chain fusions. The structures were characterized by interpretation of MS and NMR spectroscopic data. The compounds are the primary components of a fraction that deters feeding of the bluehead wrasse, *Thalassoma bifasciatum*, in aquarium assays. When the pure compounds were assayed at eight times the natural concentration, amaroxocane B (**2**) was found to be an effective antifeedant, but A (**1**) was not.

Many Caribbean marine sponges are chemically defended against reef predators by secondary metabolites. Sponges are the dominant soft-bodied benthic invertebrate on Caribbean coral reefs and are important in carbon and nutrient cycling that shapes the reef community. In a survey of Caribbean sponge chemical defenses, crude extracts obtained from approximately 70 species revealed that most (69%) deterred feeding by the bluehead wrasse, *Thalassoma bifasciatum,* a common omnivore on Caribbean reefs.[1] In follow-up studies, the secondary metabolites responsible for feeding deterrence were identified from several species, including oroidin[2] and stevensine[3] (2-amino imidazole alkaloids) from *Agelas clathrodes* and *Axinella corrugata,* respectively, the formosides[4] (triterpene glycosides) from *Erylus formosus*, and amphitoxin[5] (a polymeric pyridinium alkaloid) from *Amphimedon compressa*.

Phorbas amaranthus Duchassaing & Michelotti 1864 is a velvet-red sponge that is common in parts of the Florida Keys with more sparse distribution in the Bahamas. Crude extracts from *P. amaranthus* displayed strong feeding deterrent activity, which led us to investigate the chemical consituents of this sponge. Here we report the isolation of two new compounds, amaroxocanes A (**1**) and B (**2**), which are the major compounds responsible for feeding deterrence in the sponge extracts.

Previous investigations led to the isolation of two structurally different families of steroids of different polarities from *P. amaranthus*: from hexane extracts, ring-A contracted nonpolar steroids phorbasterones A−D (**3a**−**d**)[6] along with the known anthosterones A and B,[7] and from aqueous methanol extracts, the sulfated *N*-imidazolyl steroidal alkaloid amaranzole A (**4**).[8] The nonpolar steroids were inactive in fish feeding assays, but sufficient amounts of **4** and other polar constituents were initially unavailable for testing due to the difficulty of purification. Following a simplified isolation protocol and bioactive guided fractionation, we can now demonstrate that amaroxocanes A (**1**) and B (**2**) are responsible for the feeding deterrent activity in crude extracts of *P. amaranthus*. The characterization of **1** and **2** and evaluation of feeding deterrence of **4**, **1**, and **2** are the subjects of this report.

Results and Discussion

Feeding deterrence was assayed using paired choice tests (control and treated) with artificial food pellets presented to *T. bifasciatum* in aquarium assays as described previously.[1] Hexane extracts of *P. amaranthus* collected from the Florida Keys in 2006 were inactive, but the aqueous methanol extract exhibited strong feeding deterrent activity. The crude extract was subject to reversed-phase (C_{18}) prefractionation followed by preparative HPLC (reversed phase, C_{18}, CH_3CN/H_2O, $NaClO_4$) to a partially purified fraction that was deterrent at twice the natural concentration ("2×"). The two major compounds purified from the antifeedant fraction were new steroidal dimers amaroxocanes A (**1**) and B (**2**).[9] Compound **1** was isolated as a colorless glass of molecular formula $C_{55}H_{85}Na_3O_{17}S_3$, based on HRESIMS (*m*/*z* 1159.4764 [M − Na^+], $\Delta = +1.4$ mmu). The steroidal nature of **1** was evident in the ^{1}H NMR spectrum, which showed two deshielded methyl singlets (δ 0.64, s, C-18; 0.86, s, C-18′) and two methyl doublets (δ 0.95, d, 3H, $J = 6.7$ Hz; 1.00, d, 3H, $J = 6.6$ Hz) with chemical shifts corresponding to methyl groups H_3-18 and H_3-21, respectively. The presence of OSO_3Na and alcohol groups was supported by strong bands in the IR spectra at ν 1216 and 3453 cm^{-1}, respectively, and losses of SO_3Na (103 amu) from the negative ion ESIMS spectra. The molecular formula, with approximately twice the expected number of carbon atoms (C_{55}, cf. cholesterol C_{27}), implied that **1** was a dimeric sulfated sterol.

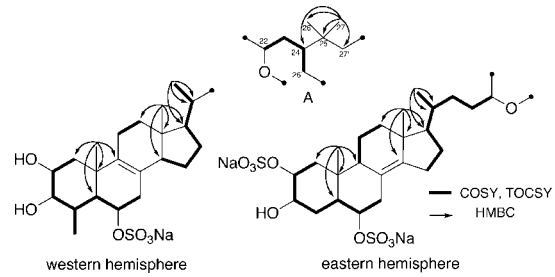
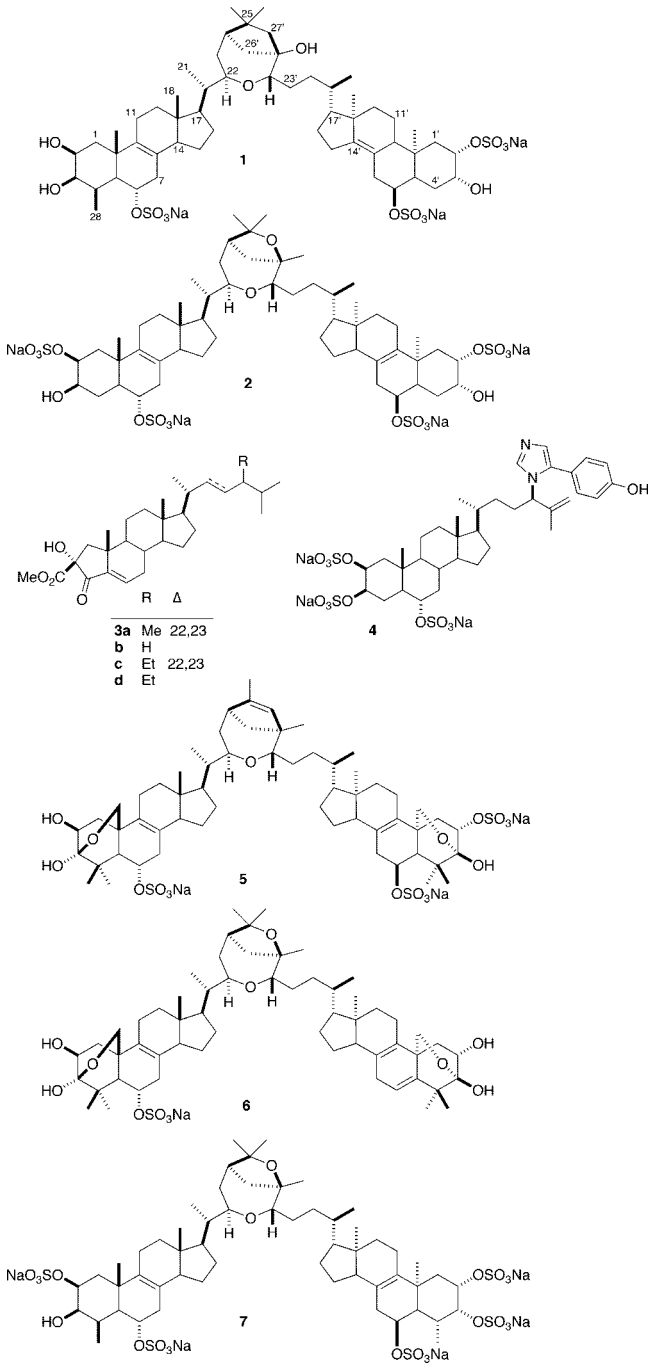


