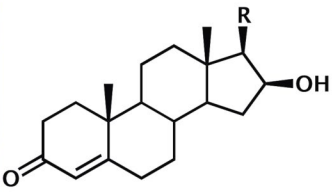
Graphical Abstract

Six cytotoxic keto-steroids have been isolated from the brown alga *Cystophora xiphocarpa*



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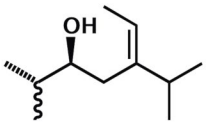

Cancer cell growth inhibition

GI_{50} 8.7 μM colon
5.6 μM breast
4.5 μM ovarian

Bioactive α,β-conjugated 3-keto-steroids from the Australian brown alga *Cystophora xiphocarpa*

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Abstract

As part of our ongoing study of the specialised metabolites present in brown algae belonging to the *Cystophora* genus, eight new steroids including three pairs of diastereoisomers were isolated from *Cystophora xiphocarpa* (Harvey) (Sargassacea, Fucales). The metabolites identified by standard spectrometric methods are (16*S*,22*S*)-16,22-dihydroxyergosta-4,24(28)-dien-3-one and (16*S*,22*R*)-16,22-dihydroxyergosta-4,24(28)-dien-3-one, (16*S*,22*S*,24*R*)-16,22,24-trihydroxyporifera-4,28-dien-3-one and (16*S*,22*S*,24*S*)-16,22,24-trihydroxystigma-4,28-dien-3-one along with (16*S*,22*S*,24*E*)-16,22-dihydroxystigma-4,24(28)-dien-3-one and (16*S*,20*S*)-16,20-dihydroxyergosta-4,24(28)-dien-3-one. (16*S*,22*S*,24*E*)-16,22-Dihydroxystigma-4,24(28)-dien-3-one possessed the most potent cytotoxicity of the steroids in this series with cell growth inhibitions of GI_{50} 8.7 ± 0.7 μM against colon cancer HT29, GI_{50} 5.6 ± 0.8 μM against the breast cancer line MCF-7 and GI_{50} 4.5 ± 0.2 μM against the ovarian cancer cell line A2780. (16*S*,22*R*)-16,22-dihydroxyergosta-4,24(28)-dien-3-one was found to be active against the ovarian cancer cell line A2780 with a GI_{50} of 6.2 ± 0.1 μM.

1. Introduction

In this paper we wish to report the isolation of six new conjugated 3-keto-steroids from the Australian brown alga *Cystophora xiphocarpa* (Harvey) (Sargassaceae, Fucales) and their cytotoxicity. This forms part of our ongoing studies of the specialised metabolites from *Cystophora* spp. (van Altena, 1988; Steinberg and van Altena, 1992; Bian and van Altena, 1998; Laird and van Altena, 2006; Laird et al., 2007, 2010). Members of this genus produce a range of specialised metabolites including simple terpenoids, cyclised terpenoids, *p*-toluquinol diterpenes, acyl-phoroglucinols and resorcinols and in the case of *C. brownii* a steroid oxidised at C-16 (Laird et al., 2010, and references cited therein). *C. xiphocarpa* is of particular interest since it is the extant putative origin species of the genus (Womersley, 1987).

Bioactive α,β-conjugated 3-keto-steroids have been isolated from both terrestrial and marine organisms but remain relatively rare (Fig. 1). The 16-hydroxy sterones tumacone B (**1**) and tumacoside B (**2**) (Fig. 1) were isolated from the South American plant *Solanum nudum* (Saez et al., 1998). While neither compound revealed bioactivity when tested, the C-16 acetate derivative tumacoside A (**3**) displayed *in vitro* antimalarial activity against *Plasmodium falciparum* chloroquine-resistant FCB-1 strain, with IC_{50} = 27 μM . In addition to hydroxylation at C-16, conjugated 3-keto-steroids with a hydroxy group at C-6 have been reported from the red algae *Jania adhaerens* from the Red Sea (Alarif et al., 2012). They included the previously undescribed compound 6β,16β-dihydroxycholest-4-en-3-one (**4**) and known compounds 6β-hydroxycholest-4-en-3-one (**5**), 6β-hydroxycholest-4,22-dien-3-one (**6**) as well as 16β-hydroxy-5α-cholestan-3,6-dione (**7**). Compound **7** was found to be protective toward induced DNA damage of human peripheral blood cells (Alarif et al., 2012). Biologically active 3-ketosteroids with hydroxy groups at C-20 and C-22 in addition to a hydroxy group at C-16 have been found in guggul, the oleogum resin of plant *Commiphora wightii* (El-Mekkawy et al., 2013). Guggulsterol I (**8**) showed weak α-glucosidase inhibitory effects (El-Mekkawy et al., 2013). Bioactive guggulsterol III (**9**), previously found in the plant *Commiphora mukul*, was isolated again from the marine gorgonian *Leptogargia sarmentosa*, along with the previously undescribed compounds 16-*epi*-guggulsterol III

(cholest-4-en-16α,20ξ-diol-3-one, **10**) and cholest-4-en-20ξ-ol-3,16-dione (**11**) (Benvegnu et al., 1982). Keto-steroids with a hydroxy group at C-7, a previously undescribed compound 7α-hydroxy-4,24(28)-ergostadien-3-one (**12**) and known compound 7,20*S*-dihydroxyergosta-4,24(28)-dien-3-one (**13**) were isolated from *Antrocaryon klaineanum*, a plant from Cameroon (Dounla et al., 2015). Compound **13** is reported to show potent inhibition of the 3D7 strain of *Plasmodium falciparum* with an IC_{50} value 21.3 ± 0.2 μM.

