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Isolation of Xestosterol Esters of Brominated Acetylenic Fatty Acids from the Marine Sponge *Xestospongia testudinaria*

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The CH_2Cl_2 extract of the marine sponge *Xestospongia testudinaria* inhibited [^{3}H]DPCPX binding to rat-brain adenosine A_1 receptors. Bioassay-guided fractionation led to the isolation of a known brominated acetylenic fatty acid **1** as the active component. Also isolated were two novel sterol esters **2** and **3**. All structures were determined on the basis of their spectroscopic data.

The adenosine A_1 receptor is a well-characterized receptor belonging to the widely distributed purinergic receptor family. Activation of adenosine A_1 receptors results in the bradycardiac,[1] cerebroprotective,[2] and antilipolytic effects of adenosine.[3] Recent progress in understanding of the physiology, pharmacology, and molecular biology of adenosine and adenosine receptors and the availability of cloned human adenosine receptors provide the foundation for the potential of adenosine receptors as drug targets and may allow for developing selective agents for human application.[4] Ligand-binding studies of adenosine receptors showed that xanthine and derivatives are classical adenosine antagonists. However, a wide variety of nonpurine ligands that bind selectively to adenosine receptors have been reported,[5] including a natural product benzofurancarbaldehyde derivative 5-(3-hydroxypropyl)-7-methoxy-2-(3′-methoxy-4′-hydroxyphenyl)-3-benzo[*b*]furancarbaldehyde.[6] Screening marine extracts for their ability to inhibit ligand binding to adenosine A_1 receptors in order to find new classes of antagonists led us to the study of *Xestospongia testudinaria* Lamarck 1814 (family Petrosiidae), which resulted in the isolation of the known acid **1**[7,8] as the constituent with A_1 adenosine receptor affinity. Two nonactive sterol esters **2** and **3** were also isolated (Chart 1). In this paper we report the isolation and structure elucidation of **1**, **2**, and **3** by 1D and 2D NMR techniques and the inhibition of **1** on the binding of [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX), an A_1 selective antagonist ligand, to adenosine A_1 receptors of rat-brain membranes.

The CH_2Cl_2 extract of *X. testudinaria* was separated into fractions by Si gel flash chromatography. Bioassay results indicated that adenosine A_1 activity was concentrated in the hexane−EtOAc (5:1) fraction. This fraction was separated into an active MeOH-soluble fraction and an inactive hexane-soluble fraction. The MeOH-soluble fraction was chromatographed on diol HPLC to give the active acid **1**. Compound **1** was determined to be the brominated *bis*-acetylenic acid, 18-bromooctadeca-(9*E*,17*E*)-diene-7,15-diynoic acid by comparison of ^{1}H and ^{13}C NMR data with reported values.[7] ^{1}H NMR of the inactive hexane-soluble fraction showed signals corresponding to **1** apart from the COOH signal. The inactive fraction was purified by reversed-phase C_{18} HPLC to give esters **2** and **3**.