Figure 1. Selected 2D NMR correlations for amaroxocane A (**1**).

The eastern and western hemisphere for **1** were assembled in a straightforward manner by analysis of COSY, TOCSY, and HMBC correlations (Figure 1). Structure determination of the western hemisphere began with an HMBC correlation from Me-19 to C-1, C-5, C-9, and C-10. COSY and TOCSY correlations delineated the spin system H-1 through H-7, which included a methyl doublet at C4 (δ 1.28, d, $J = 6.6$ Hz). The ^{13}C chemical shifts for C-2 (δ 72.8 ppm) and C-3 (δ 73.4 ppm) implied OH substitution, while the ^{13}C chemical shift for C-6 (δ 75.1 ppm) was more consistent with a sulfate half-ester $O(SO_3)Na$.[10] Proton signals due to H-7

* To whom correspondence should be addressed. Tel: +1 (858) 551-1662. Fax: +1 (858) 822-0386. E-mail: tmolinski@ucsd.edu.
[†] UC San Diego, Dept. of Chemistry and Biochemistry.
[§] UC San Diego, SSPPS.
[‡] UNC Wilmington.

10.1021/np800652v CCC: $40.75
Published on Web 01/14/2009



(δ 2.33 and 2.91 ppm), H-11 (δ 2.10 and 2.20 ppm), and H-14 (δ 1.94 ppm) showed chemical shifts consistent with allylic hydrogens and a double bond at the C-8/C-9 position that was further supported by an HMBC cross-peak from Me-19 to C-9. The H-14 to H-21 spin system was established from COSY and TOCSY correlations. HMBC correlations from Me-18 and Me-21 completed the ABCD ring system in the western hemisphere. The eastern hemisphere was assembled in a similar manner; however a few differences were observed. The methyl doublet at C-4′ (δ 28.5) was absent from the eastern hemisphere. A double bond was placed at the C-8′/C-14′ position on the basis of ^{1}H chemical shifts consistent with allylic hydrogens H-7′ (δ 1.92, m; 3.02, m), H-9′ (δ 1.87, m), and H-15′ (δ 2.26, m; 2.35, m). HMBC cross-peaks from H-7′ to C-8′ and C-14′ were also observed. The ^{13}C NMR chemical shift for C-3′ (δ 71.8, d) and a downfield shift ($\Delta\delta$ +0.14 ppm, Figure 2) upon measurement of the ^{13}C NMR specturm in deuterated solvent supported substitution by a free hydroxyl substituent, while downfield shifts observed for C-2′ (δ 79.4, d)

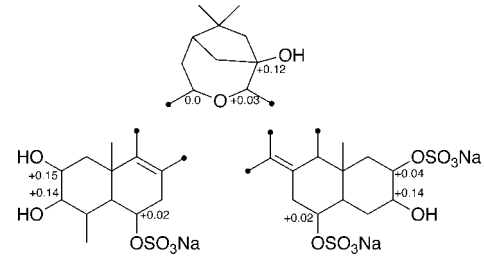


Figure 2. Selected deuterium-induced isotope shifts in the ^{13}C NMR spectrum of amaroxocane A (**1**). $\delta\Delta = [\delta_C(CD_3OD) - \delta_C(CD_3OH)]$.

and C-6′ (δ 78.7, d) and negligible ^{13}C NMR deuterium isotope shifts were consistent with *O*-sulfate esters.[11]

The two halves of **1** were shown to be linked through an oxabicycle formed through oxidative fusion of the side chains. The *gem*-dimethyl signals for Me-26 and Me-27 showed HMBC correlations to the quaternary carbon C-25 (δ 41.5, s) and also showed correlations to C-24 and C-27′. COSY and TOCSY correlations connected H-24 to the former spin system containing H-22 to H-26′, establishing substructure A (Figure 1). Reconciliation of the strong HMBC correlations from H-26′ and H-27′ to the oxygenated quaternary carbon C-25′ required a cyclopentane ring. C-25′ was connected to the eastern hemisphere by HMBC correlations from H-26′ and H-27′ to C-24′. Finally, C-22 was connected to C24′ via an ether linkage supported by an HMBC correlation from H-24′ to C-22. The complete planar structure for amaroxocane (**1**) was now secured, and attention was turned to addressing the relative and absolute configurations.