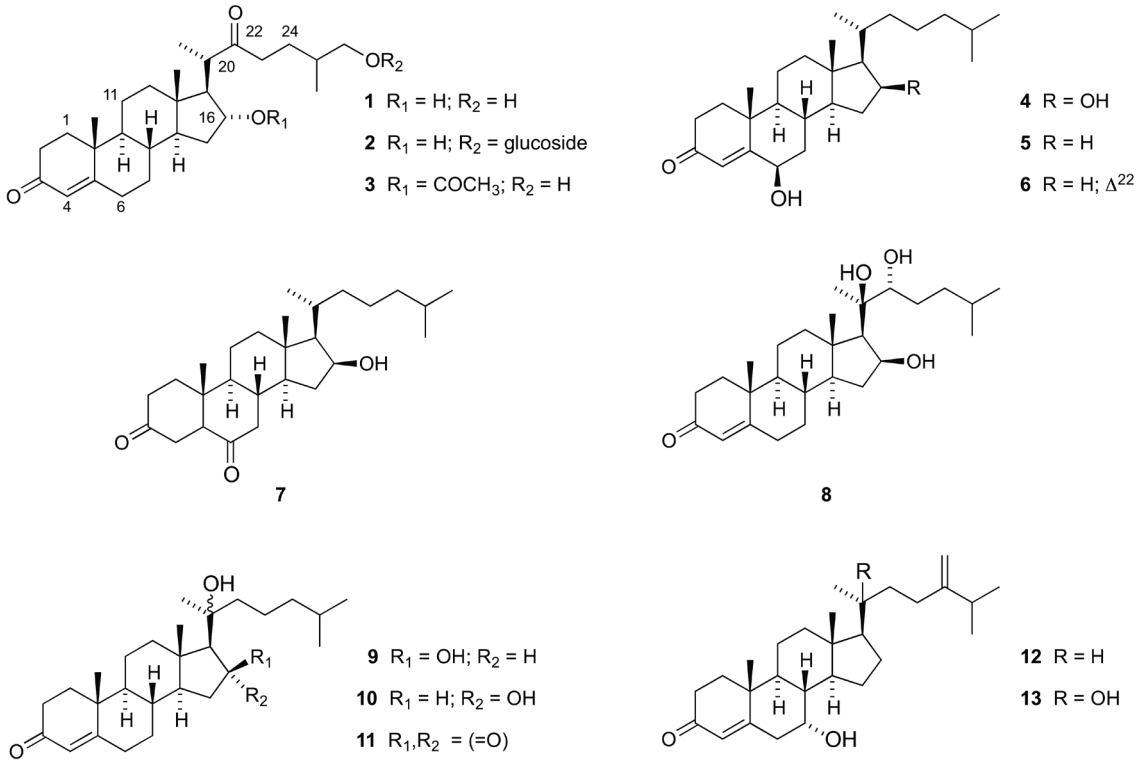


Fig. 1. Previously isolated bioactive α,β-conjugated 3-keto-steroids.

2. Results and discussion

Pure compounds from the crude acetone extract of *C. xiphocarpa* were obtained after a series of chromatographic steps which included, in order, vacuum chromatography with a stepped gradient elution (light petroleum, CH_2Cl_2, EtOH, MeOH), centripetal planar chromatography (Chromatotron®, elution with mixtures of light petroleum–EtOAc) and

semi-preparative HPLC (isooctane–CH_2Cl_2–MeOH) resulting in the isolation of six undescribed compounds **14**, **15**, **17**, **18, 21** and **22** (Fig. 2).

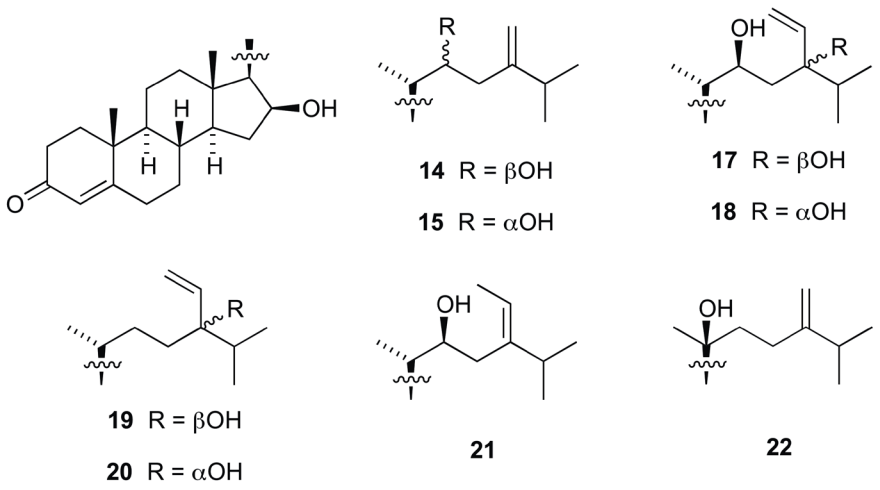


Fig. 2. α,β-Conjugated 3-keto-steroids isolated from *Cystophora xiphocarpa*.

The molecular formula of compound **14** was determined to be $C_{28}H_{44}O_3$ by high-resolution mass spectrometry, which showed a quasi-molecular ion $[M + H]^+$ at *m/z* 429.3361 (calc. for $C_{28}H_{45}O_3$ 429.3369), implying seven units of unsaturation. The infrared (IR) spectrum of **14** showed a broad absorbance at 3395 cm^{-1} indicating the presence of hydroxy group(s) and an absorbance at 3081 cm^{-1} (=C—H stretch) indicating the presence of carbon-carbon double bond(s). Also visible in the IR spectrum are distinct absorbances at 1667 cm^{-1} (s, C=O) and 1614 cm^{-1} (w, C=C) consistent with the presence of an α,β-unsaturated ketone group. The ^{13}C NMR (Table 1) and DEPT spectra of compound **14** showed 28 signals: five methyl groups, eight sp^3 and one sp^2 methylene group, six sp^3 and one sp^2 methine group, two oxymethine groups and two sp^3 and three sp^2 quaternary carbons. The ^1H (Table 2) and ^{13}C NMR spectroscopic data obtained from compound **14** revealed the presence of a terminal vinyl group with signals δ_C 110.4 (CH_2, C-28), δ_H 4.95 (1H, *t*, *J* = 1.2 Hz, H-28a) and δ_H 4.82 (1H, *br s*, H-28b). An isopropyl group from COSY correlations between δ_H 1.03 (3H, *d*, *J* = 6.8 Hz, H-26), 2.22 (1H, H-25) and 1.06 (3H, *d*, *J* = 6.8 Hz, H-27) shows HMBC correlations from both H_3-26 and H_3-27 to a quaternary sp^2 carbon δ_C 153.2

(qC, C-24) and, as well, HMBC correlations from H-28 to δ_C 37.1 (CH_2, C-23) and to δ_C 33.2 (CH, C-25) allowing a 3-methylbut-1-ene unit attached through C-24 in **14**. A long spin system was elucidated by COSY correlations from H-23 extending around to H-8, namely, between H-23b and H-22; H-22 and H-20; H-20 and H-21 and H-17; H-17 and H-16; H-16 and H-15a, H-15b; H_2-15 and H-14; H-14 and H-8 (Fig. 3, supplementary data Table S1). The only remaining discernible COSY correlations were between H_2-11 and H-12b and long range correlations between the vinylic proton at δ_H 5.71(1H, *br s*, H-4) and the protons δ_H 2.40 and 2.28 attached to C-6 (δ_C 32.9, CH_2) (Fig. 3).

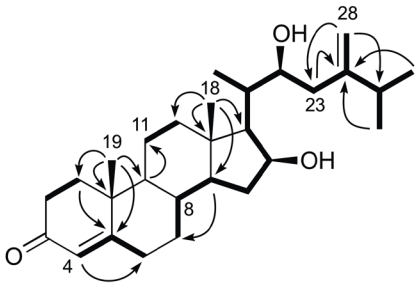


Fig. 3. Key ^{1}H,^{1}H COSY (—) and HMBC (H→C) correlations found for **14**.

