Steroids from an Australian Sponge *Psammoclema* sp.

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Investigation of an extract of the Australian marine sponge *Psammoclema* sp. for dynamin I inhibitory activity led to the isolation of four new trihydroxysterols (**1**−**4**) related to aragusterol G. These compounds were largely identified by 1D and 2D NMR spectroscopic methods. While **1** was found to be inactive in the dynamin bioassay, bioassays did reveal that compounds **1**−**4** inhibited the growth of colorectal, breast, ovarian, and prostate cancer cell lines (GI$_{50}$ 5−27 μM). The additional insight that these new compounds give to previous SAR studies is discussed briefly.

The dynamins are a group of three closely related GTPases involved in a large number of significant cellular processes, particularly implicated in cell endocytosis and phagocytosis. In our research we are targeting inhibitors of dynamin I, which is neuron specific, with the aim of developing dynamin inhibitors as new tools for cell biology and also as potentially clinically useful treatments for psychotic disorders.[1−4] As a part of this research effort we investigated the 1:1 CHCl$_3$−MeOH extract from a sponge *Psammoclema* sp. collected from Nelson Bay, Australia, which showed 97% inhibition of dynamin I at 1 mg/mL after desalting of the extract. There have been relatively few reports of secondary metabolites isolated from *Psammoclema* species. The ones that are in the literature concern halogenated alkaloids,[5] halogenated alkaloid sulfonic acids,[6] halogenated polyene amides,[7] and cytotoxic macrolide lactams[8] and diterpenoids.[9]

As a result of this investigation, we wish to report the isolation of four cytotoxic steroids (**1**−**4**) related to the aragusterols, previously isolated by Yamada et al. from the Japanese sponge *Xestospongia* sp.[10] This is the first report of steroids from the sponge genus *Psammoclema*.

Results and Discussion

Thin-layer chromatography of the crude extract enabled several compounds that were considered atypical of the standard sponge TLC profile to be identified, and these were targeted for isolation. The crude extract was submitted to vacuum liquid chromatography over silica gel,[11] with fractions containing the desired compounds being combined and further purified using Sephadex LH-20 column chromatography and HPLC to yield compounds **1**−**4**.

Compound **1** (R_f 0.43; 1:9 MeOH−CHCl$_3$) was isolated as an optically active white solid. Its molecular formula of C$_{29}$H$_{50}$O$_3$ was established by HRESIMS, which showed a pseudomolecular ion [M + Na] at *m/z* 469.3659, implying five degrees of unsaturation. The infrared spectrum of **1** showed a broad peak at 3400 cm^{-1} as the only diagnostic absorbance, consistent with the presence of a hydroxyl functionality within the molecule. The structure of compound **1** was assigned by standard one- and two-dimensional NMR spectroscopic methods. The ^{13}C NMR and DEPT spectra of **1** displayed 29 signals: five methyl, one sp^2 and 10 sp^3 methylenes,

1 R =

2 R =

3 R =

4 R =

Aragusterol G (**5**)