The relative configuration for **1** could be assigned by NOESY and analysis of coupling constant data. The equatorial disposition of H-2 in the western hemisphere was evident from the small $^3J_{HH}$ vicinal coupling to H-3, and H-3 was axial on the basis of NOESY correlations to axial protons H-1 and H-5. An equatorial orientation was assigned for H-4, which showed small vicinal couplings and NOESY correlations to H-3 (δ 3.96, dd, J = 6.1, 3.5 Hz) and H-5 (δ 2.02, dd, J = 12, 4.1 Hz), and H-6 (δ 4.87, dt, J = 12, 7.6 Hz) showed large coupling (J = 12 Hz) to H-5. A trans fused ring system was established for the C/D ring system on the basis of axial orientation of Me-18 and H-14. Assignment of the eastern hemisphere was carried out in a similar manner. H-2′ (δ 4.72, brq, J = 2.6 Hz) showed small coupling (J = 2.6 Hz) to H-3′ (δ 3.80, m), which showed NOESY correlations to H-5′ (δ 1.61 m). H-6′ was assigned as axial on the basis of large coupling to H-5′. Me-18′ showed NOESY correlations to both axial H-11′ and H-16′. The oxygenated methine H-22 (δ 3.63, dd, J = 11.8, 4.6 Hz) was informative of the relative configuration and conformation of the oxabicycle and showed NOESY correlations to H-16$_{ax/eq}$, H-23$_{ax}$, H-23′$_{ax/eq}$, and H-26′ (δ 2.05, m).

The absolute configuration of the different heterocyclic rings in **1** and **2** was addressed by relaying the configuration at C-20 to the heterocyclic linker. The natural *R* configuration for C-20 of both hemispheres can be safely assumed on the basis of biogenic principles, and the absolute configuration of the oxabicycle could be established through assignment of the relative configuration between the C-20 and C-22 bond. A dihedral angle of $\theta = \pm 90°$ was evident for H−C20−C22−H from lack of scalar coupling ($J \approx 0$ Hz) between the H-20 and H-22 protons.[12] Of the two possible signs for the dihedral angle, only $\theta = +90°$ was consistent with the ensemble of NOEs observed in **1**, and 22*R* was deduced as the most likely configuration based on NOESY correlations observed between H-17/H-23$_{eq}$ and H-16$_{ax/eq}$/H-22 (see Figure 3). Therefore the complete stereochemical assignment for **1** is as depicted.

The molecular formula for the second major constituent, amaroxocane B (**2**), was derived as $C_{54}H_{82}Na_3O_{20}S_4$ on the basis of

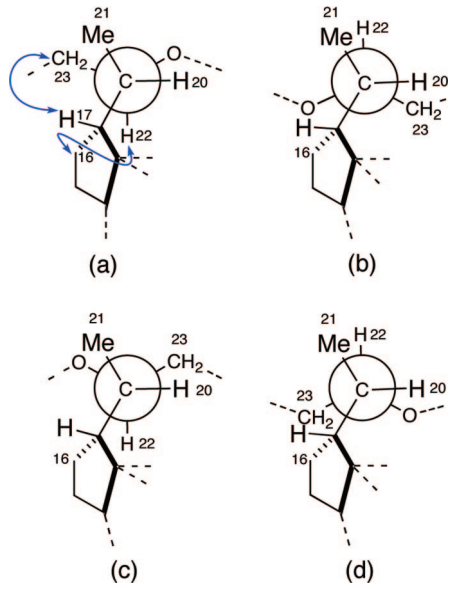


Figure 3. Newman projections along the C20−C22 bond of **1** depicting the two possible conformers for the (20*S*,22*R*) diastereomer with H20−C20−C22−H22 dihedral angles of (a) $\theta = +90°$ and (b) $\theta = -90°$ and for the (20*S*,22*S*) diastereomer with H20−C20−C22−H22 dihedral angles of (c) $\theta = +90°$ and (d) $\theta = -90°$. Ring C residues and the angular methyl group are removed for clarity. Only (a) accommodates *both* the observed NOESY cross-peaks (double-headed arrows) and ^3J(H20−H22) ≈ 0 Hz observed for **1**.

negative ion HRESIMS (*m*/*z* 1247.3965 [M − Na]$^-$, Δ = −1.0 mmu). The ^{1}H NMR and mass spectral data suggested **2** was also a sulfated sterol dimer. Most of the signals in the ^{1}H NMR appeared to be doubled or overlapping, leading us to believe the constitution and relative configuration of the sterol core in each hemisphere was the same. This was confirmed by analysis of 2D NMR (COSY, HSQC, and HMBC).

The bridged linker showed substantial differences compared to that of amaroxocane A (**1**). The *gem*-dimethyl groups Me-26 (δ 1.32, s, 3H) and Me-27 (δ 1.23, s, 3H) were now attached to an oxygenated quaternary carbon C-25 (δ 85.5, s). The H-24 signal (δ 2.15, m) was again assigned to the spin system H-22−H-25, similar to that found in compound **1**. An ether linkage was evident between C-22 (δ 71.7, d) and C-24′ (δ 86.2, d) by an HMBC correlation from H-22 to C-24′. A second oxygenated quaternary carbon attached to Me-27′ was inserted between C-24′ and C-26′, supported by HMBC correlations from the methyl singlet (Me-27′) to oxygenated quaternary carbon (C-25′), the bridgehead methylene (C-26′), and the oxygenated methine (H-24′). Finally, the molecular formula required an additional degree of unsaturation, which was satisfied by an ether linkage between C-25 and C-25′ forming a dioxabicycle identical to those found in the crellastatins[20] and hamigerols.[21] The absolute and relative configuration of **2** was assigned using similar principles used for **1**.