A downfield signal in the ^{13}C NMR spectrum at δ_C 199.6 (qC, C-3) is consistent with the presence of a ketone group, accounting for a second unit of unsaturation. Since the NMR signals of an sp^2 methine at δ_C 123.8 (CH, C-4) and δ_H 5.71 (1H, *br s*, H-4) and the quaternary sp^2 carbon at δ_C 171.4 (qC, C-5) are the only remaining signals consistent with the chemical shift of an alkene and the IR spectrum indicates that the carbonyl signal is part of an α,β-unsaturated system, these signals must constitute the α,β-unsaturation and the third unit of unsaturation.

As there are no unaccounted for unsaturated carbon atoms visible in the NMR data, the remaining four units of unsaturation have been attributed to the presence of four rings. Typical of angular methyl groups in fused ring systems, two methyl groups show strong HMBC correlations to four different carbons each, namely, H_3-18 (δ 0.96, *s*) to C-12 (δ 40.0), C-13 (δ 42.7), C-14 (δ 53.3) and C-17 (δ 57.1), and H_3-19 (δ 1.18, *s*) to C-1 (δ 35.7), C-5 (δ 171.4), C-10 (δ 38.6) and C-9 (δ 53.8). These correlations with the

HMBC correlation observed between H-9 (δ 0.90) and C-11 (δ 20.8) tie together all but one of the carbon atoms **14**, including the α,β-unsaturated ketone moiety (Fig. 3).

The molecular formula of compound **14** indicates the presence of three oxygen atoms in the structure, one of which has been attributed to the presence of the ketone group. As the IR spectrum indicates the presence of at least one hydroxy group and since there are only two sp^3 carbon atoms bonded to oxygen visible in the ^{13}C NMR spectrum, the third oxygen atom must be part of a second hydroxy group. At this stage it was evident that compound **14** is a steroid and the literature was searched for steroids with similar structures. Comparison of NMR data from compound **14** to data from a steroid nucleus and a steroidal side chain reported in the literature showed good agreement (Achenbach et al., 1996; Harding et al., 2001; Saez et al., 1998) with the values obtained from compound **14** for the steroidal nucleus ($\Delta\delta_C < 4$ppm, $\Delta\delta_H \leq 0.06$ ppm) and allowed the planar structure of compound **14** to be confidently assigned.

Compound **15** was found to be isomeric with **14** when its molecular formula was also determined to be $C_{28}H_{44}O_3$ by high-resolution mass spectrometry, showing a quasi-molecular ion $[M + H]^+$ at *m/z* 429.3363 (calc. for $C_{28}H_{45}O_3$ 429.3369). The ^{13}C NMR and DEPT spectra showed 28 signals identical in substitution and hybridisation to those found in compound **14**. Analysis of the 2D NMR spectroscopic data (supplementary data Fig. S1, Table S2) found the carbon assignments to be almost identical with those already assigned in compound **14**; The greatest differences were found at C-17, C-21 and C-23 with Δδ 2.4–4 ppm, possibly due to the two isomers being hydroxy epimers at C-22. In addition, the ^1H NMR splitting patterns for H-22 were distinctive thus supporting this hypothesis, viz. compound **14** : H-22, 3.68, *ddd*, (J = 11.1, 2.4, 2.4 Hz); compound **15**: H-22, 3.58, *ddd* (J = 10.8, 7.0, 2.2 Hz).

A series of experiments involving derivatisation of compound **14** to the cyclic carbonate (**16**), molecular mechanics calculations and splitting pattern analysis were performed to assign the configurations at C-16 and C-22. Carbonate **16** was synthesised according to the method of Achenbach et al. (1996). Formation of the carbonate was confirmed by HRESIMS ($[M + H]^+$ *m/z* 455.3150 (calc. for $C_{29}H_{43}O_4$, 455.3161), the loss of O–H stretching bands at 3395 cm^{-1} and appearance of new absorbances at 1747 (C=O), 1220 and 1064 cm^{-1} (s, C–O) in the IR spectrum, the approximately 0.5 ppm downfield shifts

of H-16 and H-22 to δ 4.87 and 4.41, respectively, in the ^{1}H NMR spectrum and the appearance of the carbonate carbonyl carbon at 153.1 ppm in the ^{13}C NMR spectrum. NMR spectra of compound **16** in both $CDCl_3$ and benzene-d_6 were fully assigned (supplementary data Table S3).

The NOESY spectrum of **16** was measured in benzene-d_6. The key NOE correlations observed are shown in Figure 4. The cyclic carbonate extends the fused ring system of the steroid limiting rotation around C-17–C-20 and C-20–C-22. The NOEs observed are consistent with the standard steroid configuration with H-9, H-14 and H-17 appearing to be on the α-face of **16** and H-8, H_3-18 and H_3-19 on the β-face. H-16 has an NOE correlation with H-17, as well as one of the protons on C-15, indicating that it is on the α-face. The cyclic carbonate has therefore been formed from a β-hydroxy group at C-16 in compound **14**. The assignment is supported by the clear difference in splitting pattern for H-16 when the hydroxy group is β compared to α. All of the previously undescribed compounds reported here have a *ddd* splitting pattern with two moderate and one smaller coupling constant (*c.* J = 8, 7, 4 Hz) compared to the C-16 α-epimer which has no coupling constants greater than 7 Hz and one between 0 and 2.5 Hz (Miyaoka et al., 1997; Holland et al., 2009).

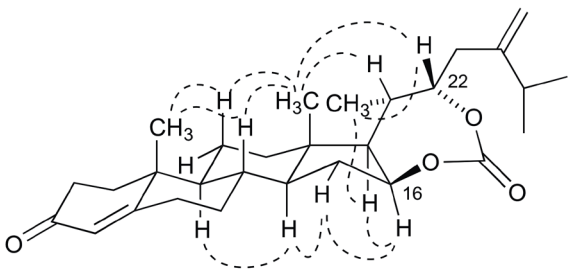


Fig. 4. Key NOESY correlations observed for the carbonate derivative of **14**, compound **16**.

H-17 has a NOESY correlation to H_3-21 while H_3-18 is correlated to the proton on C-20, as would be expected. The key NOESY correlation occurs between the angular

methyl group H_3-18 and the proton on C-22, placing it on the β-face of the ring system with the original hydroxy group, now participating in the cyclic carbonate, nominally originally on the α-face. Assuming that the normal absolute configuration for steroids applies, this makes compound **14** the 16*S*,22*S*-dihydroxy stereoisomer and compound **15** its 22*R*-epimer.

