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CHEMICAL AND BIOLOGICAL INVESTIGATION OF THE POLAR CONSTITUENTS OF THE STARFISH *LUIDIA CLATHRATA*, COLLECTED IN THE GULF OF MEXICO

MARIA IORIZZI,

Università degli Studi del Molise, Facoltà di Agraria, Via Tiberio 21/A, 86100 Campobasso, Italy

PATRICK BRYAN, JAMES MCCLINTOCK,

Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294-1170, U.S.A.

LUIGI MINALE,* ELIO PALAGIANO, STEFANO MAURELLI, RAFFAELE RICCIO, and FRANCO ZOLLO

Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli "Federico II," Via D. Montesano 49, 80131 Napoli, Italy

ABSTRACT.—Ten new [**1–10**] and three known [**11–13**] polyhydroxysteroids were isolated, along with four known asterosaponins [**14–17**], from the starfish *Luidia clathrata*, collected from the offshore waters of the northern Gulf of Mexico. The EtOH extracts of this starfish showed feeding-deterrent properties against marine fish, and inhibited the settlement of larvae of barnacles and bryozoans, as well as the growth of several bacteria. The structures of the new compounds were determined by interpretation of their nmr spectral data and by comparison with the spectral data of known compounds. The assignment of the configurations of the side-chain stereogenic centers of compounds **1** and **3–10** were based on the comparison of their nmr data with those of the stereoisomeric model compounds after derivatization with the chiral auxiliary MTPA reagent. Larval settlement assays conducted on ten isolated compounds revealed they are all potent inhibitors of settlement. Two of these isolated compounds inhibited the growth of several bacteria.

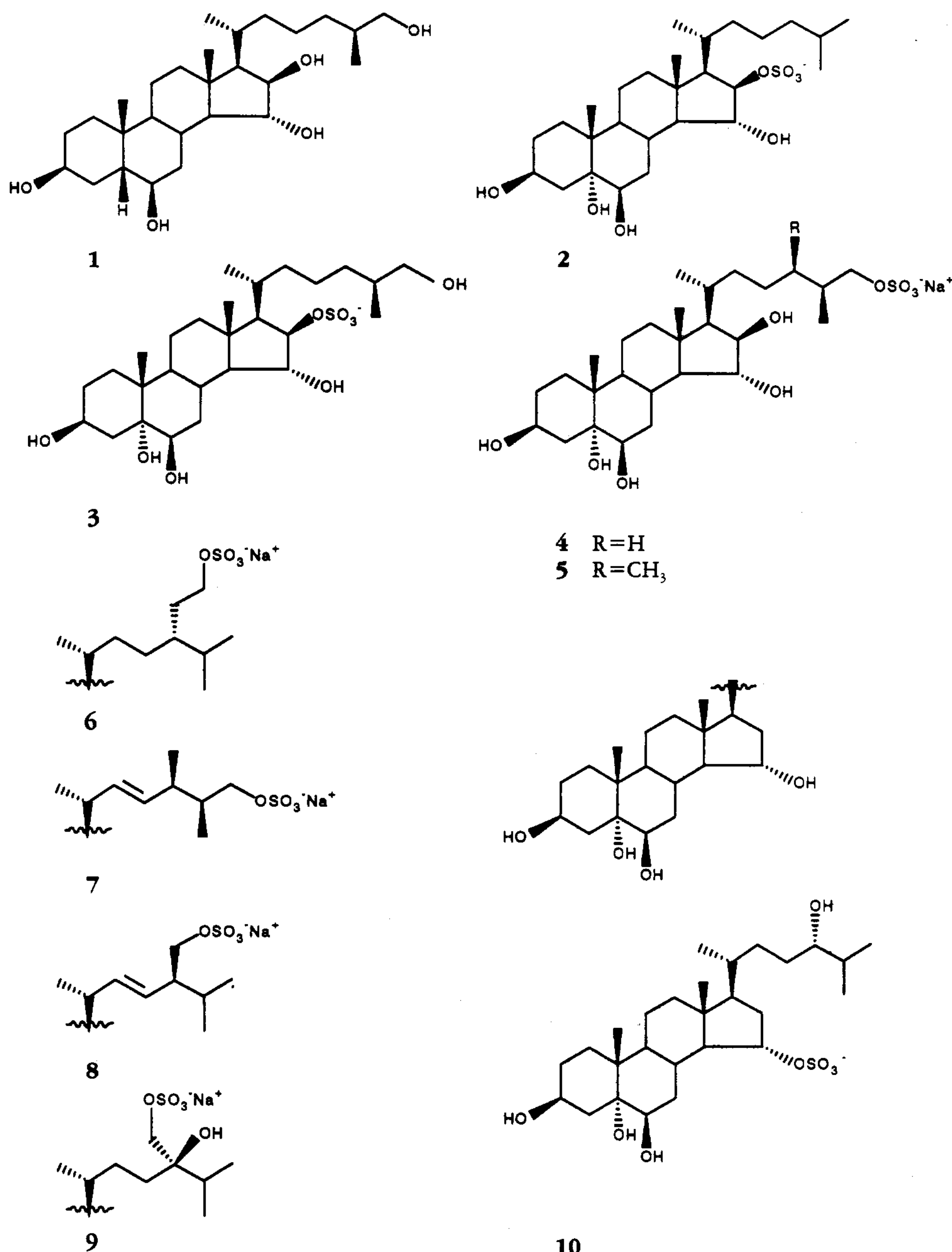
As part of a project on the investigation of the chemical ecology of starfish and other echinoderms in the northern Gulf of Mexico, we have examined the polar extracts of the body wall of the starfish *Luidia clathrata* (Say) family Luidiidae. This organism showed strong antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus*, and had significant feeding-deterrent properties against marine fish, and significantly inhibited the settlement of larvae of both barnacles and bryozoans.

Chemical investigation of the polar metabolites associated with this starfish has led to the isolation of ten new polyhydroxysteroids [**1–10**], nine in sulfated form, along with three known hydroxylated steroids [**11–13**], previously isolated from the starfish *Luidia maculata* [**11** and **12**] (1) and *Hacelia attenuata* [**13**] (2). *L. clathrata* also contained four asterosaponins [**14–17**], the highest mol wt compounds among the steroidal oligoglycosides from starfish, which are composed of a $\Delta^{9(11)}$-3β,6α-dihydroxysteroid bearing a sulfate at C-3, and of an oligosaccharide chain, commonly made up of five or six sugar units, glycosidally linked at C-6 (3). These asterosaponins were identified as thornasteroside A [**14**], the most widely distributed asterosaponin among starfish (3) and first isolated from *Acanthaster planci* (4), ophydianoside F [**15**], isolated from *Ophidiaster ophidianus* (5) and from other species (3), regularoside B [**16**], already found in *Halityle regularis* (6) and in other species (3), and marthasteroside B [**17**], the major saponin from *Marthasterias glacialis* (7), later isolated from *Luidia maculata* (8).

In this paper we describe the isolation and structural elucidation of the new polyhydroxysteroids **1–10** and also report the biological activities associated with the isolated metabolites.

RESULTS AND DISCUSSION

Specimens of *L. clathrata* were collected at 30 m depth from the northern Gulf of Mexico during June, July, and August of 1992. The freshly collected starfish were freeze-



dried and shipped to the Naples laboratory for chemical investigation. The results of our analysis are shown in Table 1.

The known non-sulfated polyhydroxysteroids **11–13** were identified by direct comparison (^{1}H-nmr and fabms) with authentic samples isolated from *L. maculata* [**11**,**12**] (1) and *Hacelia attenuata* [**13**] (2).

The new steroid [**1**] gave a fabms (negative-ion) molecular ion peak at *m/z* 451 $[M-H]^-$. The ^{13}C-nmr spectrum was consistent with the presence of 27 carbon atoms (Table 2), and DEPT measurements revealed the presence of four methyl, nine methylene, and seven methine groups; two quaternary carbons, four -OCH units, and one -OCH_2 functionality. Taken together, these data indicated a pentahydroxycholestane

11 R=OH, R′=H
12 R=H, R′=OH
13 R=H, R′=H

14 Thornasteroside A   Fuc $\xrightarrow{1-2}$ Gal $\xrightarrow{1-4}$ Xyl $\xrightarrow{1-3}$ Qui $\xrightarrow{1-6}$ **A** (Qui $\xrightarrow{1-2}$ Xyl)

15 Ophydianoside F   Fuc $\xrightarrow{1-2}$ Xyl $\xrightarrow{1-4}$ Xyl $\xrightarrow{1-3}$ Qui $\xrightarrow{1-6}$ **A** (Qui $\xrightarrow{1-2}$ Xyl)

16 Regularoside B   Fuc $\xrightarrow{1-2}$ Fuc $\xrightarrow{1-4}$ Xyl $\xrightarrow{1-3}$ Qui $\xrightarrow{1-6}$ **A** (Qui $\xrightarrow{1-2}$ Xyl)

17 Marthasteroside B   Fuc $\xrightarrow{1-2}$ Fuc $\xrightarrow{1-4}$ Qui $\xrightarrow{1-3}$ Glc $\xrightarrow{1-6}$ **B** (Qui $\xrightarrow{1-2}$ Qui)

A   **B**

structure with one of the five methyl groups oxidized to a hydroxymethylene. Examination of the ^{1}H-nmr spectrum indicated the presence of a 15α,16β-dihydroxy moiety (double doublets at δ 3.78, J=11.0 and 2.5 Hz and δ 4.00, J=8 and 2.5 Hz), and a 26-hydroxyl group (two 1H double doublets at δ 3.45, J=10.5 and 5.8 Hz and δ 3.35, J=10.5 and 4.0 Hz). These signals have been previously observed in the spectra of many polyhydroxysteroids isolated from starfish with the 15α,16β,26-hydroxylation pattern (e.g., **11–13**) (3). Also present in the ^{1}H-nmr spectrum of **1** was a sharp 1H signal at δ 4.04 characteristic of a 3α-hydroxy-5α-stanol or a 3β-hydroxy-5β-stanol, and a 1H triplet at δ 3.66 with J=2.5 Hz for a second equatorial proton. The low-field shielding to 26.1 ppm of one of the angular methyl carbons was strongly indicative of the cis-A/B ring fusion (10). In addition, because the relative rigidity of the steroidal skeleton is such that the C-18 shielding is essentially unaffected by a change of the A/B ring fusion (10),

TABLE 1. Compounds Isolated from the Starfish *Luidia clathrata* (from 2.5 kg fresh wt).