Aragusterol A
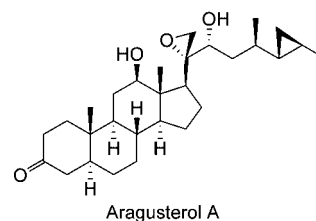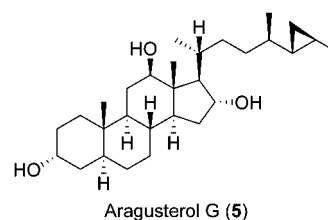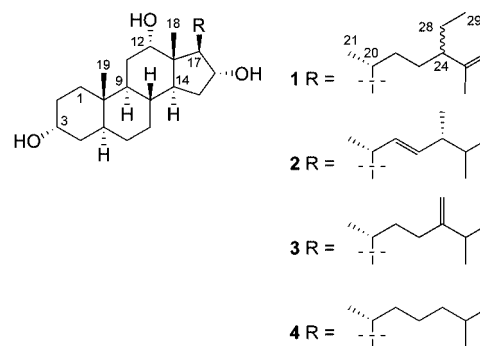
10 methines, and one sp^2 and two sp^3 quaternary carbons. Hydrogen and carbon connectivities were determined by DQF-COSY, gH-SQC, and gHMBC experiments. The only element of unsaturation seen in the NMR data was attributed to an exomethylene group with signals at δ_C 111.6 (CH$_2$, C-26), δ_H 4.72 (1H, dq, *J* = 2.7, 1.5 Hz, H-26*E*), and δ_H 4.65 (1H, bd, *J* = 2.7, H-26*Z*) and the quaternary carbon δ_C 147.6 (qC, C-25). The remaining degrees of unsaturation were thus attributed to the presence of four rings. The methyl group, δ_C 17.8 (CH$_3$, C-27), δ_H 1.56 (3H, s, H-27), is suggestive of the presence of a vinylic methyl group attached to a quaternary carbon. DQF-COSY showed a correlation between these methyl protons and H-26*E* and H-26*Z*. There is also an HMBC correlation between H-27 and C-24 (δ 49.2, CH), whose proton (δ 1.87, 1H, dt, *J* = 9.7, 5.0 Hz) showed correlations back to carbons 25 and 26. Further DQF-COSY correlations were found between H-24 and H-28 (δ 1.31), and H-28 and H-29 (δ 0.78, 3H, t, *J* =
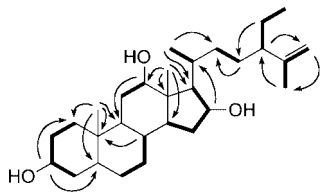
Figure 1. Key DQF-COSY (— **bold**) and gHMBC (H→C) correlations for **1**.

7.4 Hz). An HMBC correlation was also found between H-24 and the methylene at C-23 (δ 29.9, CH_2). These correlations and other key DQF-COSY and HMBC correlations are shown in Figure 1.

^{1}H and ^{13}C NMR spectra indicated the presence of three oxymethine groups, δ_H 4.01 (1H, p, $J = 2.8$ Hz, H-3) and δ_C 66.4 (CH, C-3), δ_H 3.89 (1H, t, $J = 3.0$ Hz, H-12) and δ_C 72.8 (CH, C-12), and δ_H 3.96 (1H, ddd, $J = 6.9$, 5.0, 2.3 Hz, H-16) and δ_C 76.7 (CH, C-16). Since the IR spectrum indicates the presence of only alcohol functional groups and there are three hydrogens not accounted for in the DEPT experiments, all three oxygen atoms present in the molecular formula must be accounted for as alcohol groups. The generally small coupling constants visible in the splitting patterns of the oxymethine protons after resolution enhancement are notable.

The proton NMR spectrum of **1**, which showed most of the protons as a poorly resolved group of signals between 1 and 2 ppm, as well as the distinctive methyl groups at δ_H 0.68 (3H, s, H-18), δ_C 14.3 (CH_3, C-18); δ_H 0.74 (3H, s, H-19), δ_C 11.0 (CH_3, C-19); and δ_H 0.94 (3H, d, $J = 5.8$ Hz, H-21), δ_C 17.9 (CH_3, C-21), with the partial structure C-23 to C-29 and the alcohol groups, was suggestive of a steroid. Comparison of the NMR spectroscopic data of **1** with published values revealed them to have a close match to the nucleus of the trihydroxysterol aragusterol G (**5**) (see Tables 1 and 2).[10]

Comparison of spectroscopic data indicated that two of the three alcohol groups in **1** are located at C-3 and C-16, as they are in aragusterol G. Differences in the ^{13}C NMR chemical shifts at C-9, C-12, C-14, and C-17 indicated some difference associated with the remaining alcohol group. HMBC correlations between the oxymethine H-12 and C-9 (δ 47.2, CH), and H-18 and C-12, as well as a NOESY correlation between H-12 and H-21 indicates the hydroxyl group is also at C-12, just as it is in aragusterol G. Smaller coupling constants of H-12 compared to aragusterol G and downfield shifts greater than those that would otherwise be expected for H-9 (δ 1.16, 1H, td, $J = 12.3$, 2.6 Hz) and H-14 (δ 1.84, ddd, $J = 12.1$, 10.6, 7.2) are suggestive of an axial hydroxyl group at C-12.[12] The differences observed in ^{13}C NMR shifts are also consistent with the C-12 hydroxyl group being axial.[13] In addition, this deduction is strongly supported by an NOE between the angular methyl H-18 and the now equatorial H-12.