Modelling of both the 16*S*,22*R* and 16*S*,22*S*-carbonate stereoisomers using Spartan '04® supported this assignment. The most stable conformer (>99%) of the 22*R*-epimer has a dihedral angle of about 170° between H-20 and H-22 and thus a coupling constant of ~10 Hz, as predicted by the Karplus equation calculator Sweet J (Balacco, 1996). In the case of the 22*S*-epimer, modelling revealed the presence of two major conformations, viz. different gauche conformations of H-20 and H-22, ϕ = –48° and 70°, in a ratio of 40:60, respectively. These correspond to a coupling constant $^3J_{H\text{-}20,22}$ = ~2 Hz, in line with *J* = ~4 Hz (cf. ~10 Hz) measured for compound **16**. Interestingly, if one considers the two NOESY correlations observed between both H_3-18 and H-21 to H-22, no single conformation appears to be able to explain them both, however, the presence of the two almost equally probable conformations does.

Since compounds **14** and **15**, epimeric at C-22, have such distinctive splitting patterns for H-22, the molecular modelling and coupling constant analysis used for the carbonate derivative **16** was repeated for **14** and **15** to ascertain whether these patterns might be diagnostic for the particular C-22 epimer present. Each isomer appeared to have only one major conformation (>96% conformational bias) with $\phi_{H20,22}$ = 65° and 168° for compounds **14** and **15**, respectively, similar to those calculated for the equivalent cyclic carbonate derivatives. Since the dihedral angles from H-22 to the two protons on C-23 are always one *gauche* and one *anti*, regardless of which C-22 epimer is present, it appears that the third coupling constant, viz. $^3J_{H\text{-}20,22}$, can be used to distinguish between them (**14**: C-22*S*, δ_H 3.68, *ddd*, (*J* = 11.1, <u>2.4</u>, 2.4 Hz); **15**: C-22*R*, δ_H 3.58, *ddd* (*J* = 10.8, <u>7.0</u>, 2.2 Hz). To our knowledge this is the only case where steroidal hydroxy epimers at C-22 have been co-isolated and their configurations established unambiguously.

The molecular formula of compound **17** was determined to be $C_{29}H_{46}O_4$ by high-resolution mass spectrometry, which showed a quasi-molecular ion $[M + H]^+$ at *m/z*

459.3465 (calc. for $C_{29}H_{47}O_4$ 459.3469). The IR, ^{1}H and ^{13}C 1D and 2D NMR spectra of **17** were very similar to those of compounds **14** and **15**, despite the presence of additional equivalent of CH_2O in its molecular formula. The NMR data indicates that the steroid nucleus is identical to steroids **14** and **15** with the significant differences appearing in the signals ascribed to steroid side chain (C-20 to C-29) (supplementary data Table S4, Fig. S2). COSY correlations identified a spin system extending from H_3-21 (δ 0.88, *d*, *J* = 6.9 Hz) to H_2-23 (δ 1.75, *m*; 1.54, *br d*, *J* = 10.3 Hz) which included an oxymethine proton at δ 3.80 (*ddd*, *J* = 10.3, 2.3, 2.3 Hz, H-22). HMBC spectroscopy showed that H-22 is correlated with C-24, a downfield quaternary carbon atom at δ 79.8, which was also correlated with a vinylic proton (δ 5.73, *dd*, *J* = 17.4, 11.9 Hz, H-28) which was part of a new terminal alkene accounting for the extra CH_2 equivalent found in the molecular formula. The additional oxygen atom is clearly incorporated as a quaternary alcohol at C-24. The vinylic proton H-28 is also correlated with the C-25–C27 spin system at C-25 (δ 38.4, CH) completing the structure of the steroidal side chain. Thus, the structure of compound **17**, in comparison with **14**, has a vinyl group and an additional hydroxy group substituted at C-24, replacing the sp^2 methylene group (C-28).

The molecular formula of compound **18** was determined to be $C_{29}H_{46}O_4$ by high-resolution mass spectrometry showing a quasi-molecular ion $[M + Na]^+$ at *m/z* 481.3290 (calc. for $C_{29}H_{46}O_4Na$ 481.3288), isomeric with compound **17**. All spectra (IR, NMR) were very similar to those of **17** with the only differences in the NMR spectra of **18** being attributable to differences in the steroidal side chain (C-20 to C-29). COSY and HMBC spectroscopy indicated that both compounds had the same planar structure (supplementary data Table S5, Fig. S3). The major difference in ^{13}C NMR chemical shifts appears at C-25 (Δδ 3.9) and since the splitting pattern for H-22 is practically identical, the remaining chiral centre at C-24 in the side chain must be the point of difference. Compounds **17** and **18** are epimers at C-24.

The configuration at C-24 was determined by consideration of the differences of chemical shifts of the protons surrounding C-24. Since the splitting pattern of H-22 is almost identical in both compounds, it can be inferred that the difference in chemical shift of H-22 (Δδ 0.20) will be due to differences in configuration and conformation

occurring in the C-24–C-29 part of their structures. Molecular modelling of both epimers provides lowest energy conformations consistent with this inference. The lowest energy conformation of C-24*R* epimer shows that H-22 lies within the shielding cone of the alkene (Δ^{28}) whereas in the lowest energy conformation of the corresponding C-24*S* epimer the alkene is in a conformation where it is unlikely to influence the chemical shift of H-22 (supplementary data Fig. S4). Thus the signal for H-22 of the C-24*R* epimer (δ 3.80), being in the alkene shielding cone, will be relatively upfield from the signal for H-22 in the C-24*S* epimer (δ 4.00) making compound **17** (16*S*,22*S*,24*R*)-16,22,24-trihydroxyporifer-4,28-diene-3-one and compound **18** its *24S*-epimer.

An additional pair of epimers were also isolated and tentatively identified. Compounds **19** and **20** were isolated as a mixture. The 1D and 2D NMR spectra of the mixture suggested that the compounds are closely related and that the tetracyclic nucleus of both steroids in the mixture is identical to those of the steroids isolated previously. Many of the signals for the two compounds could be distinguished from each other through correlations appearing in the 2D NMR spectra and their ^{1}H NMR integrals; They appear to be present in a 2:1 ratio. The two compounds possess an ethenyl group on the steroidal side chain, similar to that present in compounds **17** and **18**, but lack the oxymethine carbon at C-22, while retaining the downfield quaternary carbon at δ_C 77.8 (C-24).

Akihisa et al. (1999) has reported the separation and assignment of configuration of C-24 epimeric sterols bearing the same 24-ethylene-24-hydroxy functionalities on the steroidal side chain from the climbing herb *Bryonia dioica*. The chemical shift differences in the ^{1}H and ^{13}C NMR spectra are, in our opinion, too small to confidently assign the configurations at C-24 to the major and minor components in the mixture reported here, however, the major component is tentatively assigned as (16*S*,24*R*)-16,24-dihydroxyporifera-4,28-dien-3-one (**19**) and the minor component as (16*S*,24*S*)-16,24-dihydroxystigma-4,28-dien-3-one (**20**).