Compound	Amount (mg)	Optical rotation [α]D	Hplc[a] eluent MeOH-H_2O	Ref.
Non-sulfated Steroids [**1**, **11**–**13**]				
(25*S*)-5β-Cholestane-3β,6β,15α,16β-tetraol [**1**]	8.0	+28°	75:25	
(25*S*)-5α-Cholestane-3β,5,6β,15α,16β,26-hexaol [**11**]	20.0		75:25	1
(25*S*)-5α-Cholestane-3β,6β,7α,15α,16β,26-hexaol [**12**]	14.3		75:25	1
(25*S*)-5α-Cholestane-3β,6β,15α,16β,26-pentaol [**13**]	4.2		75:25	2
Sulfated Steroids [**2**–**10**]				
5α-Cholestane-3β,5,6β,15α,16β-pentaol 16-sulfate [**2**]	3.2	+14.5°	55:45[b]	
(25*S*)-5α-Cholestane-3β,5,6β,15α,16β,26-hexaol 16-sulfate [**3**]	22.0	+16.0°	1:1	
(25*S*)-5α-Cholestane-3β,5,6β,15α,16β,26-hexaol 26-sulfate [**4**]	0.6		45:55	
(24*R*,25*S*)-24-Methyl-5α-cholesta-3β,5,6β,15α,16β,26-hexaol 26-sulfate [**5**]	2.0	+22.6°	45:55[b]	
(24*R*)-24-Ethyl-5α-cholestane,3β,5,6β,15α,29-pentaol 29-sulfate [**6**]	2.0	+15.0°	55:45[b]	
(22*E*,24*R*,25*S*)-24-Methyl-5α-cholesta-22-en-3β,5,6β,15α,26-pentaol 26-sulfate [**7**]	28.0	+24.3°	55:45	
(22*E*,24*S*)-24-Methyl-5α-cholesta-22-en-3β,5,6β,15α,28-pentaol 28-sulfate [**8**]	5.2	+5.4°	55:45	
(24*R*)-24-Methyl-5α-cholesta-3β,5,6β,15α,24,28-hexaol 28-sulfate [**9**]	2.7	+17.0°	1:1	
(24*S*)-5α-Cholestane-3β,5,6β,15α,24-pentaol 15-sulfate [**10**]	7.0	+24.6°	1:1	
Asterosaponins [**14**–**17**]				
Thornasteroside A [**14**]	7.4		45:55	4
Ophydianoside F [**15**]	5.5		45:55	5
Regularoside B [**16**]	4.7		45:55	6
Marthasteroside B [**17**]	6.3		45:55	7

[a] μBondapak-C_{18} column (30 cm×7.8 mm i.d.)
[b] μBondapak-C_{18} column (30 cm×3.9 mm i.d.)

TABLE 2. [1]H- and ^{13}C-Nmr Data for Steroids **1**, **7**, and **8** in CD_3OD.

Position	Compound								
	1			**7**			**8**		
	δ_C	δ_H[a]	$J_{H\text{-}H}$/Hz	δ_C	δ_H[a]	$J_{H\text{-}H}$/Hz	δ_C	δ_H[a]	$J_{H\text{-}H}$/Hz
1	31.4	1.23 m		31.5	1.63–1.36 m		31.6	1.63–1.36 m	
2	28.1	1.54–1.48 m		33.6	1.79–1.52 m		33.5	1.79–1.52 m	
3	67.0	4.04 br s		68.2	4.04 m		68.3	4.04 m	
4	34.5	1.45–1.68 m		41.4	1.57 m		41.5	1.57 m	
					2.09 t	13.0		2.09 t	13.0
5	43.9	1.88 m		76.2			76.4		
6	74.0	3.66 t	2.5	76.4	3.50 t	2.5	76.6	3.50 br s	
7	34.9	1.98–1.50 m		35.1	1.89 m		35.3	1.87 m	
8	31.5			31.1			31.3		
9	41.4			46.4			46.6		
10	35.9			39.3			39.3		
11	21.6			22.0			22.1		
12	42.0			41.3			41.5		
13	44.7			44.7			44.8		
14	60.0	1.02 m		63.5	1.47 m		63.6	1.47 m	
15	85.0	3.78 dd	11.0, 2.5	74.0	3.89 dt	3.0, 9.0	74.2	3.88 dt	3.0, 9.0
16	83.0	4.00 dd	8.0, 2.5	42.2	1.90–1.63 m		42.3	1.72 m	
17	61.2	1.3 m		54.7	1.47 m		54.8	1.45 m	
18	15.0	0.94 s		14.2	1.20 s		14.0	0.78 s	
19	26.7	1.17 s		17.2	0.78 s		17.4	1.20 s	
20	31.0	1.92 m		39.8	2.04 m		41.1	1.90 m	
21	18.6	0.95 s		21.1	1.04 d	6.8	21.2	1.06 d	6.8
22	37.4			137.4	5.28 t	5.0	140.6	5.36 dd	15.0, 8.6
23	24.9	1.50 m		132.9	5.28 t	5.0	127.0	5.25 dd	15.0, 9.0
24	34.9	1.25–1.10 m		40.9	2.14 m		50.0	2.08 m	
25	37.0	1.60 m		39.2	1.76 m		29.3	1.75 m	
26	68.4	3.45 dd	10.5, 5.8	72.3	3.76 dd	7.5, 10.0	18.8	0.95 d	7.0
		3.35 dd	10.5, 4.0		4.06 dd	6.0, 10.0			
27	17.3	0.94 d	7.0	13.9	0.96 d	7.0	21.2	0.89 d	7.0
28				17.2	1.00 d	7.0	70.7	4.00 br d	5.7

[a]Assignments made by [1]H-[1]H 2D cross-correlation spectroscopy (COSY) at 500 MHz, which delineated the correlation of almost all the protons.

the shift of 15.0 ppm agreed with that expected for a C-18 carbon in a structural environment such as **1** [cf. **11**, $\delta_{C\text{-}18}$ 15.0 ppm (2)]. Thus, the 3β,15α,16β,26-tetrahydroxy-5β-cholestane structure could be established and the fifth hydroxyl group was located at the 6β-position in agreement with the downfield shift of the 19-methyl proton signal at δ 1.17 s [calculated for 5β-cholestane-3β,6β-diol, δ_H 1.15 ppm (11)]. This was confirmed by the 2D COSY spectrum, which established connectivities from C-1 to C-7 in which C-3 and C-6 were methines at δ_H 4.04 and 3.66 ppm, respectively. The 2D COSY spectrum also showed the ^{1}H coupling network from H-14 to H-21 and from H-23 to H-27, thus confirming the location of the remaining hydroxyl groups at C-15, C-16, and C-26. Complete assignments of the carbon signals in the spectrum of **1** were made by using 5β-cholestan-3β-ol (12) and 5α-cholestane-3β,6β,15α,16β,26-pentaol [**13**] (2) as reference compounds, confirming the 5β-cholestane-3β,6β,15α,16β,26-pentaol formulation for the new natural steroid. The stereochemistry at C-25 was determined to be 25*S* like the many 26-hydroxysteroids isolated from starfish using the MTPA method (3,13,14). In the 3,15,26-tri-(+)MTPA[1] ester of **1**, the 26-methylene protons appear in $CDCl_3$ as a 2H broad doublet at δ 4.16, as in the (25*S*)-26-hydroxysteroids; in the (+)-MTPA ester of (25*R*)-26-hydroxysteroids, the 26-

[1]The terms (+)- and (−)-MTPA ester refer to esters obtained using the acid chlorides prepared from (+)-(*R*)- and (−)-(*S*)-MTPA acid, respectively.

methylene protons are observed as well-separated double doublets at δ ca. 4.14 and 4.28 ppm (13).

Steroids with the cis-A/B ring junction are rare among starfish and **1** is the second example with such a structural feature isolated from starfish; the first example was the isomeric (25*R*)-5β-cholestane-3α,6β,15α,16β,26-pentaol, which has been recently isolated from *Tremaster novaecaledoniae* (13).

The negative-ion fabms spectrum of **2** showed a molecular anion species at *m/z* 531 [MSO_3^-]. The ^{13}C-nmr spectrum was consistent with the presence of 27 carbon atoms (Table 4) and DEPT measurements revealed the presence of five methyl groups, nine methylenes, six methines, three quaternary carbons, and four >CH-O units. Taken together, these data indicated a monosulfated pentahydroxycholestane structure. On solvolysis using dioxane/pyridine it afforded a desulfated derivative **2a**, fabms (negative-ion) *m/z* 451 [M−H]$^-$. Analysis of the ^1H-nmr signals associated with the tetracyclic nucleus of **2a** showed close similarities to those reported for 5α-cholestane-3β,5,6β,15α,16β,26-hexaol [**11**], previously isolated from *L. maculata* (1) and then from *Myxoderma platyacanthum* (15) and *Tremaster novaecaledoniae* (13). The downfield shift of the H-16 proton to δ 4.57 dd (J=8.0 and 2.5 Hz) in **2**, when compared with the signal at δ 3.76 in the desulfated derivative **2a**, suggested the location of the sulfate group at C-16. The sulfation at C-16 also caused a downfield shift of the signal for H-15 to δ 4.18 dd (J=11.0 and 2.5 Hz) (δ 3.99 in **2a**). Assignments of the carbon signals in the spectrum of **2** (Table 4) and comparison with those assigned to 5α-cholestane-3β,5α,6β,15α,16β,26-hexaol 15-sulfate isolated from the starfish *Rosaster* sp. (16) confirmed the location of sulfate at C-16 in the new steroid **2**.