Ester **2** had a molecular formula of $C_{48}H_{71}O_2Br$ as deduced from high-resolution electrospray mass spectrometry (HRESIMS) and 1D and 2D NMR data. The IR spectrum showed the presence of an acetylenic group (2250 cm^{-1}), an ester group (1727 cm^{-1}), and a terminal methylene group (1460 cm^{-1}, 908 cm^{-1}). Examination of the NMR data suggested that **2** was a steroid ester of **1**. The ^{13}C NMR spectrum (see Table 1) showed 48 carbons of which 17 had identical chemical shifts to **1** apart from a peak at 173.0 ppm compared to 178.6 ppm in **1**. This carbon was assigned to an ester linkage. The DEPT ^{13}C NMR showed that the remaining carbons included four quaternary carbons (152.6, 139.6, 36.6, 42.3 ppm), eight tertiary carbons (122.6, 31.8−56.7 ppm), 13 secondary carbons (108.7, 21.0−38.2 ppm), and five methyl carbons (11.8−19.4 ppm). The ^{1}H NMR spectrum of **2** showed five methyl signals at δ 0.67 (3H, s), 0.810 (3H, t, J = 7.2), 0.813 (3H, t, J = 7.2), 0.94 (3H, d, J = 6.6), 1.02 (3H, s); a methine signal at δ 5.37 (1H, br); and terminal double-bond signals at δ 4.76 (1H, d, J = 1.8) and 4.68 (1H, d, J = 1.8), which were assigned to C-6 (122.6 ppm) and the methylene group C-28 (108.7 ppm), respectively. From the g-COSY spectrum the sequence −CH_2 (1.58, 1.83, m, H-2) −CHOR (4.61, m, H-3) −CH_2 (2.31, d, J = 7.8, H-4) and the side chain CH_3 (0.810, t, J = 7.2, H-29) −CH_2 (1.36, m, H-26) −CHR (1.79, m, H-25) −CH_2 (1.36, m, H-27) −CH_3 (0.813, t, J = 7.2, H-30) were deduced. The HMBC spectrum showed correlations between H-4 and C-2, C-3, C-5, C-6, C-10; H-6 and C-4, C-7, C-10; H-14 and C-9, C-12, C-13, C-18; H-18 and C-12, C-13, C-14; H-17 and C-12, C-13, C-15, C-18; H-19 and C-1, C-5, C-9, C-10; H-21 and C-17, C-20, C-22; H-23 and C-20, C-22, C-24, C-25, C-28; H-26 and C-24, C-25, C-27, C-29; H-27 and C-24, C-25, C-26, C-30; H-28 and C-23, C-24, C-25; H-29 and C-25, C-26; H-30 and C-25, C-27. Thus, the side chain from C-20 to C-30 was assembled, and from the 13 units of unsaturation a tetracyclic ring system was required. From the g-COSY and HMBC data none of the rings was able to be established. The structure of **2** was resolved by the acquisition of a HMQC−TOCSY spectrum, which showed correlations from H-3 to C-1, C-2, and C-4; H-11 to C-8, C-9, and C-12; H-12 to C-7, C-8, C-9, C-11, and C-14; H-20 to C-14, C-15, C-16, C-21, C-22, and C-23; H-21 to C-16, C-17, C-20, C-22, and C-23. Therefore, it followed that the steroid part of the molecule

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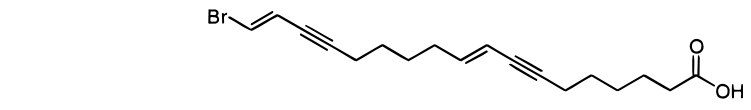
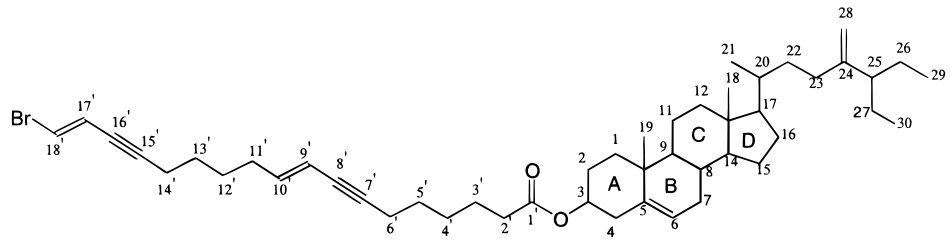
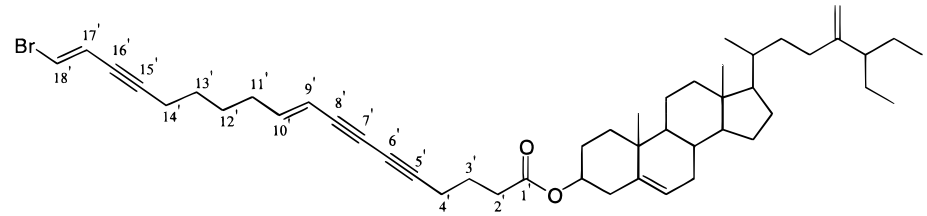
Chart 1



(1)



(2)



(3)

was the known compound xestosterol, which had previously been isolated from this sponge and its structure proposed using ^{1}H and ^{13}C NMR and partial synthesis.[9] Hence with the linkage of the acetylenic ester moiety to the xestosterol ring system, the novel ester was assigned structure **2**.