Compound **21**'s molecular formula was determined to be $C_{29}H_{46}O_3$ by high-resolution mass spectrometry, which found a quasi-molecular ion $[M + H]^+$ at *m/z* 443.3518 (calc. for $C_{29}H_{47}O_3$ 443.3520), one oxygen atom less than compounds **17** and **18**. The IR and

^{1}H and ^{13}C NMR spectra of compound **21** are also very similar to **17** and **18**, differing substantially only from C-23 onward (Tables 1 and 2). Compared to **17** and **18**, the hydroxy group at C-24 is absent (C-24, δ_C 143.8 qC), accounting of the reduction in the number of oxygen atoms in the formula, and the double bond is in its biosynthetically original position at $\Delta^{24,28}$ and C-29 is a vinylic methyl group (δ_C 13.8, CH_3; δ_H 1.65 3H, *d*, *J* = 6.7 Hz). Compound **21** is thus (16*S*,22*S*,24*E*)-16,22-dihydroxystigma-4,24(28)-dien-3-one. The 2D NMR spectroscopic data obtained supports this assignment (supplementary data Fig. S5, Table S6).

The molecular formula of Compound **22** was determined to be $C_{28}H_{44}O_3$ by high-resolution mass spectrometry, which showed a quasi-molecular ion $[M + Na]^+$ at *m/z* 451.3182 (calc. for $C_{28}H_{44}O_3Na$ 451.3189). All spectra obtained from compound **22** (supplementary data Table S7, Fig. S7) indicated that it had the same steroidal nucleus as the others with a different side chain. Notably, a singlet methyl group appeared at δ_H 1.30 (H_3-21) replacing a methyl group doublet. This methyl group was correlated by HMBC spectroscopy to a new quaternary carbon atom (δ_C 76.6, C-20), clearly attached to an oxygen atom, accounting for the third oxygen atom present in the molecular formula. An sp^2 methylene group (C-28, δ_C 106.4, $=CH_2$; dH 4.73 (*br s*), 4.69 (*d*, 1.0 Hz), similar to compounds **14** and **15**, was found to be attached at C-24 (δ_C 156.3, qC=). The configuration at C-20 was determined to be *S* due its relatively downfield chemical shift at δ_H 1.30 since this methyl group appears at δ_H 1.10–1.20 when the configuration is *R* (Wu et al. 2009). Compound **22** is (16*S*,20*S*)-16,20-dihydroxyergosta-4,24(28)-dien-3-one.

Compounds **14**, **15**, **18**, **19** and **20**, and **21** were screened against twelve cancer cell lines: colon cancer HT29 and SW480, breast cancer MCF-7, ovarian cancer A2780, lung cancer H460, skin cancer A431, prostate cancer Dul45, neuroblastoma BE2-C, glioblastoma SJ-G2, SMA (murine) and U87, and pancreas MIA. Percentage cell growth inhibitions in response to 25 μM compound(s) are reported in Table 3. Where available, compounds showing moderate or better inhibition over a number of cell lines were re-screened to obtain GI_{50} (concentration that inhibits cell growth by 50%) values (Table 4). GI_{50} data for Irinotecan, a natural product derivative mainly used in the treatment of colon cancer, has been included in Table 4 for comparison. Compound **21**

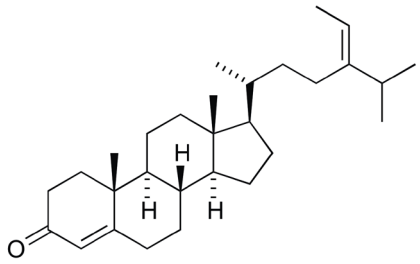
possesses the most potent cytotoxicity of the steroids in this series with GI_{50} 8.7 ± 0.7 μM against colon cancer HT29, GI_{50} 5.6 ± 0.8 μM against the breast cancer line MCF-7 and GI_{50} 4.5 ± 0.2 μM against the ovarian cancer cell line A2780 (Table 4). Significant activity was also observed for compound **15** against ovarian cancer (GI_{50} 6.2 ± 0.1 μM). Compounds **14**, **15** and **18** were found to be inactive against *Staphylococcus epidermidis*, *Enterococcus faecalis*, and *Moraxella catarrhalis* in an antibacterial disc diffusion assay.

3. Conclusions

The structures of six α,β-conjugated-3-keto-steroids hydroxylated at the C-16 position (compounds **14**, **15**, **17**, **18, 21** and **22)**, isolated from the brown alga *Cystophora xiphocarpa* (order Fucales, family Sargassaceae) have been elucidated. They are distinguished from each by oxidation and methylation of the steroidal side chain. The configuration of two isomeric steroids (**14** and **15**), epimeric at the hydroxy group on C-22 was able to be established unambiguously by combination of NMR spectroscopy, molecular mechanics calculations and derivatisation.

While these compounds were undescribed, as a class of bioactive steroids they are relatively unremarkable, joining a relatively small group isolated from a range of plant and algal sources. These compounds share hydroxylation at C-16 with the only other steroid thus far reported from the *Cystophora*, *C. brownii*.

A related steroid completely unadorned by hydroxy groups (**23**) isolated from the brown alga *Turbinaria conoides* has no significant activity against cancer cell lines, namely, HT-29, P-388, KB and A-549 (lung) (Sheu et al., 1999), while its 6β-hydroxy derivative does show significant activity, e.g. ED_{50} 2.9 μM against HT-29. From this, it appears that hydroxy group functionalisation of the steroid nucleus enhances bioactivity but their location and orientation may not be that important in these examples. The role of the hydroxy groups in enhancing cytotoxicity could be simply due to non-specific changes in steroid intermolecular non-bonding interactions.



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4. Experimental

4.1 General

Infrared spectra were recorded as casts on a NaCl disk on a Perkin-Elmer Paragon 1000 FT-IR spectrometer and Spectrum Two™ spectrometer. Optical rotations were measured with a Jasco P-2000 polarimeter fitted with a 0.98 mL volume cell. ^{1}H and ^{13}C NMR spectra were recorded either on a Bruker Avance DPX-300, Avance III 400 or 600 spectrometers in $CDCl_3$ using the standard pulse programs provided. NOESY spectra were obtained with mixing times 400, 800 and 1200 ms. High-resolution mass spectra were recorded on a Bruker BioApex 47 FT mass spectrometer with an electrospray (ESI) source; all ions were detected in positive ion mode. 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 x 250 mm, Luna 100 Å 10 mm (Phenomonex)]. Centrifugal chromatography was performed on a Chromatotron model L 7924T from Harrison Research Company, California, USA, with circular preparative layer chromatography plates coated with Merck TLC grade 7749 silica gel Kieselgel 60, PF_{254} containing gypsum (1 or 2 mm layer thickness). Acetone was re-distilled before use; Otherwise solvents were HPLC grade and used as supplied.