The steroid **3**, fabms *m/z* 547 [MSO_3^-] is the 16-sulfated derivative of the known (25*S*)-5α-cholestane-3β,5α,6β,15α,16β,26-hexaol [**11**]. On solvolysis using dioxane/pyridine, **3** afforded a desulfated derivative [**3a**], fabms *m/z* 467 [M−H]$^-$, identical with **11** (1). The location of the sulfate at C-16 in **3** was determined by comparison of the ^1H- and ^{13}C-nmr spectra of **3** (Tables 3 and 4) with those of **11** (1). The stereochemistry at C-25 was determined as 25*S* using the MTPA method as above. In the ^1H-nmr spectrum of the 3,15,26-tri-(+)-MTPA ester of the desulfated derivative **3a**, the 26-methylene protons appeared in $CDCl_3$ as 2H overlapping double doublets at δ 4.16 (J=3.0 and 13.0 Hz). While sulfation at C-15 is commonly found in steroids from starfish (3), steroids **2** and **3** are the first examples isolated from starfish with sulfation at C-16.

The steroid **4**, fabms *m/z* 547 [MSO_3^-], is isomeric with **3**, being the 26-sulfated derivative of 5α-cholestane-3β,5α,6β,15α,16β,26-hexaol. The downfield shift of the CH_2-26 signals to δ_H 3.80 dd (J=10.0 and 7.3 Hz)-3.91 dd (J=10.0 and 6.8 Hz) and δ_C 73.9 ppm in **4**, when compared with the signals at δ_H 3.45 dd-3.34 dd and δ_C 68.6 ppm in its desulfated analogue [**11**] (7), and its fabms, *m/z* 467 [M−H]$^-$, established the location of the sulfate at C-26 in **4**. The 25*S* configuration was determined on the desulfated derivative by using the MTPA method, as in **3**.

The fabms of the steroid **5** exhibited a molecular anion species at *m/z* 561 [MSO_3^-], shifted fourteen mass units relative to **4** (*m/z* 547). Spectral data (^1H-nmr and ^{13}C-nmr) indicated that **5** possessed a tetracyclic nucleus identical with that of the previous steroid [**4**].

Also present in the ^1H-nmr spectrum of **5** were signals for three methyl doublets at δ 0.98, 0.90, and 0.87, and two 1H double doublets at δ 3.85 (J=10.0 and 7.3 Hz) and 3.96 (J=10.0 and 6.8 Hz) assignable to a -CH_2-OSO_3^- grouping, which indicated that **5** is probably the 24-methyl derivative of **4**. Analysis of the ^{13}C-nmr signals assigned to the side-chain of **5** and comparison with those of 24-methyl-26-hydroxy- and 24-hydroxymethyl- model steroids (17,18), ruled out the alternative 24-sulfoxymethyl

TABLE 3. Selected 500 MHz ¹H-Nmr Signals for the Steroids **2–6**, **9**, and **10**, and Their Desulfated Derivatives **2a–6a**, **9a**, and **10a** in CD_3OD. (*J* values are shown in parentheses in Hz).

Proton	Compound													
	2	**2a**	**3**	**3a**	**4**	**4a**	**5**	**5a**	**6**	**6a**	**9**	**9a**	**10**	**10a**
3	4.04 m	4.03 m	4.04 m	4.04 m	4.03 m	4.04 m	4.04 m	4.04 m	4.04 m	4.04 m	4.04 m	4.04 m	4.04 m	4.04 m
6	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.50 t (2.5)	3.51 t (2.5)	3.51 t (2.5)
15	4.18 dd (11.0,2.5)	3.76 dd (11.0,2.5)	4.18 dd (11.0,2.5)	3.76 dd (11.0,2.5)	3.75 dd (10.0,2.5)	3.76 dd (10.0,2.5)	3.75 dd (11.0,2.5)	3.76 dd (11.0,2.5)	3.89 dt (3.0,9.0)	3.89 dt (3.0,9.0)	3.89 dt (3.0,9.0)	3.89 dt (3.0,9.0)	4.50 dt (3.0,9.0)	3.90 dt (3.0,9.0)
16	4.56 dd (8.0,2.5)	3.99 dd (8.0,2.5)	4.57 dd (8.0,2.5)	3.99 dd (8.0,2.5)	4.01 dd (7.5,2.5)	4.00 dd (7.5,2.5)	4.01 dd (8.0,2.5)	4.01 dd (8.0,2.5)						
18	0.95 s	0.94 s	0.95 s	0.94 s	0.93 s	0.94 s	0.94 s	0.94 s	0.77 s	0.77 s	0.77 s	0.77 s	0.80 s	0.77 s
19	1.21 s	1.21 s	1.21 s	1.21 s	1.20 s	1.21 s	1.21 s	1.20 s	1.20 s	1.20 s	1.20 s	1.20 s	1.20 s	1.20 s
21	0.97 d (7.0)	0.98 d (7.0)	0.97 d (7.0)	0.99 d (7.0)	0.99 d (7.0)	0.99 d (7.0)	0.98 d (7.0)	0.98 d (7.0)	0.98 d (7.0)	0.97 d (7.0)	0.98 d[b]	0.98 d (7.0)	0.99 d (7.0)	0.98 d (7.0)
24													3.25 m	3.24 m
26	0.90 d (7.0)	0.92 d (6.8)	3.44 dd (10.8,5.5)	3.44 dd (10.5,5.0)	3.80 dd (10.0,7.3)	3.45 dd (10.5,5.0)	3.85 dd (10.0,7.3)	3.51 dd (10.5,5.2)	0.91 d (7.0)	0.88 d (7.0)	0.97 d[b]	0.94 d (6.8)	0.95 d (6.8)	0.94 d (6.8)
26′			3.35 dd[a]	3.34 dd[a]	3.91 dd (10.0,6.8)	3.34 dd[a]	3.96 dd (10.0,6.8)	3.38 dd (10.5,7.0)						
27	0.90 d (7.0)	0.91 d (6.8)	0.93 d (6.8)	0.95 d (6.8)	0.99 d (7.0)	0.95 d (6.8)	0.90 d (6.8)	0.83 d (6.8)	0.88 d (7.0)	0.90 d (7.0)	0.97 d[b]	0.96 d (6.8)	0.92 d (6.8)	0.93 d (6.8)
28							0.87 d (6.8)	0.84 d (6.8)			3.98 s	3.48 d (11.0)		
28′												3.52 d (11.0)		
29									4.06 br s	3.60 m				

[a]Under solvent signals.
[b]Overlapping signals.

TABLE 4. Assignments of ^{13}C-Nmr Signals (CD_3OD) of Steroids **2–6**, **9**, and **10**.

Position	Compound						
	2	**3**	**4**	**5**	**6**	**9**	**10**
1	31.7	31.6	31.7	31.5	31.3	31.5	31.7
2	33.6	33.5	33.5	33.4	33.5	33.4	33.6
3	68.4	68.3	68.3	68.2	68.1	68.2	68.3
4	41.5	41.4	41.4	41.3	41.4	41.5	41.5
5	76.6	76.5	76.6	76.5	76.4	76.5	76.4
6	76.5	76.4	76.4	76.2	76.4	76.3	76.4
7	35.1	35.1	35.2	34.7	35.3	35.1	36.8
8	31.2	31.1	31.1	30.9	31.3	31.1	31.2
9	46.5	46.4	46.5	46.4	46.5	46.4	46.5
10	39.3	39.3	39.3	39.2	39.3	39.1	39.3
11	22.0	21.9	21.9	21.8	22.1	22.0	22.1
12	41.7	41.6	41.9	41.8	41.7	41.3	41.5
13	44.8	44.7	44.7	44.6	44.9	44.8	44.0
14	60.0	60.0	60.9	60.7	63.5	63.4	61.1
15	82.5	82.4	85.1	85.0	74.3	74.2	81.8
16	89.9	89.9	82.8	82.7	41.9	41.6	38.8
17	59.5	59.5	59.8	59.6	54.9	54.8	55.1
18	15.0	14.9	15.0	14.9	13.8	13.6	13.7
19	17.4	17.4	17.4	17.3	17.4	17.2	17.4
20	30.8	30.7	30.9	30.9	37.0	37.0	34.6
21	18.6	18.6	18.6	18.5	18.9	19.0	19.1
22	36.3	36.3	37.3	34.9	34.8	29.6	33.4
23	25.1	24.8	24.6	32.3	28.7	31.5	31.7
24	40.5	34.5	34.8	35.1	42.2	75.9	78.2
25	29.3	37.0	34.4	38.3	30.3	34.1	34.4
26	23.0	68.5	73.9	72.5	19.2	17.0	17.4
27	23.2	17.4	17.4	14.8	19.9	17.1	19.6
28	—	—	—	12.0	31.8	71.8	—
29	—	—	—	—	68.3	—	—