Our early efforts to locate the compound(s) in *P. amaranthus* responsible for feeding deterrent activity were hampered by loss of activity as purification progressed.[6,8] An HPLC fraction containing amaranzole A (**4**) and two closely related congeners did not deter feeding when tested up to 16× natural concentration. Only the HPLC fraction containing **1** and **2**, which also contained a complex mixture of sulfated sterol dimers,[13] deterred feeding at 2× natural concentration (3/10 pellets eaten), indicating that the amaroxocane "chemotype" was responsible for activity. It seemed likely that the components of the mixture in this fraction worked additively or synergistically to affect the level of activity displayed by the crude extract, much as complex mixtures of similar polar metabolites in other sponge species were found to be responsible for their chemical defense.[14] With this in mind, we tested *individual* pure samples of amaroxocanes A (**1**) and B (**2**) at higher than natural concentrations (8×). Interestingly, **2** showed significant deterrent activity (3/10 pellets eaten), while **1** elicited little feeding deterrence

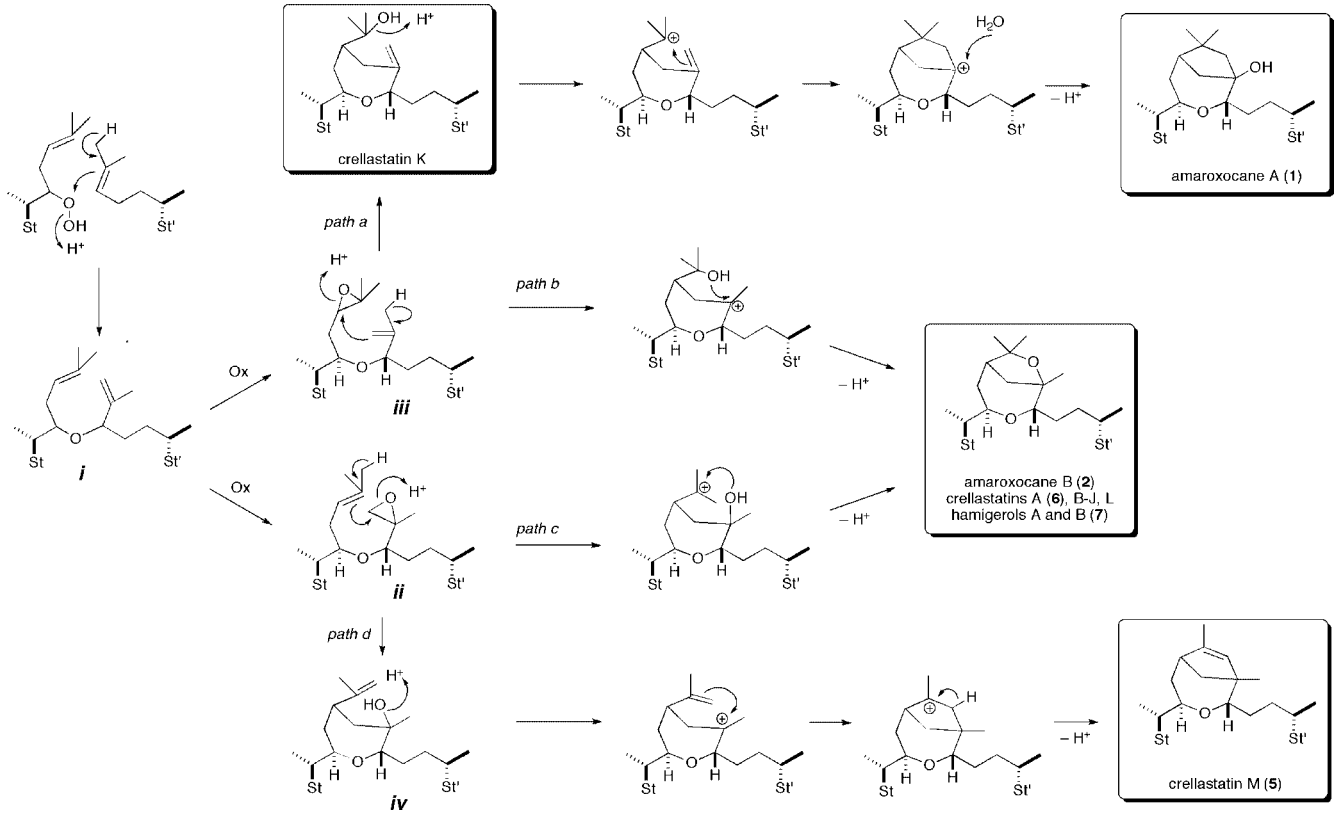


Figure 4. Possible biosynthetic pathways leading to heterobicycles in amaroxocanes, crellastatins, and hamigerols.

Table 1. NMR Data (CD_3OD) for Amaroxocane A (**1**)