4.2 Plant material

The marine brown alga *Cystophora xiphocarpa* (Harvey) (Sargassaceae, Fucales) was collected intertidally from Spikey Beach, Tasmania (42°11'14"S 148°04'06"E) by hand between 0 m and 1 m depth in February, 2010. After collection, algae were stored on ice for transportation and held in a freezer (–20 °C) prior to extraction. The alga was readily identified by one of the authors (IvA) as the brown alga *Cystophora xiphocarpa* (Harvey), a member of the order Fucales and in the Sargassaceae family, formerly in Cystoseiraceae, (Womersley, 1987) and a voucher specimen (100204ST-007) was stored in the marine specimen collection in Chemistry, The University of Newcastle, Australia.

4.3 Molecular modelling

Molecular modelling was performed using Spartan '04 (Wavefunction Inc., Irvine, CA, USA). The initial structures were minimised by molecular mechanics (MMFF) and then the native Monte-Carlo search routine employed to find the 100 lowest energy conformers for each structure, again using MMFF to determine the energy of each conformer. The conformers within each group were arranged in increasing energy. In cases where the difference in relative energy between the lowest energy conformation and first significantly different conformation is of the order of 8–10 kJ/mol, it was assumed that the lowest energy conformation is largely responsible for producing the observed NOE correlations and ^{1}H NMR coupling constants.

4.4 Extraction and isolation

The alga *Cystophora xiphocarpa* (Harvey) (Sargassaceae, Fucales) was extracted by standing covered in acetone overnight three times and the combined extracts filtered through diatomaceous earth and evaporated under vacuum to provide an oily residue. Water soluble compounds were removed from the extract by liquid-liquid separation

(H_2O-$CHCl_3$), dried (anhyd. $MgSO_4$), followed by evaporation of the $CHCl_3$ under vacuum (yield 13.9 g, 2.3%, 604.5 g, based on dry extracted mass alga). A portion of the *C. xiphocarpa* extract (4.5 g) was subjected to short column vacuum chromatography (Harwood, 1985) and eluted with a stepped series of solvents Petroleum ether (PE)–CH_2Cl_2 (1:0 to 1:9), PE–EtOAc (1:9 to 1:0) and $CHCl_3$–MeOH (49:1 to 1:9) followed by separation with centrifugal chromatography by step gradient elution with solvents PE–EtOAc (19:1 to 2:3). This was then followed by either further centrifugal chromatography and/or a series of HPLC separations. Compound **14** was eluted with MeOH–isooctane–CH_2Cl_2 (2:25:73); **15** and **18** with MeOH–isooctane–CH_2Cl_2 (5:25:70); **17** with EtOAc–isooctane gradient from 1:9 to 3:1; **19** and **20** with EtOAc–isooctane gradient from 15:85 to 1:1; **21** with MeOH–isooctane–CH_2Cl_2 gradient from 1:25:74 to 4:25:71; **22** with MeOH–isooctane–CH_2Cl_2 (0.75:25:74.25).

*(16S,22S)-16,22-Dihydroxyergosta-4,24(28)-dien-3-one (**14**)*: White solid, 19.5 mg, R_f 0.41 (eluent EtOAc–light petroleum (2:5)), $[\alpha]_D^{21}$ +28 (c 0.21, $CHCl_3$), UV (MeOH–isooctane–CH_2Cl_2 (2:25:73); HPLC PDA) λ nm: 332.8, IR (cast) ν cm^{-1}: 3395 (O–H, br m), 3081 (=C–H, w), 2946 (>C–H, s), 1771 (m), 1667 (C=O, C=CH_2, s), 1614 (C=C, m), 1448 (m), 1378 (m), 1230 (m), 1036 (m), 754 (=C–H, s), ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2, ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1, HRESIMS *m/z* 429.3361 $[M+H]^+$ (calc. for $C_{28}H_{45}O_3$, 429.3369).

*(16S,22R)-16,22-Dihydroxyergosta-4,24(28)-dien-3-one (**15**)*: White solid, 7.4 mg, R_f 0.31 (eluent EtOAc–light petroleum (2:5)), $[\alpha]_D^{21}$ +71 (c 0.055, $CHCl_3$), UV (MeOH–isooctane–CH_2Cl_2 (5:25:70); HPLC PDA) λ nm: 330.4, IR (cast) ν cm^{-1}: 3435 (O–H, br m), 3080 (=C–H, w), 2946 (>C–H, s), 1767 (m), 1666 (C=O, s), 1613 (C=C, m), 1448 (m), 1377 (m), 1331 (m), 1243 (m), 1031 (m), 754 (=CH_2, m), ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2, ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1, HRESIMS *m/z* 429.3363 $[M+H]^+$ (calc. for $C_{28}H_{45}O_3$, 429.3369).

*(16S,22S,24R)-16,22,24-Trihydroxyporifera-4,28-dien-3-one (**17**)*: White solid, 2.0 mg, R_f 0.30 (eluent EtOAc–light petroleum (2:5)), $[\alpha]_D^{21}$ +59 (c 0.080, $CHCl_3$), UV (EtOAc–(1:1); HPLC PDA) λ nm: 324.5, IR (cast) ν cm^{-1}: 3383 (O–H, br m), 3086 (=C–H, w), 2946 (>C–H, s), 1776 (m), 1667 (C=O, s), 1614 (C=C, m), 1447 (m), 1384 (m), 1244 (m), 1159 (m), 755 (=CH_2, s), ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2, ^{13}C

NMR ($CDCl_3$, 75 MHz) see Table 1, HRESIMS *m/z* 459.3465 $[M+H]^+$ (calc. for $C_{29}H_{47}O_4$, 459.3469).

*(16S,22S,24S)-16,22,24-Trihydroxystigma-4,28-diene-3-one (**18**)*: White solid, 1.1 mg, R_f 0.27 (eluent EtOAc–light petroleum (2:5)), $[\alpha]_D^{21}$ +70 (c 0.05, $CHCl_3$), UV (MeOH–isooctane–CH_2Cl_2 (5:25:70); HPLC PDA) λ nm: 335.2, IR (cast) ν cm^{-1}: 3400 (O–H, br s), 3088 (=C–H, w,), 2945 (>C–H, s), 1769 (m), 1660 (C=O, s), 1613 (C=C, m), 1447 (m), 1383 (m), 1243 (m), 1032 (m), 753 (=CH_2, s), ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2, ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1, HRESIMS *m/z* 481.3290 $[M+Na]^+$ (calc. for $C_{29}H_{46}O_4Na$, 481.3288)