side-chain for **5**. Particularly diagnostic were the shifts of the C-27 and C-28 methyl carbons at 14.8 and 12.0 ppm; in 24-hydroxymethyl-steroids the C-26 and C-27 methyl carbon signals were observed at δ 19.2 and 20.4 (24*R*) and 19.3 and 19.9 (24*S*) ppm (18). The stereochemistry at C-24 and C-25 was assigned in **5** by comparison of ^{1}H-nmr data of the desulfated derivative **5a**, fabms, *m/z* 481 $[M-H]^-$, with those of stereospecifically synthesized model compounds (17), which identified the stereochemistry in **5a** as threo. This was followed by comparison of the ^{1}H-nmr spectrum of the derived 26-(+)-MTPA ester of **5a** with those of the 26-(+)-MTPA esters of the threo pair of model compounds (i.e., the 24*R*,25*S*- and 24*S*,25*R*-isomers), which identified the absolute configuration in **5a** as 25*S* and allowed the 24*R*,25*S* configuration to be assigned to the natural steroid. Specifically, the chemical shifts of the C-27 and C-28 methyl protons in **5a** (Table 3) are close to those reported for the threo model compounds [δ_H 0.83 d, 0.81 d (24*R*,25*S*) and 0.81 d, 0.81 d (24*S*,25*R*)], but different from those of the erythro model compounds [δ_H 0.93 d, 0.92 d (24*S*,25*R*) and 0.91 d, 0.91 d (24*R*,25*S*)] (17). In the ^{1}H-nmr spectrum of the derived 3,15,26-tri-(+)-MTPA ester, the 26-methylene protons appeared as a doublet at δ 4.25 very close to those of the 26-(+)-MTPA ester of the 24*R*,25*S*-isomer (δ_H 4.23, br d) and distinct from those of the 26-(+)-MTPA ester of the 24*S*,25*R*-isomer (δ_H 4.14 dd–4.34 dd) (17).

Spectral data (^{1}H- and ^{13}C-nmr; Tables 2–4) indicated that steroids **6–9** possessed

the same nuclear 3β,5,6β,15α-hydroxylation pattern, previously encountered in a group of polyhydroxysteroids isolated from *Myxoderma platyacanthum* (15). Steroid **10** also had the same nucleus, but with a sulfate conjugated on the 15-hydroxy group. It remained to determine the structure and the stereochemistry of their side-chains.

The fabms of the steroid **6** gave a molecular anion peak at *m/z* 559 [MSO_3^-]. The presence of a sulfate group was indicated by its polarity and confirmed by solvolysis in dioxane/pyridine, affording the desulfated derivative **6a**, fabms *m/z* 479 $[M-H]^-$. Thus, the mol wt of 480 daltons determined for **6a** corresponded to a saturated C-29 sterol with five hydroxyl groups. The 24-(β-hydroxyethyl) side-chain, already found in several steroid glycosides (3), and as the 29-sulfated derivative in a polyhydroxysteroid from the starfish *Poraster superbus* (19), accounted for the spectral data. The ^{13}C-nmr spectrum of **6**, along with the comparison of those for model 29-hydroxysteroids (20), confirmed the location of the sulfate at C-29 ($\delta_{C\text{-}29}$ 68.3, $\delta_{C\text{-}28}$ 31.8 ppm in **6**, in 3β,29-dihydroxy-5α-stigmast-7-ene, $\delta_{C\text{-}29}$ 62.2, $\delta_{C\text{-}28}$ 34.3 ppm) and also suggested the 24*R* configuration. In the ^{13}C-nmr spectrum of the 24*S* model compound, the 26- and 27-methyl carbons appear as very close signals at 19.2 and 19.3 ppm, while in the 24*R* isomer they are shifted by ca. 1 ppm (18.6–19.7 ppm) (20). Our values (19.2 and 19.9 ppm) compared better with those of the 24*R* isomer.

Steroids **7** and **8** are isomeric; the fabms of both **7** and **8** gave a molecular anion species at *m/z* 543 [MSO_3^-], and after solvolysis the fabms spectra of the desulfated materials **7a** and **8a** both exhibited a pseudomolecular ion peak at *m/z* 463 $[M-H]^-$. The ^{1}H-nmr spectra of both compounds contained olefinic signals, at δ 5.28 m (2H) in **7** and at 5.25 dd (1H, J=15.0 and 9.0 Hz) and 5.36 dd (J=15.0 and 8.6 Hz) in **8**, which could be assigned to the Δ^{22}-protons.

In the ^{1}H-nmr spectrum of **7**, we also observed two 1H double doublets at δ 3.76 dd (J=10.0 and 7.5 Hz) and 4.06 dd (J=10.0 and 6.0 Hz) shifted upfield to δ 3.29 dd and 3.59 dd in the desulfated **7a**; analogous signals were observed in the ^{1}H-nmr spectrum of **8** at δ 4.00 (2H, br d, J=5.7 Hz) shifted upfield to δ 3.51 dd (J=9.5 and 5.5 Hz)-3.57 dd (J=9.5 and 6.7 Hz) in the ^{1}H-nmr spectrum of the desulfated **8a**. Present in the ^{1}H-nmr spectra of both **7** and **8** were also three methyl doublets at δ 1.04, 1.00, and 0.96 in **7** and δ 1.06, 0.95, and 0.89 in **8**. These data corresponded to a Δ^{22},24-methylcholesterol side-chain in which one of the methyl groups has been oxidized to a hydroxymethylene. Analysis of the 2D COSY spectra of **7** and **8** (Table 2) indicated in both cases the sequence H-20 to H-28, and allowed a Δ^{22E},24-methyl-26-sulfoxy and a Δ^{22E},24-methyl-28-sulfoxy side-chain, respectively, to be assigned to **7** and **8**.

The trans stereochemistry of the $\Delta^{22(23)}$ double bond in **7** ($\delta_{H\text{-}22,H\text{-}23}$ 5.28 m) was assigned from the coupling constant of 15.0 Hz between H-22 and H-23 observed in the ^{1}H-nmr spectrum of the desulfated derivative **7a**. The configurations at C-24 and C-25 were assigned by spectral comparison of **7a** with stereoselectively synthesized stereoisomers of Δ^{22},24-methyl-26-hydroxysteroids (17). ^{1}H-Nmr spectra differentiate between the threo and erythro isomers, whereas differentiation between individual stereoisomers of a threo and erythro pair is achieved by ^{1}H-nmr analysis of the MTPA derivatives (17). In the nmr spectra of the threo and erythro isomers, major differences relate to the chemical shifts at the C-26, C-27, and C-28 protons (threo: δ_H 3.28 dd–3.60 dd, 0.90, 0.95 (or 0.97); erythro: δ_H 3.34 dd–3.52 dd, 0.87, 1.02). Our values, δ_H 3.29 dd–3.59 dd (H_2-26), 0.90 d and 0.95 d (H_3-27, -28) are very close to those of the threo isomers. Treatment of the desulfated **7a** with (+)-MTPA chloride and measurement of the ^{1}H-nmr spectrum of the resulting 3,15,26-tri-(+)-MTPA ester showed two close overlapping double doublets at δ 4.21 and 4.23 for the -CH_2O-MTPA protons, in agreement with a 25*S* configuration. The 26-proton resonances are expected to be much

more highly resolved (e.g., δ_H 4.04–4.38) in the case of the 25*R* configuration (17). Thus, we assigned the 24*R*,25*S* configuration to the steroid **7**.

The 24*S* configuration was assigned to **8** after preparation of the 3,15,28-tri-(+)-MTPA ester from **8a** and ^{1}H-nmr measurements. In the ^{1}H-nmr spectrum, the resonances of the C-28 protons (two 1H doublet doublets at δ 4.24, *J*=10.0 and 7.5 Hz, and δ 4.39, *J*=10.0 and 5.0 Hz) were in good agreement with the corresponding resonances observed in the 28-(+)-MTPA ester of the 24*S*-synthetic model, and different from those of the 24*R*-isomer, which were observed as a 2H doublet at δ 4.34 (18).

Steroid **9**, fabms *m/z* 561 [MSO_3^-], had a mol wt 18 mass units larger than **8**. The ^{1}H- and ^{13}C-nmr data indicated that the side-chain of **8** was saturated and also revealed the presence of an oxygenated quaternary carbon, δ_C 75.9 ppm, three *sec*-methyls, two methylenes, two methines, and one -$CH_2OSO_3^-$ grouping located on the quaternary carbon in the side-chain, in addition to the 3β,5,6β,15α-tetrahydroxylated steroid nucleus. The 24-hydroxy-24-(sulfoxymethyl)cholesterol side-chain was thus evident. The location of the sulfate at C-28 was confirmed by solvolysis of **9** affording a desulfated material, **9a**, fabms *m/z* 481 [M−H]$^-$, which showed, in the ^{1}H-nmr spectrum, the H_2-28 signal shifted upfield to δ 3.48 dd-3.52 dd (δ 3.98 s in **9**). The 24*R* configuration was assigned to **9** after the enantioselective synthesis of (24*R*)- and (24*S*)-24-hydroxymethyl-24-hydroxycholesterols and spectral comparison with the desulfated **9a** (Figure 1). The isomeric model steroids were synthesized from 24-methylenecholesterol by Sharpless osmium-catalyzed asymmetric dihydroxylation (21), using AD-mix-α and AD-mix-β reagents, which are based on the phthalazine class of ligands (22). By using the AD-mix-β (dihydroquinidine-1,4-dichlorophthalazine ligands) the 24*R*- [**18**] and the 24*S*-