In keeping with the most common steroid configuration, NOE experiments indicated that the angular methyl groups, C-20 and H-8, are all on the β-face of the steroid nucleus. The *trans*-AB ring junction is confirmed by the observation of NOEs between H_3-19 and the protons on C-2 and C-4, which would not be possible if the ring junction is *cis*. The unusual all-axial hydroxyl group orientation is further supported by the lack of *trans*-diaxial coupling to H-3, H-12, and H-16, with all coupling constants being less than 7 Hz. An NOE between H-18 and H-16 is consistent with the α-hydroxyl configuration at C-16. Key NOE correlations are shown in Figure 2.

Comparison of ^{13}C NMR spectroscopic data for steroids with the same side chain as found in **1** (C-20 to C-29), and epimeric at C-24, suggests the 24*S* configuration for **1**.[14,15] Significant differences occur in the chemical shifts of carbons 25 to 28 in compounds epimeric at C-24. However, since there is insufficient precedence for the use of ^{13}C NMR shifts to distinguish between 24*R* and *S*

epimers, the configuration at C-24 is left formally undefined. Compound **1** is $3\alpha,12\alpha,16\alpha$-trihydroxy-$24\xi$-ethylcholest-25-ene.

Compound **2** (R_f 0.43; 1:9 MeOH−CHCl$_3$) was isolated as an optically active white solid. The molecular formula of $C_{28}H_{48}O_3$, equivalent to compound **1** less CH_2, was established by high-resolution mass spectrometry with a pseudomolecular ion [M + Na] at m/z 455.3491. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to **1**, with the steroid nucleus being identical as indicated, for example, by the three oxymethine signals δ_H 4.01 (1H, m, H-3), δ_H 3.85 (1H, t, $J = 2.8$ Hz, H-12), and δ_H 3.95 (1H, m H-16) and their carbon shifts δ_C 66.5 (CH, C-3), δ_C 72.8 (CH, C-12), and δ_C 76.8 (CH, C-16) (see Tables 1 and 2).

The only difference between compounds **1** and **2** appears to be in the steroidal side chain, C-20 to C-28. Signals at δ_C 135.3 (CH, C-22) and δ_H 5.39 (1H, m, H-22), and δ_C 134.5 (CH, C-23) and δ_H 5.42 (1H, m, H-23), indicate that the side chain of **2** has a 1,2-disubstituted double bond, thus accounting for the fifth degree of unsaturation implied by its molecular formula. In compound **2** the ethyl group found at C-24 in **1** has been replaced by a methyl group, thus accounting for the difference in molecular formula between the two molecules. DQF-COSY spectroscopy correlated all of the protons H-16 through H-28 and allowed the entire structure between C-16 and C-28 to be formulated. These assignments are consistent with published data, in particular the configuration of the double bond at C-22 (*E*) and of C-24 (*R*).[16−18] On the basis of the foregoing discussion **2** is $3\alpha,12\alpha,16\alpha$-trihydroxy-24*R*-methyl-cholest-22*E*-ene.