*(16S,24R)-16,24-Dihydroxyporifera-4,28-dien-3-one and (16S,24S)-16,24-dihydroxystigma-4,28-dien-3-one (**19** and **20**)*: White solid, 6.3 mg, R_f 0.44 (eluant MeOH–isooctane–CH_2Cl_2 (5:25:70)), $[\alpha]_D^{21}$ +50 (c 0.06, $CHCl_3$); UV (EtOAc–isooctane (2:5); HPLC PDA) λ nm: 322.1, IR (cast) ν cm^{-1}: 3417 (O–H, s), 3084 (=C–H, w), 2933 (>C–H, s), 1770 (m), 1731 (m), 1667 (C=O, s), 1614 (C=C, m), 1448 (m), 1232 (s), 1032 (m), 755 (=CH_2, s); ^{1}H NMR ($CDCl_3$, 300 MHz) δ (major/minor) 5.71 (*br s*, H-4), 5.84/5.72 (*dd*, *J* = 17.4/17.5, 10.8 Hz, H-28), 5.19/5.17 (*dd*, *J* = 17.4/17.6, 1.5 Hz, H-29$_Z$); 5.13/5.14 (*dd*, *J* = 10.8, 1.5 Hz, H-29$_E$), 1.84 (*m*, H-20), 1.78/1.71 (*m*, H-25), 0.94 (*s*, H-18), 1.17 (*s*, H-19), 0.87 (*d*, *J* = 6.9 Hz, H-27), 0.84 (*d*, *J* = 6.9 Hz, H-26). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (major/minor) 199.6 (*s*, C-3), 171.4 (*s*, C-5), 142.5 (*d*, C-28), 123.8 (*d*, C-4), 113.7 (*t*, C-29), 77.8 (*s*, C-24), 72.4 (*d*, C-16), 61.4 (*d*, C-17), 53.8/53.7 (*d*, C-9, 14), 42.2/42.1 (*s*, C-13), 39.7/39.6 (*t*, C-12), 38.6 (*s*, C-10), 36.4 (*d*, C-20), 36.1/35.9 (*t*, C-23), 35.7 (*t*, C-1), 35.2 (*d*, C-8, 25), 35.0 (*t*, C-15), 34.0 (*t*, C-6), 32.8 (*t*, C-2), 32.0 (*t*, C-7), 29.2/29.4 (*t*, C-22), 20.7 (*t*, C-11), 17.7/17.6 (*q*, C-26), 17.4 (*q*, C-19, 21), 16.5/16.6, (*q*, C-27), 13.1/13.2 (*q*, C-18).

*(16S,22S,24E)-16,22-Dihydroxystigma-4,24(28)-dien-3-one (**21**)*: White solid, 3.0 mg, R_f 0.29 (MeOH–isooctane–CH_2Cl_2 (2:25:73)), $[\alpha]_D^{21}$ +34 (c 0.050, $CHCl_3$), UV (MeOH–isooctane–CH_2Cl_2 (2:25:73); HPLC PDA) λ nm: 331.3, IR (cast) ν cm^{-1}: 3389 (O–H, m), 2951 (C–H, s), 1771 (m), 1668 (>C=O, s), 1614 (C=C, m), 1463 (m), 1382 (m), 1355 (m); ^{1}H NMR ($CDCl_3$, 300 MHz) see Table 2, ^{13}C NMR ($CDCl_3$, 75 MHz) see Table 1, HRESIMS *m/z* 443.3518 $[M + H]^+$ (calc. for $C_{29}H_{47}O_3$ 443.3520).

*(16S,20S)-16,20-Dihydroxyergost-4,24(28)-dien-3-one (**22**)*: White solid, 2.0 mg, R_f 0.42 (EtOAc–petroleum ether (3:7), $[\alpha]_D^{22}$ +30 (c 0.08, $CHCl_3$), UV (MeOH–isooctane–CH_2Cl_2 (0.75:25:74.25); HPLC PDA) λ nm: 331.3, IR (cast) ν cm^{-1}: 3329 (O–H, m), 3081 (=C–H), 2946 (C–H, s), 1661 (>C=O, s), 1615 (C=C, m), 1434 (m), 1230 (m), 1038 (m), 754 (m); ^{1}H NMR ($CDCl_3$, 600 MHz) see Table 2, ^{13}C NMR ($CDCl_3$, 150 MHz) see Table 1, HRESIMS *m/z* 451.3182 $[M + Na]^+$ (calc. for $C_{28}H_{44}O_3Na$ 451.3189).

*4.5 Derivatisation of compound **14** to form carbonate compound **16***

Compound **14** (5 mg) was dissolved in toluene (2 mL). To this phosgene ($COCl_2$, 250 μL, 20% in toluene) was added dropwise over 1 minute while stirring vigorously at room temperature. The reaction was monitored by TLC and after 3 hours the emergence of two products was observed. Additional phosgene solution (250 μL) was added dropwise over 1 minute and the reaction stirred over night. The products were purified by normal phase HPLC (EtOAc–2,2,4-trimethylpentane, gradient from 1:19 to 1:4 over 80 min) to yield compound **16** and a major by-product which appeared to be the carbonate dimer at C-16, as determined by HRESIMS ($[M + Na]^+$: 905.6264 (found), 905.6266 (calc. for $C_{57}H_{86}O_7Na$) and ^{1}H NMR spectroscopy. The only significant change observed in the ^{1}H NMR spectrum of compound **16** from that of compound **14** was the downfield shift of H-16 to δ 4.87 (1H, *m*) from δ 4.33 and H-22 to δ 4.41 (*ddd*, *J* = 9.0, 3.9, 3.9 Hz) from δ 3.68.

*(16S,22S)-Ergost-4,24(28)-dien-3-one-16,22-diyl carbonate (**16**)*: White solid; 2.2 mg, 42 % yield. R_f 0.66, eluent EtOAc–light petroleum (2:5), $[\alpha]_D^{21}$ –5.0 (c 0.10, $CHCl_3$). UV (HPLC PDA) λ_{max} 334.0 nm. IR (cast) ν cm^{-1}: 3083 (w,=C–H), 2937 (s), 1747 (s, C=O), 1673 (s, C=O, C=C), 1615 (C=C), 1449, 1354, 1220 (s, C–O), 1064 (s, C–O), 755 (m). ^{1}H NMR (300 MHz, C_6D_6): δ 0.32 (1H, *ddd*, *J* = 13.6, 10.7, 7.0 Hz, H-14), 0.45 (1H, *m*, H-9), 0.56 (1H, *m*, H-7b), 0.57 (3H, *d*, *J* = 6.7 Hz, H-21), 0.63 (3H, *s*, H-18), 0.64 (1H, *m*, H-12α), 0.70 (3H, *s*, H-19), 0.89 (1H, *dd*, *J* = 10.2, 7.2 Hz, H-17), 0.98 (1H, *m*, H-11α), 1.03 (3H, *d*, *J* = 6.9 Hz, H-26), 1.07 (1H, *m*, H-11β), 1.08 (3H, *d*,