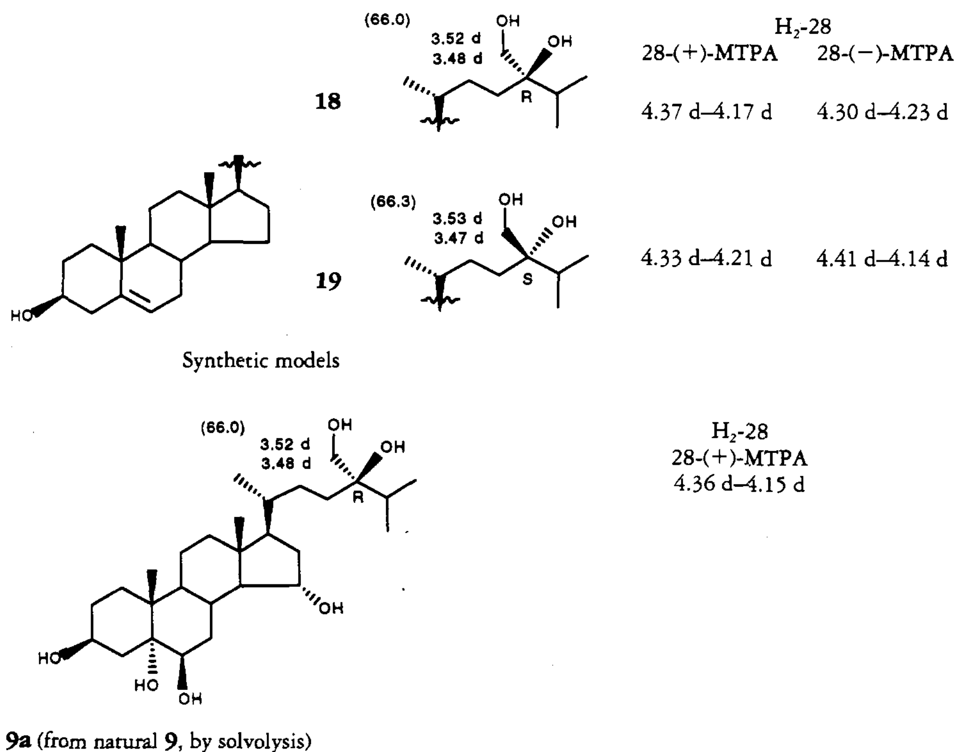


FIGURE 1. ^{1}H- and ^{13}C-nmr data (δ values) for model (24*R*)- and (24*S*)-24-hydroxymethyl-24-hydroxycholesterols and the natural derived **9a**.

isomers [**19**] were obtained in a ratio of *ca.* 7:3, while with AD-mix-α (dihydroquinine-1,4-dichlorophthalazine ligands), the reverse happened. The differences in the ^{1}H- and ^{13}C-nmr spectra of the two synthetic stereoisomers **18** and **19** were very small but still significant for assigning the 24*R* configuration to the natural derived **9a** (Figure 1). Greater confidence could be had by relying on ^{1}H-nmr spectral data of the 28-(+)-MTPA and 28-(−)-MTPA derivatives; indeed, clearly different chemical shifts were shown by the H_2-28 signals in the ^{1}H-nmr spectra of the 28-(+)-MTPA ester derivatives of **18** (24*R*) and **19** (24*S*). These appeared as two well-separated doublets at δ 4.37 (J=11.2 Hz) and 4.17 (J=11.3 Hz) in the ^{1}H-nmr spectrum of the 24*R*-isomer and closer, δ 4.33 (J=11.2 Hz) and 4.21 (J=11.3 Hz) in the ^{1}H-nmr spectrum of its 24*S*-epimer. The reverse was apparent in the ^{1}H-nmr spectra of the 28-(−)-MTPA ester derivatives. In the ^{1}H-nmr spectrum of the 3,15,28-tri-(+)-MTPA ester of the natural product **9a**, the 28-methylene proton signals appeared at δ 4.36 d (J=11.2 Hz) and δ 4.15 d (J=11.0 Hz), in close agreement with the 24*R*-configuration. Additional confirmation could also be obtained from the ^{1}H-nmr chemical shifts of the isopropyl methyls in the spectra of the MTPA esters; these appeared more highly separated in the ^{1}H-nmr spectrum of the 28-(+)-MTPA ester derivative of the 24*R* isomer (in the 500 MHz spectrum they appeared as an apparent triplet at δ 0.90, because of the coincidental overlap of the lower field arm of one doublet, δ 0.89, with the higher field arm of the other, δ 0.91) than in that of the 24*S* isomer (overlapping doublets at δ 0.910 and 0.915). In the ^{1}H-nmr spectrum of the 3,15,28-tri-(+)-MTPA ester of **9a**, they appeared as separated doublets at δ 0.87 and 0.90 ppm. Thus, the steroid **9** can be formulated as (24*R*)-24-methyl-5α-cholestane-3β,5,6β,15α,24,28-hexaol 28-sulfate. The 24-hydroxy, 24-(hydroxymethyl) side-chain has previously been found in indicoside A, a polyhydroxysteroidal glycoside from *Astropecten indicus*, in which the sugar moiety was glycosidally linked at C-28 (23).

The fabms of **10** exhibited a molecular anion peak at *m/z* 531 [MSO_3^-], corresponding to a monosulfated derivative of a cholestane-pentaol. Examination of the ^{1}H- and ^{13}C-nmr spectra of **10** (Tables 3 and 4) indicated the presence of the common 3β,5α,6β-trihydroxy moiety. The ^{1}H-nmr spectrum of **10** also contained a 1H double triplet (J=3.0 and 9.0 Hz) at δ 4.50, shifted upfield to δ 3.90 dt (J=3.0 and 9.0 Hz) in the desulfated **10a**, fabms *m/z* 451 [M−H]$^-$, with the shape for a 15α-hydroxy substituent, and one 1H multiplet at δ 3.25, already observed in the ^{1}H-nmr spectra of the many 24-hydroxysteroids encountered in starfish (3,24). The ^{13}C-nmr shifts at δ 78.2 ppm (C-24), 19.6, and 17.4 (C-26,27) in **10** and comparison with analogous spectra of the synthetic (24*R*)- and (24*S*)-hydroxycholesterols (25) supported the hydroxylation at C-24 in the side-chain. The differences in the ^{1}H- and ^{13}C-nmr spectra of the synthetic (24*R*)- and (24*S*)-hydroxycholestanes are very small for an unambiguous configurational assignment, but the two isomers can be easily differentiated by the ^{1}H-nmr spectra of their MTPA esters, even when only a single stereoisomer is available (3,26). Thus, we have prepared the 3,15,24-tri-(+)-MTPA ester from **10a**, whose ^{1}H-nmr isopropyl methyl proton signals at δ 0.82 d and 0.84 d matched closely those found in the (+)-MTPA ester of the 24*S* model isomer (δ 0.83 and 0.85); in the (+)-MTPA derivative of the 24*R*-model isomer these signals are observed downfield shifted to δ 0.91 (6H, d). Thus, the (24*S*)-5α-cholestane-3β,5,6β,15α,24-pentaol-15-sulfated structure could be assigned for the steroid **10**.

The known compounds thornasteroside A [**14**], ophydianoside F [**15**], regularoside B [**16**], and marthasteroside B [**17**] were identified by fabms and 500 MHz ^{1}H-nmr spectroscopy and by comparison with authentic samples isolated from *Ophidiaster ophidianus* [**14** and **15**] (5), *Halityle regularis* [**16**] (6), and *Marthasterias glacialis* [**17**] (7).

The body-wall tissues of *L. clathrata* were rejected by the pinfish *Lagodon rhomboides*

significantly more frequently than control tissues ($p<0.05$). Pellets containing EtOH body-wall extract (3 mg/ml agar) and krill were rejected significantly more often than control pellets containing only krill ($p<0.05$). Pellets containing body-wall extracts at 0.75 mg/ml agar were consumed with equal frequency to control pellets. The fish antifeedant activity noted for pellets containing natural tissue concentrations of body-wall extract of *L. clathrata* indicates the presence of compounds that deter fish predators. The specific compound(s) responsible for fish antifeedant activity are likely to be polyhydroxysteroids or asterosaponins isolated from *L. clathrata*, but the comparatively large amount of compound required in the fish antifeedant assay precluded this determination.

The EtOH body-wall extract of *L. clathrata* significantly ($p<0.05$) inhibited attachment of competent cyprid larvae of the barnacle *Balanus amphitrite* and the larvae of the bryozoan *Bugula neritina* at concentrations of 3.0, 0.6, and 0.12 mg/ml of sea water (Figure 2). Significant inhibition of bryozoan larval attachment ($p<0.05$) was observed for concentrations as low as 0.0048 mg/ml. This indicates that bryozoan larvae were more sensitive to the body-wall compounds in the EtOH extract than barnacle larvae.

The results of pure compounds tested at three concentrations are presented in Table 5. Compounds **11** and **15** were the most potent, significantly inhibiting barnacle cyprid attachment at a concentration of 0.008 mg/ml ($p<0.05$). All compounds tested were significantly effective ($p<0.05$) inhibiting most attachment of barnacle larvae at 0.2 mg/ml, while compounds **1** and **14–17** completely inhibited all larvae from attaching.

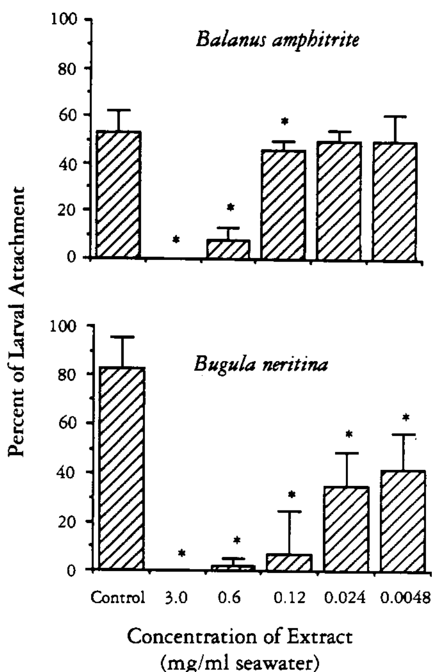


FIGURE 2. Antifouling activity of EtOH body-wall extracts of the starfish *Luidia clathrata* against larvae of the barnacle *Balanus amphitrite* and the bryozoan *Bugula neritina*. Asterisks indicate values that are significantly different from control values ($p<0.05$).