Compound **3** (R_f 0.43; 1:9 MeOH−CHCl$_3$) was isolated as an optically active white solid. Its molecular formula as determined by HRESIMS showed it to be isomeric with compound **2** and, according to its ^{1}H and ^{13}C NMR spectra, differing once again only in the C-20 to C-28 portion of its structure. Signals at δ_C 106.3 (CH_2, C-28), δ_H 4.72 (1H, s, H-28a) and 4.68 (1H, s, H-28b), and δ_C 156.0 (qC, C-24) and the loss of signals corresponding to the 1,2-disubstituted double bond and the methyl group at C-28 in **2**, supported by COSY correlations between H-16/H-23 and H-24/H-28, allowed compound **3** to be assigned as $3\alpha,12\alpha,16\alpha$-trihydroxy-24-methylcholest-24(28)-ene. The assignments for C-20 to C-28 are consistent with published data.[17]

Compound **4** (R_f 0.43; 1:9 MeOH−CHCl$_3$) was isolated as an optically active white solid. The molecular formula of $C_{27}H_{48}O_3$ was established by HRESIMS, which showed a pseudomolecular ion at m/z 443.3488 [M + Na]$^+$. The ^{1}H and ^{13}C NMR spectra of **4** were once again very similar to **1** and indicated that the structure of **4** possessed the same steroid nucleus but with the C-20 to C-27 portion identical with that found in cholesterol. Thus compound **4** is $3\alpha,12\alpha,16\alpha$-trihydroxycholestane.

Biological tests undertaken with **1** in our dynamin I bioassay revealed it to be inactive (IC$_{50}$ > 300 μM). The other fractions produced during isolation of the steroids also proved to be inactive in this assay, indicating possible degradation of the originally active material during chromatography. The obvious structural similarity of compounds **1**−**4** to the aragusterols did, however, point to them having potential antitumor activity. Aragusterols A and C, in particular, have elicited considerable interest due to their strong in vitro inhibition of cell proliferation of colon, breast, and ovarian cancer cell lines, among others, and in vivo activity against P388 and L1210 cells in mice.[10,19,20] With this in mind compounds **1**−**4** were screened against colorectal (HT29), breast (MCF-7), ovarian (A2780), and prostate (DU145) cancer cell lines. The results of these assays (see Table 3) show that all four compounds inhibit cell proliferation (GI$_{50}$ 5−27 μM), with **4** being 3 times more potent than the other compounds at inhibiting cell growth. Considering our results in light of those from an SAR study by Sato et al.,[21] who compared the anticancer activity of two tetraoxygenated sclaranes with aragusterols, confirms that a 3-keto group enhances activity over a 3α- or 3β-hydroxyl functional group. Oxygenation at C-3 appears to be necessary, as well as placement of