no.	δ_C, mult.[a]	δ_H, mult. (*J* in Hz)[b]	DQF-COSY[b]	HMBC[b] (H→C)	no.	δ_C, mult.[a]	δ_H, mult. (*J* in Hz)[b]	DQF-COSY[b]	HMBC[b] (H→C)
1	42.3, CH_2	1.85, dd (14.6, 3.0) 1.97, m	2	5, 10, 19	1′	41.8, CH_2	1.63, m 2.32, dd (14.6, 3.2)	2′	3, 5, 10, 19
2	72.8, CH	4.16, brq (3.0)	1, 3	10	2′	79.4, CH	4.72, brq (2.6)	1′, 3′	10
3	73.4, CH	3.96, dd (6.1, 3.5)	2, 4	4, 28	3′	71.8, CH	3.80, m	2′	
4	34.6, CH	2.50, m	3, 5, 28		4′	28.5, CH_2	1.60, m 2.05, m	3′, 5′	5′
5	48.7, CH	2.02, dd (12.0, 4.1)[d]	4, 6	4, 6, 10, 19, 28	5′	50.5, CH	1.61, m	4′, 6′	
6	75.1, CH	4.87, dt (12.0, 7.6)	5, 7	5, 10	6′	78.7, CH	4.09, td (10.7, 5.2)	5′, 7′	
7	37.2, CH_2	2.33, m 2.91, dd (18.2, 6.8)	6		7′	37.8, CH_2	1.92, m 3.02, dd (13.6, 5.2)	6′	6′, 8′, 9′, 14′
8	126.8, C				8′	125.9, C			
9	138.1, C				9′	51.0, CH	1.87, m	11′	
10	38.8, C				10′	38.2, C			
11	23.0, CH_2	2.10, m 2.20, m	12		11′	20.8, CH_2	1.56, m 1.69, m	9′, 12′	
12	38.2, CH_2	1.36, m 2.00, m	11	9, 13, 14	12′	39.3, CH_2	1.13, m 1.97, m	11′	
13	43.2, C				13′	43.7, C			
14	53.0, CH[c]	1.94, m	15		14′	146.1, C			
15	25.2, CH_2	1.38, m 1.62, m	14, 16		15′	27.0, CH_2	2.26, m 2.35, m	16′	
16	30.8, CH_2	1.29, m 2.13, m	15, 17		16′	28.5, CH_2	1.41, m 1.83, m	15′, 17′	
17	52.4, CH[c]	1.37, m	16, 20		17′	56.1, CH	1.29, m	16′, 20′	
18	11.3, CH_3	0.64, s		12, 13, 14, 17	18′	18.5, CH_3	0.86, s		12, 13′, 14′, 17′
19	23.4, CH_3	1.27, s		1, 5, 9, 10	19′	16.5, CH_3	0.91, s		1′, 5′, 9′, 10′
20	44.1, CH	1.24, m	17, 21	21	20′	34.9, CH[c]	1.68, m	17′, 21′, 22′	
21	13.4, CH_3	0.95, d (6.7)	20	17, 20, 22	21′	19.7, CH_3	1.00, d (6.6)	20′	17′, 20′, 22′
22	72.0, CH	3.63, dd (11.8, 4.6)	23	17, 20, 21, 24′	22′	37.1, CH_2	1.51, m 1.68, m	20′, 23′	
23	35.0, CH_2[c]	1.16, m 1.93, m	22, 24	22, 24, 25	23′	25.4, CH_2	1.39, m 1.79, m	22′, 24′	
24	46.3, CH	1.81, m	23, 26′	25′	24′	87.1, CH	3.26, d (7.9)	23′	22, 25′, 26′, 27′
25	41.5, C				25′	85.1, C			
26	27.3, CH_3	1.03, s		24, 25, 27, 27′	26′	37.8, CH_2	1.93, m 2.05, m	24	24, 25, 24′, 25′
27	35.5, CH_3	1.14, s		24, 25, 26, 27′	27′	57.1, CH_2	1.62, m 1.76, m		24, 25, 26, 27, 24′, 25′ 26′
28	10.4, CH_3[d]	1.28, d (6.6)	4	3, 4, 5					

[a] 100 MHz. [b] 600 MHz. [c] Peaks may be interchanged. [d] Multiplicity assigned from 1D TOCSY.

(8/10 pellets eaten). This surprising difference suggests that structural differences in the heterocycle or the degree of sulfation may be responsible for differential activites, at least in the pure compounds. More extensive assays with defined compositions of mixtures of major and minor components to resolve additive or synergistic effects[15] were precluded by the limited availablity of pure **1** and **2**.

There have been a number of steroidal dimers isolated from marine sources including the cephalostatins[16] and ritterazines[17] (highly cytotoxic pyrazine-containing steroidal alkaloids from the worm *Cephatodisens gilchriti* and the tunicate *Ritterella tokioka*), bistheonellasterone[18] and bisconicasterone[19] (Diels−Alder adducts from the sponge *Theonella swinhoei*), and crellastatins[20] and hamigerols[21] (dioxabicyclic sulfated sterols from the sponges *Crella* sp. and *Hamigera hamigera*). The crellastatins and hamigerols are unique among the dimeric steroids possessing a 3,8-dioxabicyclo-[4.2.1]nonane system formed by oxidative fusion of unsaturated sterol side chains. Amaroxocane B (**2**) contains the same heterocycle found in the crellastatins and hamigerols,[22] and amaroxocane A (**1**) is similar to crellastatin M in that the dimer formed by oxidative cyclization of the side chains contains C_5 carbocycles (based on 3-oxabicyclo[4.2.1]nonane) by variations in their biosynthetic pathways.

Our studies suggest an ecological role for amaroxocane B (**2**) as a chemical defense against fish predators. Amaroxocane A (**1**) may also be involved in the chemical defense in an additive or synergistic capacity (see above), or it may be an inactive byproduct of the biosynthesis of B (**2**). Amaroxocanes are water soluble, comparable to the feeding deterrent saponins found in *Erylus formosus*,[4] and would diffuse readily from the sponge upon tissue injury. Although ritterazines, cephalostatins, and crellastatins exhibit cytotoxic activity against cultured cancer cells, no ecological function for these compounds has been assigned. Antifeedant activity has also been reported from crude extracts of *Hamigera hamigera*; however the compounds responsible for this activity were not identified.[23]

The biosynthesis of oxabicyclic systems present in the crellastatins, hamigerols, and now the amaroxocanes poses interesting questions regarding mechanism. Dimerization reactions in sterol biosynthesis are rare, but have been observed between A rings in the cephalastatin−ritterazine family and others (see above). The most relevant to the present discussion are the side-chain-cyclized crellastatins and hamigerols. The key theme in the biosynthesis of the latter compounds is tightly coupled dimerization of steroidal carbon skeletons through oxidation and electrophilic substitutions and additions at their side chains. Formation of the heterocyclic bridge appears to be initiated by stereoselective oxidation, most likely by hydroperoxidation or hydroxylation mediated by a cytochrome P_{450}-dependent enzyme, as typical for oxygenated sterol side chains. A plausible precursor that unifies biosynthesis for all the compounds in this class appears to be either lanosterol or desmosterol ($\Delta^{24,25}$ olefin). We are in agreement with D'Auria and co-workers, that the C-22−O−C-24′ ether bond is probably formed first to give linked dimer ***i*** by a substitution of the electrophilic oxygen of C-22 hydroperoxide on the C═C double bond of a second molecule of sterol to position the new C═C bond at the C-25/C26 terminus (Figure 4). Stereoselective epoxidation of one or the other C═C double bond in ***i*** would give ***ii*** or ***iii*** followed by electrophilic substitution of the C25−C26 double bond (*path a*) to provide the tertiary alcohol (cf. crellastatin K). Protonation of the tertiary OH group followed by elimination of H_2O could initiate C−C bond