Ester **3** was isolated as a white powder, and the molecular formula, $C_{48}H_{67}O_2Br$, was established by HRESIMS and NMR data. Its formula indicated a loss of four protons compared to **2**. The IR spectrum showed the presence of an acetylenic group (2246 cm^{-1}), a carbonyl group (1727 cm^{-1}), and a terminal double bond (1460 cm^{-1}, 908 cm^{-1}). The ^{13}C NMR spectrum (see Table 2) showed 48 carbon resonances of which 30 were assigned to the xestosterol system and eight to the acid part (C-11′−C-18′), suggesting that **2** and **3** had a similar structure. The main difference between esters **2** and **3** was the presence of two acetylenic carbons (66.0, 82.3 ppm) along with the absence of two methylene carbons (28.2, 28.3 ppm). There were also changes in ^{13}C chemical shifts of C-3, C-1′−C-4′, and C-7′−C-10′ (Table 2). The ^{1}H NMR spectrum showed a clear difference in chemical shifts of H-2′ (2H, δ 2.41, t, J = 7.8) and H-10′ (1H, δ 6.26, dt, J = 7.2, 15.6) compared to ester **2**. The HMBC spectrum established an enediyne conjugation system with the correlation of H-3′ to C-1′, C-2′, C-4′, and C-5′; H-4′ to C-2′, C-3′, C-5′, C-6′, C-7′, and C-8′ (five-bond coupling [10,11]); H-9′ to C-7′ and C-11′. Therefore, the acid moiety was 18-bromooctadeca-(9*E*,17*E*)-diene-5,7,15-triynoic acid, which had been previously reported.[12] Hence, with the linkage of this acetylenic ester portion to the xestosterol system, the second novel ester was assigned structure **3**. The acid **1** inhibited [^{3}H]DPCPX binding to rat-brain adenosine A_1 receptors with an IC_{50} of 106 μM and a Hill slope factor of −2.7, while esters **2** and **3** were tested as a mixture and had no effect up to the concentration of 10^{-2} M.

Experimental Section

General Experimental Procedures. Solvents used were Omnisolv MeOH, EtOAc, and CH_3CN; distilled and filtered CH_2Cl_2 and hexane, and milli-Q filtered H_2O. The flash column (50 mm i.d. × 400 mm) was packed with Davisil Si gel (30−40 μm). Rainin C_{18} (3 μm, 4.6 mm i.d. × 50 mm) and YMC Diol (5 μm, 4.6 mm i.d. × 150 mm) columns were used for HPLC. A Waters 600 pump equipped with a 996 PDA detector was used for analytical and semipreparative HPLC separations. NMR spectra were recorded in $CDCl_3$ on a Varian INOVA 600 MHz NMR spectrometer with ^{1}H and ^{13}C chemical shifts referenced to the solvent peaks δ 7.26 and 77.0 ppm, respectively. LRESIMS was recorded on a single quadrupole VG platform II mass spectrometer with MassLynx Version 1 used for data acquisition. HRESIMS were measured on a Bruker BioAPEX 47e mass spectrometer. 2-Chloroadenosine (2-CADO) was purchased from Research Biochemical International; [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX), from Dupont New Products; and adenosine deaminase type VI, from Sigma Chemical Co.

Sponge Material. The sponge was collected by scuba diving (−11 m) on the northwest side of Bird Island, Wreck Reef (22°09.9′ S, 155°27.1′ E), Coral Sea, Australia. It was identified as *Xestospongia testudinaria* Lamarck 1814 (phylum Porifera, class Demospongiae, order Haplosclerida, family Petrosiidae). A voucher specimen (QMG306555) has been deposited at the Queensland Museum, South Brisbane, Queensland, Australia.

Extraction and Isolation. A sample (10 g) of freeze-dried *X. testudinaria* was extracted exhaustively with CH_2Cl_2. After evaporation of the solvent, the crude extract (100 mg) was purified through a flash Si gel column with hexane, hexane−EtOAc (5:1), and EtOAc. The hexane−EtOAc (5:1) fraction (40 mg) was partitioned between hexane and MeOH. The MeOH-soluble material was purified by diol HPLC eluted with hexane−EtOAc (9:1) to give acid **1** (10 mg, 0.1% dry wt). The hexane fraction was purified by reversed-phase HPLC, gradient from CH_3CN−CH_2Cl_2 (9:1) to CH_3CN−CH_2Cl_2 (4:1) in 10 min, to give ester **2** (5 mg, 0.05% dry wt) and ester **3** (1 mg, 0.01% dry wt).

Receptor Binding Assays. Membranes were prepared from adult rat brains, which were homogenized in approximately 10 volumes of ice-cold 50 mM Tris HCl buffer (pH 7.4) and centrifuged (25 000 *g* × 20 min, 4 °C). The pellets were washed again with buffer and centrifuged twice more. Adenosine deaminase (1 unit/1 mg protein) was incubated with