Table 1. ^{13}C NMR Spectroscopic Data (75 MHz, $CDCl_3$) for Compounds **1−5**

position	1 δ, mult.	2 δ, mult.	3 δ, mult.	4 δ, mult.	aragusterol G (5) δ, mult.[a]
1	31.9, CH$_2$	32.0, CH$_2$	31.9, CH$_2$	31.9, CH$_2$	32.1, CH$_2$
2	28.9, CH$_2$	29.0, CH$_2$	29.0, CH$_2$	28.9, CH$_2$	28.9, CH$_2$
3	66.4, CH	66.5, CH	66.5, CH	66.5, CH	66.4, CH
4	35.8, CH$_2$	35.9, CH$_2$	35.9, CH$_2$	35.8, CH$_2$	35.8, CH$_2$
5	39.1, CH	39.1, CH	39.1, CH	39.1, CH	39.0, CH
6	28.4, CH$_2$	28.4, CH$_2$	28.4, CH$_2$	28.4, CH$_2$	28.4, CH$_2$
7	31.7, CH$_2$	31.6, CH$_2$	31.7, CH$_2$	31.7, CH$_2$	31.5, CH$_2$
8	35.0, CH	35.1, CH	35.0, CH	35.0, CH	33.6, CH
9	47.2, CH	47.5, CH	47.2, CH	47.2, CH	53.1, CH
10	35.7, qC	35.8, qC	35.7, qC	35.7, qC	36.0, qC
11	28.0, CH$_2$	28.0, CH$_2$	28.0, CH$_2$	28.0, CH$_2$	30.7, CH$_2$
12	72.8, CH	72.8, CH	72.8, CH	72.8, CH	79.9, CH
13	48.4, qC	48.3, qC	48.4, qC	48.4, qC	49.2, qC
14	45.3, CH	45.6, CH	45.5, CH	45.3, CH	51.4, CH
15	36.2, CH$_2$	35.9, CH$_2$	36.5, CH$_2$	36.4, CH$_2$	36.1, CH$_2$
16	76.7, CH	76.8, CH	77.2, CH	76.8, CH	74.1, CH
17	58.7, CH	58.3, CH	58.5, CH	58.9, CH	67.8, CH
18	14.3, CH$_3$	14.4, CH$_3$	14.3, CH$_3$	14.3, CH$_3$	9.1, CH$_3$
19	11.0, CH$_3$	11.1, CH$_3$	11.1, CH$_3$	11.0, CH$_3$	11.1, CH$_3$
20	33.4, CH	39.1, CH	33.8, CH	33.9, CH	32.3, CH
21	17.9, CH$_3$	19.8, CH$_3$	18.0, CH$_3$	18.0, CH$_3$	22.6, CH$_3$
22	33.3, CH$_2$	135.3, CH	34.1, CH$_2$	35.8, CH$_2$	32.7, CH$_2$
23	29.9, CH$_2$	134.5, CH	31.9, CH$_2$	24.5, CH$_2$	36.2, CH$_2$
24	49.2, CH	42.7, CH	156.0, qC	39.4, CH$_2$	38.7, CH
25	147.6, qC	33.0, CH	33.7, CH	29.7, CH	27.3, CH
26	111.6, CH$_2$	20.0, CH$_3$	22.0, CH$_3$	22.5, CH$_3$	12.8, CH
27	17.8, CH$_3$	19.7, CH$_3$	21.8, CH$_3$	22.8, CH$_3$	11.5, CH$_2$
28	26.6, CH$_2$	17.4, CH$_3$	106.3, CH$_2$		20.0, CH$_3$
29	12.0, CH$_3$				19.1, CH$_3$

[a] Spectrum recorded at 100 MHz in $CDCl_3$.[10]

Table 2. ^1H NMR Spectroscopic Data ($CDCl_3$) for Compounds **1−5**

position	1 δ (J in Hz)[a]	2 δ (J in Hz)[b]	3 δ (J in Hz)[b]	4 δ (J in Hz)[b]	aragusterol G (5) δ (J in Hz)[c]
1	1.34[d]	1.34	1.34	1.35	
2	1.61, 1.37	1.60, 1.35	1.64, 1.38	1.61, 1.38	
3	4.01, p (2.8)	4.01, m	4.02, m	4.02, m	4.00, m
4	1.46, 1.35	1.49, 1.37	1.45, 1.39	1.45, 1.35	
5	1.50	1.50	1.50	1.50	
6	1.17, 1.08	1.16	1.16	1.17	
7	1.62, m, 1.00, qd (12.3, 4.1)	1.60, 0.95	1.64, 0.99	1.61, 1.02	
8	1.38	1.38	1.38	1.39, m	
9	1.16, td (12.3, 2.6)	1.16	1.19	1.20	
11	1.63, 1.49	1.60, 1.50	1.62	1.64	
12	3.89, t (3.0)	3.86, t (2.8)	3.90, t (2.7)	3.90, t (2.9)	3.53, dd (4.6, 10.9)
14	1.84, ddd (12.1, 10.6, 7.2)	1.85, m	1.87, m	1.84, m	
15	1.56	1.53, m	1.57, m	1.57, m	
16	3.96, ddd (2.3, 5.0, 6.9)	3.95, m	3.98, m	3.97, m	4.32, t (6.8)
17	1.53	1.63, m	1.57, m	1.58, m	
18	0.68, s	0.72, s	0.70, s	0.70, s	0.71, s
19	0.74, s	0.75, s	0.75, s	0.75, s	0.78, s
20	1.49	2.15	1.57, m	1.43, m	1.84, m
21	0.94, d (5.8)	1.07, d (6.0)	1.00, d (5.7)	0.95, d (5.7)	1.09. d (6.9)
22	1.08	5.39, m	1.82, m, 1.31, m	1.38, m	
23	1.42, 1.24	5.42, m	2.21, m, 1.92, m	1.35, m	
24	1.87, dt (9.7, 5.0)	1.91		1.23, m	0.65, m
25		1.52, m	2.23, sept (7.0)	1.44, m	0.15, m
26	4.72, dq (2.7, 1.5), 4.65, bd (2.7)	0.84, d (6.8)	1.02, d (6.6)	0.84, d (6.6)	0.46, m
27	1.56, s	0.84, d (6.8)	1.01, d (6.6)	0.85, d (6.6)	0.09, m, 0.15, m
28	1.31	0.92, d (6.9)	4.72, s, 4.68, s		0.91, d (6.7)
29	0.78, t (7.4)				1.00, d (6.0)