Table 2. NMR Data (CD_3OD) for Amaroxocane B (**2**)

no.	δ_C, mult.[a]	δ_H, mult. (J in Hz)[b]	DQF-COSY[b]	HMBC[b] (H→C)	no.	δ_C, mult.[a]	δ_H, mult. (J in Hz)[b]	DQF-COSY[b]	HMBC[b] (H→C)
1	40.8, CH_2	1.46, m 2.45, dd (14.6, 3.1)	2	2, 3, 5, 10, 19	1′	40.3, CH_2	1.69, m 2.36, dd (14.6, 3.1)	2′	2′, 3′, 5′, 10′, 19′
2	79.1, CH	4.72, brq (3.1)	1, 3	10	2′	79.4, CH	4.74, brq (3.1)	1′, 3′	10′
3	71.0, CH	4.01, dd (11.5, 4.3)	2, 4	2	3′	71.4, CH	3.94, m	2′, 4′	2′
4	28.0, CH_2	1.64, m 2.51, m	3, 5	2, 3, 5, 10, 19	4′	28.0, CH_2	1.61, m 2.16, m	3′, 5′	2′, 3′, 5′, 10′, 19′
5	47.6, CH	1.68, m	4, 6	6, 10, 19	5′	46.9, CH	1.75, td (12.4, 2.2)	4′, 6′	6′, 10′, 19′
6	76.2, CH	4.48, m	5, 7	10	6′	76.2, CH	4.48, m	5′, 7′	10′
7	36.7, CH_2	2.00, m 2.70, m	6	5, 9	7′	36.7, CH_2	2.07, m 2.70, m	6′	5′, 9′
8	nd[c]				8′	nd[c]			
9	137.5, C				9′	137.2, C			
10	39.2, C				10′	39.0, C			
11	23.5, CH_2	2.17, m 2.22, m	12		11′	23.5, CH_2	2.17, m 2.22, m	12′	
12	38.3, CH_2	1.37, m 2.04, m	11		12′	38.3, CH_2	1.37, m 2.04, m	11′	
13	42.6, C				13′	42.4, C			
14	52.6, CH	2.26, m	15		14′	53.4, CH	2.23, m	15′	
15	24.5, CH_2	1.36, m 1.58, m	14, 16		15′	25.0, CH_2	1.40, m 1.67, m	14′, 16′	
16	29.4, CH_2	1.31, m 2.15, m	15, 17		16′	30.6, CH_2	1.40, m 1.90, m	15′, 17′	
17	52.7, CH[c]	1.51, m	16, 20	20	17′	53.9, CH	1.29, m	16′, 20′	
18	11.1, CH_3	0.65, s		12, 13, 14, 17	18′	10.8, CH_3	0.68, s		12′, 13′, 14′, 17′
19	22.1, CH_3	1.24, s		1, 5, 9, 10	19′	22.1, CH_3	1.22, s		1′, 5′, 9′, 10′
20	43.7, CH	1.33, m	17, 21		20′	36.0, CH	1.65, m	17′, 21′, 22′	
21	12.9, CH_3	0.99, d (7.4)	20	17, 20, 22	21′	19.2, CH_3	0.98, d (7.4)	20′	17′, 20′, 22′
22	71.7, CH	3.67, dd (11.4, 4.3)	23	17, 20, 21, 24′	22′	36.9, CH_2	1.51, m 2.03, m	20′, 23′	
23	33.8, CH_2	1.27, m 2.03, m	22, 24		23′	25.4, CH_2	1.42, m 1.51, m	22′, 24′	
24	45.5, CH	2.15, m	23, 26′	23, 27, 25′, 26′	24′	86.2, CH	3.16, d (6.6)	23′	22, 25′, 26′
25	85.5, C				25′	88.0, C			
26	25.4, CH_3	1.32, s		24, 25, 27,	26′	36.2, CH_2	2.05, m 2.26, m	24	23, 24, 25
27	32.5, CH_3	1.23, s		24, 25, 26	27′	27.0, CH_3	1.15, s		24′, 25′, 26′

[a] Assigned from HSQC and HMBC. [b] 600 MHz. [c] Correlations to C8 were not observed.

formation between C-25/C-27′ and closure of the cyclopentane ring leading to **1**. Alternative carbocation-initiated reactions of ***ii*** (*path b*, *path c*) would lead to an oxygen-bridged oxocane (3,8-dioxabicyclo[4.2.1]octane) present in **2**, crellastatins A−J and L, and hamigerols A and B (**7**). The carbocycle in crellastatin M (**5**) may be formed by an alternate cascade (*path d*) initiated by simple *endo-trig* electrophilic substitution of the C-24/C-25 double bond by a tertiary carbocation derived from ***iv***, giving a cyclopentene with a $\Delta^{25,27'}$ olefin.[24]

We have isolated and characterized two new oxocane-bridged sterol dimers, amaroxocane A (**1**) and amaroxocane B (**2**), from *Phorbas amaranthus*. Compound **2** is responsible for the feeding deterrent activity against a common reef predator, the bluehead wrasse. Feeding deterrence was observed at higher concentrations of **2** (8× natural concentration) than that seen with partially purified fractions containing **1**, **2**, and other dimeric sterols, suggesting additive or synergistic activities observed with mixtures. Plausible mechanisms are advanced that rationalize and unify the biosynthesis of amaroxocanes, crellastatins, and hamigerols.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Jasco P2010 polarimeter. ^1H, ^{13}C, and 2D NMR spectra were recorded on a Bruker Avance III DRX-600 (600 MHz, ^1H, 1.7 mm TCI cryoprobe) or Varian Mercury 400 (400 MHz) spectrometers in CD_3OD using residual solvent [δ_H CHD_2OH 3.31 ppm; δ_C 49.00 ppm] as internal standards. UV spectra were measued on a Jasco J600 double-beam UV−vis spectrometer using spectroscopic grade solvents (Fluka) and a 2 mm quartz cell with 50 nm/min scan rate and 1 nm slit. FTIR was measured using a Jasco FT-4100 spectrometer equipped with a Pike MIRacle ZnSe ATR plate. LR ESI mass spectra were obtained on a ThermoElectron MSQ single quad mass spectrometer coupled to an Accela UPLC. HRESI mass spectra were provided by the University of California, Riverside mass spectrometry facility. HPLC was carried out using two Dynamax model SD-200 pumps and a Dynamax UV-1 detector operating at 220 nm. For semipreparative HPLC, a Phenomenex Luna C18 5 μm column (10 × 250 mm) was used, and for preparative HPLC a Waters RCM Prep Nova-Pak HR C18, 6 μ (25 × 100 mm) column was used. HPLC grade solvents were used for HPLC (EMD Chemicals). TLC was performed on silica gel coated 0.25 mm aluminum-backed plates (Whatman AL SIL G/UV) with visualization by vanillin/H_2SO_4/EtOH or aqueous ceric ammonium nitrate.