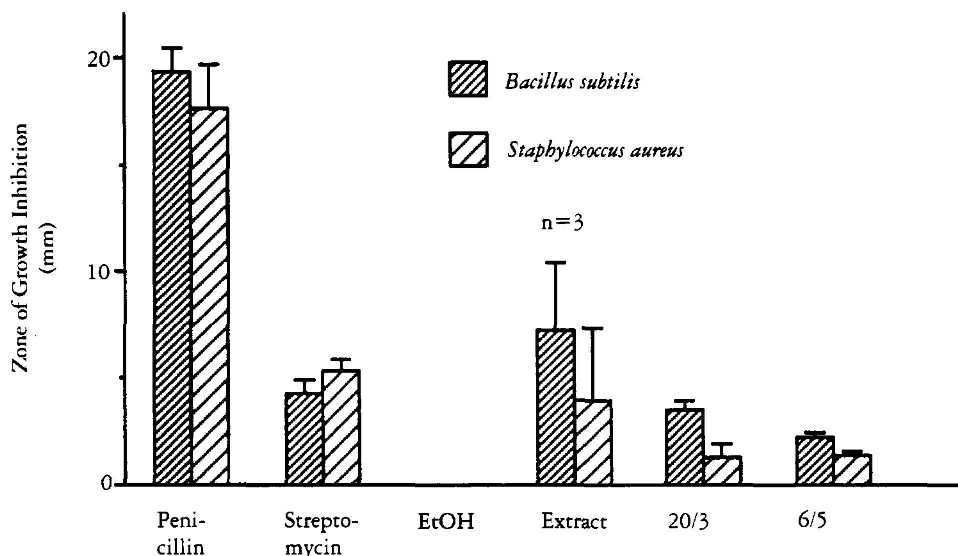


FIGURE 3. Antimicrobial activity of crude body-wall extract and two isolated body-wall compounds of the starfish *Luidia clathrata*.

These larvae appeared to be dead at the end of the incubation period, suggesting that at the highest concentration tested a toxic effect is most likely responsible for inhibition of barnacle attachment. This is the first demonstration that bioactive echinoderm compounds may play a role in the prevention of fouling. There are very few reports of fouled echinoderms (27), indicating echinoderms have evolved an effective mechanism of protection from fouling. The compounds demonstrated in this study to be effective at inhibiting barnacle attachment are likely to play a major role as antifoulants in *L. clathrata*.

Of the 19 microbial species utilized, the EtOH body-wall extract of *L. clathrata* inhibited the growth of two species of Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, at a concentration of 0.75 mg per disk (Figure 3). Of the 10 compounds isolated from *L. clathrata* which were tested, two [**10** and **12**] inhibited the growth of *B. subtilis* and *S. aureus*. At a concentration of 50 μg per disk, compound **10** inhibited the growth of both bacterial species, with 1- and 3-mm zones of growth inhibition for *S. aureus* and *B. subtilis*, respectively. At a concentration of 50 μg per disk, compound **12** caused 1- and 2-mm zones of inhibition for *S. aureus* and *B. subtilis*, respectively.

Although all ten pure compounds tested inhibited the settlement of barnacle larvae, only two compounds inhibited growth of bacteria. This indicates that while some polyhydroxysteroids and asterosaponins may display a broad spectrum of bioactivity, others may have specific functional roles.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra, Bruker AMX-500 instrument (^1H at 500 MHz, ^{13}C at 125 MHz) δ (ppm), *J* in Hz, spectra referenced to the CHD_2OD signal at 3.34 ppm and to the central carbon CD_3OD signal at 49.0 ppm; mass spectra, VG ZAB mass spectrometer equipped with fab source [in glycerol-thioglycerol (3:1) matrix; Xe atoms of 2–6 kV]; optical rotations, Perkin-Elmer model 241 polarimeter; reversed-phase hplc, C_{18} μ-Bondapak column (30 cm×7.8 mm i.d.; flow rate 5 ml/min^{-1}), and C_{18} μ-Bondapak column (30 cm×3.9 mm i.d.; flow rate 2 ml/min^{-1}), Waters model 6000A pump equipped with U6K injector and a differential refractometer, model 401; dccc, DCC-A apparatus manufactured by Tokyo Rikakikai Co., equipped with 250 tubes, and Buchi apparatus equipped with 300 tubes.

ANIMAL MATERIAL.—The animals (*L. clathrata*) were collected in the northern Gulf of Mexico and were identified by Dr. T. Hopkins of the University of Alabama; a voucher specimen is preserved at the Department of Biology, University of Alabama.

EXTRACTION AND ISOLATION.—The lyophilized animals (1.2 kg) were chopped and soaked in H_2O (three times, 2 liters for 4 h each): the aqueous extracts were decanted and passed through a column of Amberlite XAD-2 (1 kg). This column was washed with distilled H_2O (1 liter) and eluted with MeOH (7 liters) to give, after removal of the solvent, a glassy material (4.2 g). The residual solid mass, after H_2O extraction, was extracted again with Me_2CO (2 liters for 24 h). The Me_2CO extracts were evaporated *in vacuo* and partitioned between H_2O and Et_2O. The aqueous residue was then extracted with *n*-BuOH. Evaporation of the *n*-BuOH extracts afforded 2.2 g of a residue, which was combined with the above MeOH eluate from an Amberlite XAD-2 column and chromatographed on a column of Sephadex LH-60 (4×100 cm) with MeOH-H_2O (2:1) as eluent. Fractions (5 ml) were collected and analyzed by tlc on SiO_2 with *n*-BuOH-HOAc-H_2O (12:3:5) and $CHCl_3$-MeOH-H_2O (80:18:2).

Fractions 47–71 contained the crude asterosaponins (1.0 g), the successive fractions 72–80 (810 mg) contained a complex mixture of sulfated polyhydroxysteroids, and the last fractions 81–113 (1.2 g) contained a mixture of polyhydroxysteroids.

Fractionation of asterosaponins was continued by droplet counter-current chromatography (dccc) with *n*-BuOH-Me_2CO-H_2O (3:1:5) (descending mode; the upper phase was used as stationary phase: flow 16 ml/h; 6 ml fractions were collected and analyzed by tlc on SiO_2 with *n*-BuOH-HOAc-H_2O, 12:3:5) to give three main fractions. Fractions 60–71 (85 mg) contained thornasteroside A [**14**] and ophydianoside F [**15**]; fractions 72–81 (91 mg) contained regularoside B [**16**] and marthasteroside B [**17**]. All fractions were submitted to hplc on a C_{18} μ-Bondapak column (30 cm×7.8 mm i.d.) with MeOH-H_2O (45:55) (flow rate 5 ml/min), to give pure asterosaponins.

Fractions 81–113 from LH-60 (1.2 g) were submitted to dccc using $CHCl_3$-MeOH-H_2O (7:13:8) in the ascending mode (the lower phase was the stationary phase). Fractions (3 ml) were collected and monitored by tlc on SiO_2 with $CHCl_3$-MeOH-H_2O (80:18:2) to give four main fractions. Fractions 11–22 (250 mg) were mixtures of sulfated compounds, fractions 23–30 (430 mg) contained the polyhydroxysteroid **11** and the fractions 31–34 (28.5 mg) and 35–42 (25.1 mg) were constituted by polyhydroxysteroids **1**, **12**, and **13**. Each of the above fractions was purified by hplc with MeOH-H_2O (75:25) on a C_{18} μ-Bondapak column (30 cm×7.8 mm i.d.) to give pure compounds.

Fractions 72–80 (810 mg) eluted from the Sephadex LH-60 column contained more polar compounds. This material was combined with the first fractions (11–22; 250 mg) derived from the above dccc separation and were then submitted to dccc using *n*-BuOH-Me_2CO-H_2O (3:1:5) in the ascending mode (the lower phase was the stationary phase; flow rate 8 ml/h; 4 ml fractions were collected and monitored by tlc). The purification of each fraction was achieved by hplc on a C_{18} μ-Bondapak column with MeOH-H_2O eluent composition ranging from 45:55 to 55:45. The results are summarized in Table 1.

SOLVOLYSIS OF POLYHYDROXYSTEROIDS.—Compounds **2–10** (1.0 to 2.0 mg each) were submitted to solvolysis in pyridine (0.2 ml) and dioxane (0.2 ml) and heated at 130° (145° for **9**) for 2 h in stoppered reaction vials. After the solutions were cooled, H_2O (1 ml) was added to each mixture and the solutions were extracted with *n*-BuOH (3×1 ml). The combined extracts were evaporated to dryness under reduced pressure. Each residue was purified by hplc [C_{18} μ-Bondapak column, MeOH-H_2O (7:3) and (75:25) for **9**] to give the desulfated derivatives **2a–10a**. ^{1}H-nmr data (CD_3OD) of **2a–6a** and **9a**, **10a** are presented in Table 3.

Compound **7a**.—^{1}H nmr (CD_3OD) δ_H 0.78 (3H, s, Me-18), 0.90 (3H, d, J=7 Hz, Me-27), 0.95 (3H, d, J=7 Hz, Me-28), 1.04 (3H, d, J=7 Hz, Me-21), 1.20 (3H, s, Me-19), 3.29 (1H, dd, J=10.5 and 7 Hz, H-26), 3.59 (1H, dd, J=10.5 and 6.2 Hz, H'-26), 3.50 (1H, dd, J=15 and 7.5 Hz, H-23), 5.28 (1H, dd, J=15 and 7.2 Hz, H-22).