[a] Spectrum recorded at 600 and 300 MHz. [b] Spectrum recorded at 300 MHz. [c] Spectrum recorded at 400 MHz in $CDCl_3$.[10] [d] Where no multiplicities are recorded, chemical shift data were obtained from 2D correlations.

at least one hydroxyl group at the other end of the steroid nucleus at C-16, -20, or -22. If there is no hydroxyl group at any of these latter positions, aragusterols become inactive,[22] while multiple oxidation (hydroxyl and/or epoxide functionality) seems to enhance biological activity. There does not seem to be a necessity for a cyclopropyl group at C-25, -26, alkylation at C-24, or a particular configuration of the hydroxyl group at C-12, since these do not seem to have an effect on activity. Oxidation at C-12 may not be necessary at all. Indeed, it may be that that the entire steroid nucleus is not absolutely critical either, merely acting as a substantial hydrophobic spacer.[23]

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Jasco P-2000 polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 600 with a cryoprobe or a Bruker Avance DPX-300 spectrometer. Spectra were, in general, recorded as $CDCl_3$ solutions and the solvent signals used as the internal standard for chemical shifts (^{13}C 77.0 ppm, residual ^1H 7.24 ppm). High-resolution mass spectra were recorded on a Bruker BioApex 47 FT mass spectrometer with an electrospray (ESI) source; ions were detected in positive mode. Thin-layer chromatography was performed using
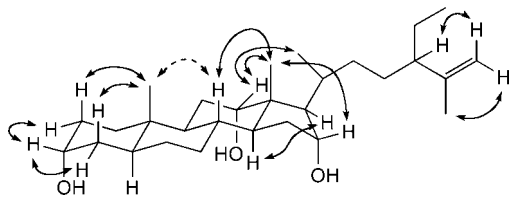
Figure 2. Key NOE observations for **1**. The dashed arrow indicates data obtained in C_5D_5N instead of $CDCl_3$.

Table 3. Growth Inhibition (GI_{50}) Response Determined in HT29 (human colorectal), MCF-7 (human breast), A2780 (human ovarian), and DU145 (human prostate) Cancer Cell Lines by the MTT Cytotoxicity Assay after 72 h Continuous Exposure to the Compound

compound	HT29 GI_{50} (μM)[a]	MCF-7 GI_{50} (μM)	A2780 GI_{50} (μM)	DU145 GI_{50} (μM)
1	7.1 ± 0.4	10 ± 2	11 ± 1	13 ± 1
2	7.0 ± 0.1	17 ± 8	10 ± 1	27 ± 1
3	18 ± 2	18 ± 1	19 ± 4	27 ± 1
4	5.0 ± 0.1	5.5 ± 0.5	5.4 ± 0.2	6.7 ± 0.2

[a] The GI_{50} value is the concentration that inhibits cell growth by 50% relative to untreated cells.

aluminum-backed plates (silica gel 60 F_{254}, 0.2 mm, Merck), vacuum liquid chromatography (VLC)[11] used silica gel H (Merck), and size exclusion and partition chromatography used Sephadex LH-20 (Pharmacia). High-performance liquid chromatography (HPLC) was performed using a Waters 600 controller fitted with a Waters 600 pump, photodiode array detector (Waters 996), and a normal-phase semi-preparative column [10 × 250 mm, Luna 100 Å 10 μm (Phenomenex)]. Solvents for HPLC were HPLC grade; all other solvents were distilled from glass prior to use.