Animal Material. *Phorbas amaranthus* Duchassaing & Michelotti 1864 was collected in November 2006 from Dry Rocks Reef, Key Largo, Florida (25°7.850′ N, 80°17.521′ W), at a depth of 20−25′, using scuba. Specimens were collected and identified by J.R.P. The sample was stored at −20 °C until lyophilized. Voucher samples are archived at UCSD.

Extraction and Isolation. A freeze-dried sample of *P. amaranthus* (06-04-004b, wet sponge vol 1005 mL, lyophilized wt = 131.2 g) was extracted by standing in water/MeOH (1:1, 1 L, 23 °C, overnight). The extract was decanted and extraction repeated with 1:1 water/MeOH (1 L), then MeOH (1 L). The animal was then blended and extracted once more with MeOH (1 L, 23 °C, overnight). The combined extracts were concentrated under reduced pressure to remove solvent, and the residual animal material was extracted with CH_2Cl_2 (2 × 1 L). The CH_2Cl_2 extract was dried and partitioned between hexane and 1:9 water/MeOH. The aqueous MeOH layer was dried and combined with the previous MeOH extracts and concentrated under reduced pressure to give a crude aqueous MeOH extract (48.6 g). A portion of the aqueous MeOH crude extract (33.9 g) was filtered through C_{18} reversed-phase silica (conditioned with 19:1 water/MeOH) and eluted with water/MeOH mixtures of increasing organic composition (9:1 then 1:9), followed by *i*-PrOH to provide three fractions, *a*, *b*, and *c*, respectively. A portion (1.16 g) of fraction *b* (5.82 g) was subjected to gradient preparative C_{18} reversed-phase HPLC (25 × 200 mm, flow rate 25 mL/min; mobile phase water/

CH_3CN containing 1.5 M $NaClO_4$; gradient 73:27 isocratic 10 min to 23:77 over 30 min; $\lambda = 240$ nm) to give seven fractions. The sixth fraction (337.3 mg, 72.5 mg further purified) was subjected to gradient reversed-phase HPLC (flow rate 2.5 mL/min; mobile phase water/CH_3CN containing 1.5 M $NaClO_4$; gradient 11:9 isocratic 15 min to 1:3 over 30 min; $\lambda = 220$ nm) to give amaroxocane A (**1**, 17.9 mg, 0.46% dry wt) and amaroxocane B (**2**, 6.5 mg, 0.17% dry wt).

Amaroxocane A (1): colorless glass; $[\alpha]^{21}_D$ +51.2 (*c* 1.0, MeOH); IR (ATR, ZnSe) ν_{max} 3453, 2949, 1637, 1457, 1383, 1216, 1061, 959, 914 cm^{-1}; UV (MeOH) λ_{max} 218 nm (ϵ 6700); ^1H NMR, ^{13}C NMR, see Table 1; HRESITOFMS *m/z* 1159.4764 $[M - Na]^-$ calcd for $C_{55}H_{85}Na_2O_{17}S_3$, 1159.4744.

Amaroxocane B (2): colorless glass; $[\alpha]^{21}_D$ +49.8 (*c* 0.92, MeOH); IR (ATR, ZnSe) ν_{max} 3480, 2945, 1652, 1456, 1376, 1220, 1062, 968, 913 cm^{-1}; UV (MeOH) λ_{max} 218 nm (ϵ 6900); ^1H NMR, ^{13}C NMR, see Table 2; HRESITOFMS *m/z* 1247.3965 $[M - Na]^-$ calcd for $C_{54}H_{82}Na_3O_{20}S_4$, 1247.3975.

Fish Feeding Deterrent Assays. Fish feeding assays employing the bluehead wrasse, *Thalassoma bifasciatum*, were carried out in paired tests using artificial food pellets (treated and control) as previously described.[1] Briefly, artificial food pellets were formulated with test fractions or pure compounds at natural concentrations found in the sponge (1×) or higher concentrations (2−16×). Groups of 3−5 fish maintained in separate "cells" in flow-through aquaria were offered, alternately, a control pellet (solvent treatment) followed by treated pellet followed by another control pellet. The assay was repeated for 10 different groups of fish. Assays were conducted at UNCW's Center for Marine Science or onboard the *R/V Seward Johnson* (Bahamas). Results were scored as the number of treated pellets, *N*, eaten, out of an offering of 10. A score of 0/10 corresponded to complete deterrence and 10/10 was the same as the control. Treatments were considered "deterrent" if $N \leq 6$ per Fisher's exact test.

Acknowledgment. We thank J. Cowart, W. Leong, and T.-L. Loh for assistance with sponge collections and assays, T. Hong for preliminary work on *Phorbas amaranthus*, A. Jansma and E. Rogers for assistance with NMR experiments, and R. New (UC Riverside Mass Spectrometry Facility) for HRMS measurements. This work was supported by grants from NIH (CA122256 to T.F.M), the National Undersea Research Program at UNCW (NOAA NA96RU-0260 to J.R.P.), and the NSF Biological Oceanography Program (OCE-0095724, 0550468 to J.R.P). We are grateful to the captain and crew of the *RV Seward Johnson* for logistical support during collecting expeditions and in-field assays.