Compound **8a**.—^{1}H nmr (CD_3OD) δ_H 0.79 (3H, s, Me-18), 0.87, 0.93 (3H each, d, J=7 Hz, Me-26 and Me-27), 1.06 (3H, d, J=7.0 Hz, Me-21), 1.20 (3H, s, Me-19), 3.50 (1H, t, J=2.5 Hz, H-6), 3.51 (1H, dd, J=9.5 and 5.5 Hz, H-28), 3.57 (1H, dd, J=9.5 and 6.7 Hz, H'-28), 5.21 (1H, dd, J=14.0 and 8.6 Hz, H-23), 5.34 (1H, dd, J=14.0 and 8.6 Hz, H-23).

Compound **9a**.—^{13}C nmr (CD_3OD) δ_C C-1 (31.7), C-2 (33.6), C-3 (68.3), C-4 (41.7), C-5 (76.6), C-6 (76.4), C-7 (35.3), C-8 (31.3), C-9 (46.6), C-10 (39.3), C-11 (22.1), C-12 (41.1), C-13 (44.9), C-14 (63.6), C-15 (74.3), C-16 (41.8), C-18 (13.8), C-19 (17.4); the remaining side-chain signals are presented in Table 6.

SYNTHESIS OF MODEL COMPOUNDS.—*(24R) and (24S)-24-Hydroxymethyl-24-hydroxycholesterols* **18** *and* **19**.—A 5-ml round-bottomed flask equipped with magnetic stirrer was charged with 0.6 ml of *tert*-butyl alcohol, 0.6 ml of H_2O, and 168 mg of AD-mix-α or AD-mix-β (Aldrich Chemical Company). Stirring at

room temperature produced two clear phases; the lower aqueous phase appeared bright yellow. A 0.050-mmol (20 mg) quantity of olefin, 24-methylenecholesterol, was added at once, and the heterogeneous slurry was stirred vigorously at 25° for 72 h (progress monitored by tlc). Then, sodium sulfite (90 mg) was added and the mixture was stirred for 60 min. Two ml of EtOAc were added to the reaction mixture, and after separation of the layers, the aqueous phase was further extracted with the organic solvent (3×2 ml). The combined organic extracts were dried over anhydrous $MgSO_4$ and concentrated to give the diol and the ligand. Each crude product was purified by Si gel cc (a Pasteur pipette filled with a slurry of Si gel) using as eluent a mixture of hexane/EtOAc of increasing polarity (from 100% hexane to 1:1). The ligand does not move in this solvent system and afforded the 1,2-diols **18** and **19**. By using AD-mix β, **18** was the major component (7:3), and by using AD-mix α, **19** was the major component (7:3), as assessed by ^{1}H-nmr studies.

(24R)-24-Hydroxymethyl-24-hydroxycholesterol [**18**].—*m/z* 432 $[M]^+$; ^{1}H nmr (CD_3OD) δ_H 0.76 (3H, s, Me-18), 1.00 (3H, d, J=6.8 Hz, Me-21), 1.05 (3H, s, Me-19), 3.42 (1H, m, H-3α), 5.37 (1H, br d, H-6); remaining side-chain signals presented in Table 6; ^{13}C nmr (CD_3OD) δ_C C-1 (38.5), C-2 (32.3), C-3 (72.5), C-4 (43.0), C-5 (142.3), C-6 (122.5), C-7 (33.0), C-8 (33.3), C-9 (51.8), C-10 (37.7), C-11 (22.2), C-12 (41.2), C-13 (43.5), C-14 (58.2), C-15 (25.3), C-16 (29.3), C-18 (12.3), C-19 (19.8); the remaining side-chain signals are presented in Table 6.

(24S)-24-Hydroxymethyl-24-hydroxycholesterol [**19**].—*m/z* 432 $[M]^+$; ^{1}H nmr (CD_3OD) δ_H side-chain signals are presented in Table 6; the remaining signals were identical to those reported for **18**; ^{13}C-nmr (CD_3OD) side-chain signals are presented in Table 6, and the remaining signals were identical to those reported for **18**.

MTPA ESTERS OF MODEL COMPOUNDS.—The synthetic models **18** and **19** were treated with (+)- and (−)-α-methoxy-α-(trifluoromethyl)phenylacetyl (MTPA) chloride (3–5 μl) in dry pyridine (30 μl) for 1 h at room temperature. After removal of the solvent, the products were analyzed by ^{1}H-nmr spectroscopy (see Table 6).

3,28-Di-(+)-MTPA ester of model **18**.—*m/z* 864 $[M]^+$; ^{1}H nmr ($CDCl_3$) δ_H 0.65 (3H, s, Me-18), 0.86 (3H, d, J=7 Hz, Me-21), 0.89, 0.91 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 1.00 (3H, s, Me-19), 4.88 (1H, m, H-3α), 5.40 (1H, br d, J=4.5 Hz, H-6); the remaining signals are shown in Figure 1.

3,28-Di-(−)-MTPA ester of model **18**.—*m/z* 864 $[M]^+$; ^{1}H nmr ($CDCl_3$) δ_H 0.64 (3H, s, Me-18), 0.85 (3H, d, J=7 Hz, Me-21), 0.910, 0.915 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 0.99 (3H, s, Me-19), 4.88 (1H, m, H-3α), 5.42 (1H, br d, J=4.5 Hz, H-6); the remaining signals are shown in Figure 1.

3,28-Di-(+)-MTPA ester of model **19**.—*m/z* 864 $[M]^+$; ^{1}H nmr ($CDCl_3$) δ_H 0.65 (3H, s, Me-18), 0.85 (3H, d, J=7 Hz, Me-21), 0.910, 0.915 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 1.00 (3H, s, Me-19), 4.89 (1H, m, H-3α), 5.42 (1H, br d, J=4.5 Hz, H-6); the remaining signals are shown in Figure 1.

3,28-Di-(−)-MTPA ester of model **19**.—*m/z* 864 $[M]^+$; ^{1}H nmr ($CDCl_3$) δ_H 0.66 (3H, s, Me-18), 0.86 (3H, d, J=7 Hz, Me-21), 0.90, 0.91 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 1.00 (3H, s, Me-19), 4.89 (1H, m, H-3α), 5.42 (1H, br d, J=4.5 Hz, H-6); the remaining signals are shown in Figure 1.

MTPA ESTERS OF COMPOUND **1** AND OF DESULFATED DERIVATIVES **3a–5a**, **7a–10a**.—Each compound (ca. 1 mg) was treated with *R*-(+)-MTPA chloride (6–8 μl) in dry pyridine (30 μl) for 1 h at room temperature. After removal of the solvent each product was analyzed by ^{1}H-nmr spectroscopy.

3,15,26-Tri-(+)-MTPA ester of **1**.—^{1}H nmr ($CDCl_3$) δ_H 0.64 (1H, s, Me-18), 0.68 (1H, s, Me-19), 0.86 (3H, d, J=6.8 Hz, Me-21), 0.91 (3H, d, J=6.8 Hz, Me-27), 3.76 (1H, br t, H-6), 3.98 (1H, dd, J=8.0 and 2.5 Hz, H-16), 4.16 (2H, br d, H_2-26), 4.22 (1H, dd, J=11.0 and 2.5 Hz, H-15), 5.30 (1H, br s, H-3).

3,15,26-Tri-(+)-MTPA ester of **3a**.—^{1}H nmr ($CDCl_3$) δ_H 0.90 (3H, d, J=6.8 Hz, Me-27), 0.92 (3H, d, J=6.8 Hz, Me-21), 0.96 (3H, s, Me-18), 1.18 (3H, s, Me-19), 4.06 (1H, dd, J=8.0 and 2.5 Hz, H-16), 4.16 (2H, two overlapping double doublets, H_2-26), 4.55 (1H, dd, J=11.0 and 2.5 Hz, H-15), 5.41 (1H, m, H-3α).

3,15,26-Tri-(+)-MTPA ester of **4a**.—^{1}H nmr (CD_3OD) δ_H 0.95 (3H, d, J=7.0 Hz, Me-27), 0.97 (3H, d, J=7.0 Hz, Me-21), 1.01 (3H, s, Me-18), 1.16 (3H, s, Me-19), 4.10 (1H, dd, J=8.0 and 2.5 Hz, H-16), 4.19, 4.23 (1H each, dd, J=10.5, 5.2 and 10.5, 7.0 Hz, H_2-26), 5.00 (1H, dd, H-15), 5.44 (1H, m, H-3α).

3,15,26-Tri-(+)-MTPA ester of **5a**.—^{1}H nmr (CD_3OD) δ_H 0.84, 0.88 (3H each, d, J=7.0 Hz, Me-27 and Me-28), 0.95 (3H, d, J=7.0 Hz, Me-21), 1.01 (3H, s, Me-18), 1.16 (3H, s, Me-19), 4.10 (1H, dd, J=8.0 and 2.5 Hz, H-16), 4.25 (2H, d, J=6.0 Hz, H_2-26), 5.02 (1H, dd, J=11.0 and 2.5 Hz, H-15), 5.50 (1H, m, H-3α).

3,15,26-Tri-(+)-MTPA ester of **7a**.—^{1}H nmr (CD_3OD) δ_H 0.83 (3H, s, Me-18), 0.90, 0.93 (3H each,

TABLE 5. Percent of Barnacle Larvae (*Balanus amphitrite*) Exhibiting Substrate Attachment Following Exposure to Pure Compounds Isolated from the Starfish *Luidia clathrata*.

Compound	Percent Larval Attachment Concentration (mg/ml)			
	0.2	0.04	0.008	Control
1	0	27.9	74.1	76.5
3	21.4	57.6	68.7	65.9
7	12.1	25.5	73.4	68.7
10	45	47	67.2	66.7
11	0.93	5.5	58.7	68.7
12	1.4	5.3	62.1	76.6
14	0	52.1	71	66.7
15	0	0	54.4	76.6
16	0	34.8	65.1	65.9
17	0	68.7	68.3	76.5

d, J=6.8 Hz, Me-27 and Me-28), 1.02 (3H, d, J=6.8 Hz, Me-21), 1.16 (3H, s, Me-19), 4.21, 4.23 (1H each, dd, overlapped H_2-26), 4.85 (1H, dt, H-15), 5.12 (1H, dd, J=14.0 and 8.5 Hz, H-23), 5.17 (1H, dd, J=14.0 and 8.0 Hz, H-22), 5.45 (1H, m, H-3α).