Animal Material. The sponge *Psammoclema* sp. 1183 (phylum Porifera, class Demospongiae, order Poecilosclerida, family Chondropsidae) was collected at −8 m by divers using scuba, in Nelson Bay, New South Wales, Australia (32°43′0.5″ S, 152°08′25.0″ E) on October 26, 2006. The sponge was identified by Dr Merrick Ekins, The Queensland Museum (Grey St, South Brisbane, Qld, Australia), where the voucher specimen is held (specimen no. G326650). The sponge was stored on ice for transportation and held in a freezer (−40 °C) prior to extraction.

Extraction and Isolation. The frozen sponge was blended in 1:1 $CHCl_3$–MeOH (3 × 1 L) and filtered through diatomaceous earth, and the solvent removed under vacuum to leave an aqueous mixture. The aqueous mixture (500 mL) was extracted using dichloromethane (3 × 300 mL), which was then dried under vacuum to yield 12.74 g of crude extract (7.8% of dry mass of extracted sponge). The extract was stored at −4 °C in the dark until required. Crude extract (12.7 g) was fractionated by means of VLC commencing with 3:97 MeOH−$CHCl_3$ (15 × 50 mL), followed by 1:19 MeOH−$CHCl_3$ (15 × 50 mL) and finally 7:93 MeOH−$CHCl_3$ (5 × 50 mL). Fractions 19−25 were combined, and the solvent was removed under vacuum and submitted to Sephadex LH-20 column chromatography (10:10:1 $CHCl_3$−light petrol−EtOH, 3 mL fractions). Fractions 82−87 were combined and evaporated under vacuum, yielding 50 mg of crude steroidal material. Further purification by careful normal-phase HPLC (MeOH−CH_2Cl_2 ramped from 1:99 to 5:95) afforded four pure steroids: **1** (12.7 mg, 7.8 × 10^{-3} %), **2** (1.1 mg, 6.7 × 10^{-4} %), **3** (1.0 mg, 6.1 × 10^{-4} %), and **4** (1.4 mg, 8.5 × 10^{-4} %). HPLC fractions were combined on the basis of their ^{1}H NMR spectra.

3α,12α,16α-Trihydroxy-24ξ-ethylcholest-25-ene (1): white, amorphous solid; $[\alpha]^{21}_D$ 24.0 (*c* 0.33, $CHCl_3$); IR (cast) ν_{max} 3304 (br), 3066, 2918, 2871, 1446, 1351 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) see Table 2; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1; HRESIMS *m/z* 469.3659 [M + Na]$^+$ (calcd for $C_{29}H_{50}O_3Na$, 469.3652).

3α,12α,16α-Trihydroxy-24R-methylcholest-22E-ene (2): white, amorphous solid; $[\alpha]^{21}_D$ 22 (*c* 0.06, $CHCl_3$); IR (cast) ν_{max} 3382 (br), 3074, 2916, 2850 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1; HRESIMS *m/z* 455.3491 [M + Na]$^+$ (calcd for $C_{28}H_{48}O_3Na$, 455.3496).

3α,12α,16α-Trihydroxy-24-methylcholest-24(28)-ene (3): white, amorphous solid; $[\alpha]^{21}_D$ 21 (*c* 0.05, $CHCl_3$); IR (cast) ν_{max} 3406, 2923, 2850, 1454, 1380, 1157 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1; HRESIMS *m/z* 455.3490 [M + Na]$^+$ (calcd for $C_{28}H_{48}O_3Na$, 455.3496).