Supporting Information Available: ^1H and ^{13}C NMR and 2D NMR spectra for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Pawlik, J. R.; Chanas, B.; Toonen, R. J.; Fenical, W. *Mar. Ecol.: Prog. Ser.* **1995**, *127*, 183–194.
(2) Assmann, M.; Lichte, E.; Pawlik, J. R.; Köck, M. *Mar. Ecol.: Prog. Ser.* **2000**, *207*, 255–262.
(3) Wilson, D. M.; Puyana, M.; Fenical, W.; Pawlik, J. R. *J. Chem. Ecol.* **1999**, *25*, 2811–2823.
(4) Kubanek, J.; Pawlik, J. R.; Eve, T. M.; Fenical, W. *Mar. Ecol.: Prog. Ser.* **2000**, *207*, 69–77.
(5) Albrizio, S.; Ciminiello, P.; Fattorusso, E.; Magno, S.; Pawlik, J. R. *J. Nat. Prod.* **1995**, *58*, 647–652.
(6) Masuno, M. N.; Pawlik, J. R.; Molinski, T. F. *J. Nat. Prod.* **2004**, *67*, 731–733.
(7) Tischler, M.; Ayer, S. W.; Anderson, R. J.; Mitchell, J. F.; Clardy, J. *Can. J. Chem.* **1988**, *66*, 1173–1178.
(8) Morinaka, B. I.; Masuno, M. N.; Pawlik, J. R.; Molinski, T. F. *Org. Lett.* **2007**, *9*, 5219–5222.
(9) Amaroxocane A and B each constituted ~25% of this antifeedant fraction based on HPLC analysis.
(10) Selected ^{13}C chemical shifts for the western hemisphere of hamigerol B: δ 79.4, d, C-2; 73.2, d, C-3; 74.6, d, C-6. See ref 21.
(11) Newmark, R. A.; Hill, J. R. *Org. Magn. Reson.* **1980**, *13*, 40–44.
(12) H-22 appeared as a doublet of a doublet, coupled only to the adjacent diastereotopic methylene group (H-23). No cross-peaks were observed between H-20 and H-22 in the COSY or TOCSY spectra.
(13) Mass spectra and NMR of these minor constituents showed they are most likely double-bond isomers with sulfate groups at the same or different positions.
(14) Kubanek, J.; Pawlik, J. R.; Fenical, W. *Nat. Prod. Lett.* **2001**, *15*, 275–285.
(15) Jones, A. C.; Blum, J. E.; Pawlik, J. R. *J. Exp. Mar. Biol.* **2005**, *322*, 67–81.
(16) (a) Pettit, G. R.; Inoue, M.; Kamano, Y.; Herald, D. L.; Arm, C.; Dufresne, C.; Christie, N. D.; Schmidt, J. M.; Doubek, D. L.; Krupa, T. S. *J. Am. Chem. Soc.* **1988**, *110*, 2006−2007. (b) Pettit, G. R.; Inoue, M.; Kamano, Y.; Dufresne, C.; Christie, N. D.; Niven, M. L.; Herald, D. L. *J. Chem. Soc., Chem. Commun.* **1988**, 865–867. (c) Pettit, G. R.; Kamano, Y.; Dufresne, C.; Inoue, M.; Christie, N. D.; Schmidt, J. M.; Doubek, D. L. *Can. J. Chem.* **1989**, *67*, 1509–1513. (d) Pettit, G. R.; Kamano, Y.; Inoue, M.; Dufresne, C.; Boyd, M. R.; Herald, C. L.; Schmidt, J. M.; Doubek, D. L.; Christie, N. D. *J. Org. Chem.* **1992**, *57*, 429–431. (e) Pettit, G. R.; Xu, J. P.; Williams, M. D.; Christie, N. D.; Doubek, D. L.; Schmidt, J. M.; Boyd, M. R. *J. Nat. Prod.* **1994**, *57*, 52–63. (f) Pettit, G. R.; Ichihara, Y.; Xu, J. P.; Boyd, M. R.; Williams, M. D. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1507–1512. (g) Pettit, G. R.; Xu, J. P.; Ichihara, Y.; Williams, M. D. *Can. J. Chem.* **1994**, *72*, 2260–2267. (h) Pettit, G. R.; Xu, J. P.; Schmidt, J. M. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2027–2032. (i) Pettit, G. R.; Tan, R.; Xu, J. P.; Ichihara, Y.; Williams, M. D.; Boyd, M. R. *J. Nat. Prod.* **1998**, *61*, 955–958.
(17) (a) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1994**, *59*, 6164–6166. (b) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1995**, *60*, 608–614. (c) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *Tetrahedron* **1995**, *51*, 6707–6716. (d) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *J. Org. Chem.* **1997**, *62*, 4484–4491.
(18) Kobayashi, M.; Kawazoe, K.; Kator, T.; Kitagawa, I. *Chem. Pharm. Bull.* **1992**, *40*, 1773–1780.
(19) Inouye, Y.; Sugo, Y.; Kusumi, T.; Fusetani, N. *Chem. Lett.* **1994**, 419–420.
(20) (a) D'Auria, M. V.; Giannini, C.; Zampella, A.; Minale, L.; Debitus, C.; Roussakis, C. *J. Org. Chem.* **1998**, *63*, 7382–7388. (b) Zampella, A.; Giannini, C.; D'Auria, M. V.; Debitus, C.; Roussakis, C. *Eur. J. Org. Chem.* **1999**, 949–953. (c) Giannini, C.; Zampella, A.; Debitus, C.; Menou, J. L.; Roussakis, C.; D'Auria, M. V. *Tetrahedron* **1999**, *55*, 13749–13756.
(21) Cheng, J.-F.; Lee, J.-S.; Sun, F.; Jares-Erijman, E. A.; Cross, S.; Rinehart, K. L. *J. Nat. Prod.* **2007**, *70*, 1195–1199.
(22) Crellastatins K and M contain different bridging oxabicycles.
(23) Hassan, W.; Edrada, R.; Ebel, R.; Wray, V.; Proksch, P. *Mar. Drugs* **2004**, *2*, 88–100.
(24) These hypothetical reactions for oxidative dimerization at the side chains account for the heterocycles in *most* of this family of dimeric sulfated sterols; however, alternate mechanisms may be drawn that trigger the cascade with *initial* formation of the carbocyclic rings in **7** and crellastatin M, respectively.

NP800652V