3,15,28-Tri-(+)-MTPA ester of **8a**.—^{1}H nmr (CD_3OD) δ_H 0.80 (3H, s, Me-18), 0.83, 0.89 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 1.04 (3H, d, J=7.0 Hz, Me-21), 1.16 (3H, s, Me-19), 4.24 (1H, dd, J=7.5 and 10.0 Hz, H-28), 4.39 (1H, dd, J=5.0 and 10.0 Hz, H-28′), 4.89 (1H, dt, H-15), 5.26 (1H, dd, J=8.2 and 15.0 Hz, H-23), 5.39 (1H, dd, J=15.0 and 8.0 Hz, H-22), 5.45 (1H, m, H-3α).

3,15,28-Tri-(+)-MTPA ester of **9a**.—^{1}H nmr ($CDCl_3$) δ_H 0.70 (3H, s, Me-18), 0.83 (3H, d, J=7.0 Hz, Me-21), 0.87, 0.90 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 1.09 (3H, s, Me-19), 4.83 (1H, dt, J=3.0 and 9.0 Hz, H-15), 5.41 (1H, m, H-3α); other signals are shown in Figure 1.

3,15,24-Tri-(+)-MTPA ester of **10a**.—^{1}H nmr ($CDCl_3$) δ_H 0.72 (3H, s, Me-18), 0.82, 0.84 (3H each, d, J=6.8 Hz, Me-26 and Me-27), 0.92 (3H, d, J=6.8 Hz, Me-21), 1.09 (3H, s, Me-19), 4.84 (1H, dt, J=3.9 Hz, H-15), 4.92 (1H, m, H-24), 5.41 (1H, m, H-3α).

FISH FEEDING DETERRENCE ASSAYS.—Cubes of fresh *Luidia clathrata* body-wall tissue (3×3×3 mm) were proffered to groups of the pinfish *Lagodon rhomboides*. Controls consisted of presenting *L. rhomboides* similarly sized pieces of fish tissue. EtOH extracts of body-wall tissues of *L. clathrata* were embedded in agar pellets (4% Sigma gum agar dissolved in deionized H_2O containing 1.5% dry krill) at a concentration approximating natural body-wall tissue level (3.0 mg/ml agar) and at a four-fold lower concentration (0.75 mg/ml agar). Carmine particles (0.5% of pellet wet wt) were added to color the pellets and assist in feeding observations. Pellets were cylindrical, measuring 1 mm in diameter and 5 mm in height. Control pellets were composed of agar containing a 1.5% dry krill powder.

Each laboratory feeding trial consisted of introducing either a piece of fresh tissue or an agar pellet into one of six 15-gallon aquaria and observing the feeding responses of adult pinfish over a 1-min period. Five individual *Lagodon rhomboides* were held in each aquarium, as they tend to feed more effectively when grouped. Feeding behaviors were recorded by scoring whether tissues or pellets were ultimately accepted (ingested) or rejected (mouthed and spit out). Twenty control and experimental tissues or pellets were each presented in a random sequence to groups of fish. Fish were conditioned to feeding on control pellets for several weeks prior to experimentation. A Fisher's Exact-test was used to determine if there was a statistically significant difference between the consumption of experimental and control pellets.

BARNACLE SETTLEMENT INHIBITION ASSAYS.—Larvae of the barnacle *Balanus amphitrite* were cultured at the Duke University Marine Laboratory in Beaufort, North Carolina. The nauplii were raised to a competent three-day-old cyprid stage. Approximately 35 cyprid larvae in a volume of 100–450 μl sea water were each added to a Falcon 50×9-mm covered polystyrene petri dish containing either 5 ml of 100 kilodalton-filtered sea water (control) or filtered sea water containing body-wall extract of *L. clathrata*. The crude EtOH body-wall extract was evaluated at five concentrations, beginning at a concentration occurring naturally in the body wall, and then at four half-log dilutions of that concentration (3.0, 0.6, 0.12, 0.024, 0.0048 mg/ml). Pure compounds were tested at three concentrations (0.2, 0.04, 0.008 mg/ml). Three replicate assays of each concentration were conducted along with similar numbers of controls. The assay was initiated upon the addition of cyprid larvae to the final dish. The dishes were incubated at 28° under

TABLE 6. Selected Nmr Data (CD_3OD) for Side-Chain Signals in Synthetic 24-Hydroxymethyl-24-hydroxycholesterols [**18**,**19**] and Natural **9a**.

Compound	^{1}H nmr			^{13}C nmr									
	H_2-28	H_3-26	H_3-27	C-17	C-20	C-21	C-22	C-23	C-24	C-25	C-26	C-27	C-28
Synthetic models													
18 (24*R*)	3.48 d (11.4) 3.52 d (11.2)	0.92 d (7.0)	0.94 d (7.0)	57.5	37.8	19.2	30.1	31.6	77.1	33.8	17.27	17.37	66.0
19 (24*S*)	3.47 d (11.2) 3.53 d (11.0)	0.94 d (7.0)	0.96 d (7.0)	57.5	37.6	19.3	30.0	31.7	77.1	33.6	17.34	17.41	66.3
Natural compound													
9a	3.48 d (11.0) 3.52 d (11.0)	0.94 d (7.0)	0.96 d (7.0)	55.0	37.3	19.1	30.0	31.4	77.1	33.8	17.30	17.38	66.0

a 15 l: 9 d photoperiod for 24 h. Following incubation, several drops of a 10% formalin solution were added to each dish to kill larvae, stopping the bioassay. The dishes were viewed under a dissecting microscope and the number of attached and unattached cyprid larvae were counted. Larvae that were permanently attached and/or metamorphosed on the polystyrene dish were considered settled. The differences in the percent of settlement of cyprid larvae in experimental and control treatments were tested for significance using a G-test for independence (28).

BRYOZOAN SETTLEMENT INHIBITION ASSAYS.—Adult bryozoans, *Bugula neritina*, were collected near the Duke University Marine Laboratory in November 1992. Colonies were held in the laboratory with aeration in the dark for approximately 24 h. Adult bryozoans were shocked with light to trigger larval release. The larvae were concentrated with a fiber optic light source and were used immediately in the settlement inhibition assays. From 40 to 80 larvae each were added to 50×9 mm polystyrene dishes containing 5 ml of 100-kilodalton filtered sea water (control) or filtered sea water containing one of five concentrations of EtOH body-wall extract of *L. clathrata* (3.0, 0.6, 0.12, 0.024, 0.0048 mg/ml). The dishes were incubated at 20±2° for 30 min. Assays were terminated with the addition of several drops of formalin to each dish. Dishes were viewed under a dissecting microscope and larvae that had attached and were deciliated were considered settled. The differences in the percent of settlement of bryozoan larvae in experimental and control treatments were tested for significance using a G-test for independence (28).

ANTIMICROBIAL ASSAYS.—The EtOH body-wall extract of *L. clathrata* was assayed for growth suppression of single species cultures of five marine bacteria (*Alteromonas luteo-violacea, Deleya marina, Pseudomonas piscicida, Listonella anguillarium, Vibrio haloplanktis*), five Gram-positive non-marine bacteria (*Streptococcus lactis, Staphylococcus aureus, Staphylococcus epidermis, Bacillus subtilis, Micrococcus roseus*), five Gram-negative non-marine bacteria (*Escherichia coli, Serratia marcescens, Enterobacter aerogenes, Proteus vulgaris, Pseudomonas aeruginosa*), and four species of fungi (*Sordaria fimicola, Rhizopus stolonifer, Sporothrix schebckii, Candida albicans*). Pure compounds were tested against *S. aureus* and *B. subtilis*. Two of the marine species listed above (*A. luteo-violacea* and *V. haloplanktis*) were collected and cultured from sea water samples taken at a site where *L. clathrata* was collected in the Gulf of Mexico. All bacterial cultures, except the two marine isolates, were obtained from the American Type Culture Collection, Rockville, Maryland.

Bacterial cultures were inoculated into nutrient or marine broth and incubated for 22 h at 28°. Fungal cultures were maintained on Sabouraud Dextrose agar and were inoculated into Sabouraud broth. The broth cultures were incubated for 22 h and then 100 μl of broth culture were aseptically pipetted onto agar petri dishes (10 cm diameter) and spread evenly over the media.

Antimicrobial activity was assessed following standard disk-assay methodology (29). The EtOH extract was resolublized in EtOH-H_2O (4:1). A 50-μl aliquot of each solublized extract was applied to a sterile filter-paper disk (6-mm diameter) to a final concentration of 75 mg of body-wall extract per filter-paper disk. Pure compounds were tested against *Staphylococcus aureus* at a concentration of 50 μg per disk. After solvent evaporation, each treated disk was placed on either Mueller-Hinton (non-marine bacteria), Marine (marine bacteria), or Sabouraud Dextrose (fungi) media inoculated previously with the appropriate microorganism. Three replicate petri dishes were prepared for each microbial species such that each body-wall extract or pure compound was tested in triplicate. Penicillin G (10 units) and streptomycin (10 μg) were used as standard control disks. Solvent control disks had 50 μl of EtOH-H_2O (4:1) applied to them and was allowed to dry. Treated petri dishes were incubated for 72 h at 28°. Zones of growth inhibition surrounding filter-paper disks were measured to the nearest mm after microbial cultures had reached asymptotic growth (24–72 h).

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