3α,12α,16α-Trihydroxycholestane (4): white, amorphous solid; $[\alpha]^{21}_D$ 7.8 (*c* 0.07, $CHCl_3$); IR (cast) ν_{max} 3382 (br), 3101, 2923, 2854, 1450, 1380, 1157 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2; ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1; HRESIMS *m/z* 443.3488 [M + Na]$^+$ (calcd for $C_{27}H_{48}O_3Na$, 443.3496).

Bioassays. Dynamin Assay:[4] The assay measures inhibition of the GTPase activity of dynamin I. In brief, samples were made up as stock solutions (1 mg/mL for impure extracts or fractions, 300 μM for pure compounds) in 10% DMSO and diluted in 10% (v/v) DMSO/20 mM Tris-HCl, pH 7.4, before use in the assay. Dynamin I was purified from sheep brain by extraction from the peripheral membrane fraction of whole brain followed by affinity purification on GST-Amph2-SH3-Sepharose. GTP (0.3 mM), GTPase buffer (10 mM Tris-HCl, 10 mM NaCl, 2 mM Mg^{2+}, 0.05% Tween 80, pH 7.5), 1 μg/mL leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride were preincubated in the presence of the test sample (15 μL) for 12 min at 37 °C to bring the reaction mixture to temperature, after which dynamin I (20 nM), diluted in dynamin diluting buffer (6 mM Tris-HCl, 20 mM NaCl, and 0.02% Tween 80; pH 7.5), was added and the mixture incubated for 30 min at 37 °C. The assay (total volume: 150 μL) was conducted in round-bottomed 96-well plates and incubated in a dry heating block with shaking at 300 rpm (Eppendorf Thermo-mixer; Eppendorf South Pacific, North Ryde, Australia). Dynamin activity was measured as phospholipid stimulated with addition of different concentrations of phosphatidylserine liposomes. The reaction was terminated with 10 μL of 0.5 M EDTA, pH 8.0. A 40 μL amount of filtered (0.45 μm) malachite green solution (2% (w/v) ammonium molybdate tetrahydrate, 0.15% (w/v) malachite green in 4 M HCl) was added to each well. Color was allowed to develop for 5 min, and the absorbance of the samples in each plate was determined on a microplate spectrophotometer at 650 nm (VERSA$_{max}$ microplate reader; Molecular Devices, Sunnyvale, CA). Phosphate release was quantified by comparison with a standard curve of sodium dihydrogen orthophosphate monohydrate.

Anticancer Assay: All cell lines were purchased from the American Type Culture Collection (ATCC). Cell culture and stock solutions were prepared as follows. A 20 mM stock solution in DMSO was prepared for each compound and stored at −20 °C. All cancer cell lines were cultured at 37 °C, under 5% CO_2 in air, and were maintained in Dulbecco's modified Eagle's medium (Trace Biosciences, Australia) supplemented with 10% fetal bovine serum, 10 mM sodium bicarbonate penicillin (100 IU/mL), streptomycin (100 μg/mL), and glutamine (4 mM).

Cells in logarithmic growth were transferred to 96-well plates. Cytotoxicity was determined by plating cells in duplicate in 100 mL of medium at a density of 2000−3000 cells/well. On day 0 (24 h after plating), when the cells were in logarithmic growth, 100 μL of medium with or without the test agent was added to each well. After 72 h drug exposure growth inhibitory effects were evaluated using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay, and absorbance was read at 540 nm. A dose−response curve was produced for each test compound using eight drug concentrations in the range of 0.25−50 μM. The GI value was determined as the concentration (μM) of agent that induced 50% growth inhibition relative to untreated cells; the lower the value, the greater the growth inhibition. The assay was performed in duplicate and replicated on two separate occasions.

Supporting Information Available: A table of all correlations observed in ^{1}H, ^{1}H DQF-COSY, gHMBC, and NOE NMR spectroscopic measurements for compound **1**, obtained in both $CDCl_3$ and C_5D_5N. An analysis of ^{13}C NMR chemical shifts relating to 24-ethyl and 24-methyl configurations of steroids. This material is available free of charge via the Internet at http://pubs.acs.org.

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