Table 1. NMR Data of Ester **2**[a]

position	^{13}C δ	^{1}H δ (mult., J in Hz)	HMBC (C no.)	HMQC−TOCSY[b] (C no.)
1	37.0	1.13 (m)		2, 3, 4
		1.84 (m)		
2	27.8	1.58 (m)		
		1.83 (m)		1, 3, 4
3	73.8	4.61 (m)		1, 2, 4
4	38.2	2.31 (d, 7.8)	2, 3, 5, 6, 10	1, 2, 3, 6, 7
5	139.6			
6	122.6	5.37 (b)	4, 7, 10	4, 7, 8, 9, 14
7	31.8	1.56 (m)	5, 6	
		1.97 (m)	5, 6, 9	
8	31.9	1.47 (m)		
9	50.0	0.96 (m)		11, 12
10	36.6			
11	21.0	1.45 (m)		8, 9, 12
12	39.7	1.18 (m)		8, 9, 11, 14
		2.01 (m)		8, 9, 11, 14
13	42.3			
14	56.7	1.01 (m)	9, 12, 13, 18	
15	24.3	1.10 (m)		
16	28.2	1.28 (m)		8, 14, 15
17	56.0	1.14 (m)	12, 13, 15, 18	
18	11.8	0.67 (s)	12, 13, 14	
19	19.4	1.02 (s)	1, 5, 9, 10	
20	35.8	1.42 (m)		14, 15, 16, 17, 21, 22, 23
21	18.8	0.94 (d, 6.6)	17, 20, 22	15, 16, 17, 20, 22, 23
22	34.4	1.56 (m)		
23	29.3	1.76 (m)	20, 22, 24, 25, 28	22
		1.96 (m)	22, 24, 28	20, 21, 22
24	152.6			
25	50.2	1.79 (m)	23, 24, 26, 27, 28, 29, 30	26, 27, 29, 30
26	26.3	1.36 (m)	24, 25, 27, 29, 30	25, 29
27	26.4	1.36 (m)	24, 25, 26, 29, 30	25, 30
28	108.7	4.76 (d, 1.8)	23, 24, 25	22, 23
		4.68 (d, 1.8)	23, 25	22, 23
29	11.9	0.81 (t, 7.2)	25, 26	25, 26
30	12.0	0.81 (t, 7.2)	25, 27	25, 27
1′	173.0			
2′	34.6	2.28 (t, 7.8)	1′, 3′, 4′	3′, 4′, 5′, 6′
3′	24.6	1.64 (dt, 7.8)	1′, 2′, 4′	2′, 4′, 5′, 6′
4′	28.2	1.50 (m)		
5′	28.3	1.55 (m)		
6′	19.3	2.30 (m)	5′	2′, 3′, 4′, 5′
7′	88.6			
8′	79.2			
9′	110.2	5.45 (dt, 15.6, 1.8)	7′, 11′	10′, 11′, 12′, 13′, 14′
10′	142.6	6.02 (dt, 15.6, 6.6)	8′, 11′, 12′	9′, 10′, 12′, 13′, 14′
11′	32.4	2.10, 2.28 (dd, 6.6)	9′, 10′, 12′	
12′	28.0	1.49 (m)	11′, 13′	
13′	27.7	1.45 (m)		
14′	19.2	2.26 (dt, 7.2, 2.4)	13′, 15′, 16′, 17′	9′, 10′, 11′, 12′, 13′
15′	92.7			
16′	77.4			
17′	117.9	6.17 (dt, 13.8, 2.4)	15′, 18′	14′
18′	117.1	6.57 (d, 13.8)	16′, 17′	

[a] Spectra recorded in $CDCl_3$ at 30 °C. [b] Data obtained from HMQC-TOCSY experiment with mixing time of 75 ms.

brain membranes for 20 min at 37 °C before the binding assay. Mixture of membranes (100 μg/well), radioligands ([^{3}H]-DPCPX, 0.2 nM), acid **1** which was dissolved in DMSO, 2-CADO (for nonspecific binding assays, 1×10^{-4} M), and buffer (50 mM Tris HCl, pH 7.4) were incubated for 120 min at room temperature (approximately 25 °C). The final concentration of DMSO in the incubation volume was 2%. The binding was terminated by rapid filtration over Whatman GF/B filters using ice-cold 50 mM Tris HCl buffer (pH 7.4, 4 °C). Two experiments (each with triplicate determinations) were performed for each concentration of acid **1**. Data were analyzed using a nonlinear, least-squares regression program (Prism 2.0) to determine the IC_{50} and Hill slope values.

18-Bromooctadeca-(9*E*,17*E*)-diene-7,15-diynoic acid (1): white powder; (−)-LRESMS *m/z* 351 [M−H, $C_{18}H_{23}O_2{}^{81}Br$], 349 [M−H, $C_{18}H_{23}O_2{}^{79}Br$], 80 [^{81}Br−H], 78 [^{79}Br−H]; ^{1}H and ^{13}C NMR data were identical with published data.[7]

Xestosterol ester of 18-bromooctadeca-(9*E*,17*E*)-diene-7,15-diynoic acid (2): white powder; $[\alpha]^{25}_{D}$ −7.8° (*c* 0.19 in CH_2Cl_2); UV (hexane) λ_{max} (ϵ) 228 (19 300), 237 (20 800), 250 nm (12 900); IR (film) 2980, 2840, 1727, 1460, 1378, 907, 732 cm^{-1}; ^{1}H and ^{13}C ($CDCl_3$) data, see Table 1; (+)-LRESMS *m/z* 781 (100) [M + Na, $C_{48}H_{71}O_2{}^{79}BrNa$] and 783 (100) [M + Na, $C_{48}H_{71}O_2{}^{81}BrNa$]; (+)-HRESIMS *m/z* 781.4563 (calcd for M + Na, $C_{48}H_{71}O_2{}^{79}BrNa$, 781.4529) and 783.4563 (calcd for $C_{48}H_{71}O_2{}^{81}BrNa$, 783.4529).

Xestosterol ester of 18-bromooctadeca-(9*E*,17*E*)-diene-5,7,15-triynoic acid (3): white powder; $[\alpha]^{25}_{D}$ −2.6° (*c* 0.25 in CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (ϵ) 232 (8500), 239 (9200), 245 nm (8200); IR (film) 2980, 2840, 2253, 1727, 1460, 1378, 1200, 908, 733 cm^{-1}; ^{1}H and ^{13}C ($CDCl_3$) data, see Table 2; (+)-LRESMS *m/z* 778 [M + Na, $C_{48}H_{67}O_2{}^{79}BrNa$] and 780 [M + Na, $C_{48}H_{67}O_2{}^{81}BrNa$]; (+)-HRESIMS *m/z* 777.4205 (calcd for

Table 2. NMR Data of Ester **3**[a]

position	^{13}C δ	^{1}H δ (mult., J in Hz)	HMBC (C no.)
1	37.0	1.13 (m)	
		1.84 (m)	
2	27.8	1.58 (m)	
		1.83 (m)	
3	74.1	4.62 (m)	1, 2, 4, 5
4	38.2	2.32 (b)	2, 3, 5, 6
5	139.6		
6	122.6	5.36 (b)	4, 7, 8
7	31.8	1.56 (m)	5, 6
		1.97 (m)	5, 6, 9
8	31.8	1.47 (m)	
9	50.1	0.96 (m)	7, 11
10	36.6		8, 11
11	21.0	1.45 (m)	9, 10, 12
12	39.7	1.18 (m)	
		2.01 (m)	
13	42.4		
14	56.7	1.06 (m)	13, 15, 16, 17
15	24.3	1.10 (m)	
16	28.2	1.29 (m)	
17	56.0	1.14 (m)	
18	11.8	0.69 (s)	16, 17
19	19.3	1.02 (s)	1, 5, 9, 10
20	35.8	1.42 (m)	
21	18.8	0.94 (d, 6.6)	
22	34.4	1.56 (m)	
23	29.5	1.76 (m)	24, 25
		1.96 (m)	24, 25
24	152.7		
25	50.2	1.79 (m)	
26	26.3	1.36 (m)	
27	26.4	1.36 (m)	
28	108.7	4.76 (d, 1.8)	
		4.68 (d, 1.8)	
29	11.9	0.81 (t, 7.2)	
30	12.0	0.81 (t, 7.2)	
1′	172.3		
2′	33.3	2.41 (t, 7.8)	1′, 3′, 4′
3′	23.6	1.87 (m)	1′, 2′, 4′, 5′
4′	18.8	2.39 (t, 7.2)	2′, 3′, 5′, 6′, 7′, 8′
5′	82.3		
6′	66.0		
7′	72.9		
8′	74.2		
9′	109.1	5.50 (d, 15.6)	7′, 11′
10′	147.5	6.26 (dt, 15.6, 7.2)	8′, 11′
11′	32.4	2.14 (ddd, 7.2, 7.2, 1.8)	9′, 10′, 12′
12′	27.4	1.51 (m)	
13′	27.7	1.51 (m)	
14′	19.0	2.27 (dt, 6.6, 2.4)	13′, 15′, 16′, 17′
15′	92.5		
16′	77.5		
17′	117.9	6.17 (dt, 13.8, 2.4)	
18′	117.1	6.58 (d, 13.8)	16′, 17′

[a] Spectra recorded in $CDCl_3$ at 30 °C.

$C_{48}H_{67}O_2{}^{79}BrNa$, 777.4216) and 779.4205 (calcd for $C_{48}H_{67}O_2{}^{81}BrNa$, 779.4124).

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