J = 6.9 Hz, H-27), 1.11 (1H, *m*, H-8), 1.26 (3H, *m*, H-1b, H-6b,H-15α), 1.29 (1H, *m*, H-7a), 1.48 (1H, *m*, H-12β), 1.49 (1H, *m*, H-1a), 1.70 (1H, *m*, H-6a), 1.72 (1H, *ddd*, *J* = 13.6, 8.1, 7.4 Hz, H-15β), 2.03 (1H, *m*, H-20), 2.07 (1H, *m*, H-23b), 2.14 (1H, *m*, H-23a), 2.22 (1H, *m*, H-2b), 2.27 (1H, *m*, H-25), 2.32 (1H, *m*, H-2a), 4.21 (1H, *ddd*, *J* = 8.5, 4.0, 4.0 Hz, H-22), 4.49 (1H, *ddd*, *J* = 8.4, 7.3, 4.8 Hz, H-16), 4.99 (1H, *br s*, H-28$_E$), 5.01 (1H, *br s*, H-28$_Z$), 5.84 (1H, *br s*, H-4). ^{13}C NMR (75 MHz, C_6D_6,): δ 13.4 (q, C-18), 15.9 (q, C-21), 17.3 (q, C-19), 20.9 (t, C-11), 22.28 (q, C-27), 22.33 (q, C-26), 32.3 (t, C-7), 32.77 (t, C-15), 32.79 (d, C-20), 32.9 (t, C-6), 33.9 (d, C-25), 34.6 (t, C-2), 35.1 (d, C-8), 35.4 (t, C-23), 36.3 (t, C-1), 38.7 (s, C-10), 39.5 (t, C-12), 42.3 (s, C-13), 53.3 (d, C-14), 54.2 (d, C-9), 55.0 (d, C-17), 79.9 (d, C-16), 82.9 (d, C-22), 110.7 (t, C-28), 125.0 (d, C-4), 151.8 (s, C-24), 152.7 (s, O(C=O)O), 168.4 (s, C-5), 197.3 (s, C-3). HRESIMS *m/z* 455.3150 $[M + H]^+$ (calc. for $C_{29}H_{43}O_4$, 455.3161).

4.6 Growth Inhibition Assay

All cell lines were purchased from the American Type Culture Collection. 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 μL of medium at a density of 2,000–3,000 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 hrs 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. Initially, percentage growth inhibition was determined at a fixed drug concentration of 25 μM. A value of 100% is indicative of complete cell growth inhibition. Those compounds showing appreciable percentage growth inhibition underwent further analysis in order to calculate the concentration (μM) that induced

50% growth inhibition (GI_{50}) relative to untreated cells. To achieve this a dose-response curve was produced using eight drug concentrations in the range of 0.25-50 μM.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary data

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Table 1

^{13}C NMR Spectroscopic Data (75 MHz, $CDCl_3$) for the compounds **14**–**18**, **21**, and **22** (150 MHz, $CDCl_3$)

	14	**15**	**16**	**17**	**18**	**21**	**22**
C	δ, mult.	δ, mult.	δ, mult	δ, mult.	δ, mult.	δ, mult.	δ, mult.
1	35.7, CH_2	35.7, CH_2	35.7, CH_2	35.7, CH_2	35.7, CH_2	35.7, CH_2	35.6, CH_2
2	34.0, CH_2	34.0, CH_2	33.9, CH_2	32.9, CH_2	34.0, CH_2	34.0, CH_2	34.0, CH_2
3	199.6, qC	199.6, qC	199.3, C=O	199.6, qC	199.6, qC	199.5, qC	199.6, qC
4	123.8, CH	123.8, CH	124.1, CH	123.8, CH	123.8, CH	123.8, CH	123.9, CH
5	171.4, qC	171.4, qC	170.4, qC	171.5, qC	171.5, qC	171.9, qC	171.1, qC
6	32.9, CH_2	32.9, CH_2	32.6, CH_2	34.0, CH_2	32.9, CH_2	32.9, CH_2	32.8, CH_2
7	32.0, CH_2	32.0, CH_2	31.9, CH_2	32.0, CH_2	32.0, CH_2	32.0, CH_2	31.8, CH_2
8	35.3, CH	35.2, CH	34.8, CH	35.3, CH	34.7, CH	35.3, CH	34.6, CH
9	53.8, CH	53.7, CH	53.8, CH	53.9, CH	53.8, CH	53.9, CH	53.8, CH
10	38.6, qC	38.6, qC	38.6, qC	38.6, qC	38.6, qC	38.6, qC	38.6, qC

11	20.8, CH_2	20.8, CH_2	20.5, CH_2	20.8, CH_2	20.8, CH_2	20.8, CH_2	20.7, CH_2
12	40.0, CH_2	40.2, CH_2	39.1, CH_2	40.0, CH_2	40.0, CH_2	40.0, CH_2	40.2, CH_2
13	42.7, qC	43.0, qC	42.1, qC	42.7, qC	42.6, qC	42.8, qC	43.0, qC
14	53.8, CH	53.6, CH	53.3, CH	53.8, CH	53.8, CH	53.8, CH	53.9, CH
15	37.1, CH_2	35.3, CH_2	32.6, CH_2	35.1, CH_2	35.2, CH_2	35.1, CH_2	37.3, CH_2
16	71.7, CH	72.5, CH	80.2, CH	71.6, CH	71.6, CH	71.9, CH	74.0, CH
17	57.1, CH	61.1, CH	54.6, CH	57.6, CH	57.2, CH	57.2, CH	60.4, CH
18	12.9, CH_3	13.0, CH_3	13.1, CH_3	13.0, CH_3	13.0, CH_3	12.9, CH_3	14.9, CH_3
19	17.4, CH_3	17.4, CH_3	17.4, CH_3	17.3, CH_3	17.4, CH_3	17.4, CH_3	17.3, CH_3
20	33.4, CH	35.9, CH	34.9, CH	34.3, CH	34.7, CH	33.5, CH	76.6, qC
21	17.0, CH_3	14.7, CH_3	15.8, CH_3	17.4, CH_3	16.8, CH_3	17.1, CH_3	26.6, CH_3
22	74.6, CH	73.1, CH	83.4, CH	75.1, CH	73.8, CH	75.9, CH	42.4, CH_2
23	37.1, CH_2	40.4, CH_2	34.6, CH_2	36.7, CH_2	38.7, CH_2	30.8, CH_2	29.3, CH_2
24	153.2, qC	153.3, qC	150.8, qC	79.8, qC	78.8, qC	143.8, qC	156.3, qC

25	33.2, CH	33.2, CH	32.3, CH	38.4, CH	34.5, CH	33.6, CH	33.9, CH
26	22.3, CH_3	22.3, CH_3	21.8, CH_3	17.4, CH_3	17.8, CH_3	22.9, CH_3	21.9, CH_3
27	21.5, CH_3	21.5, CH_3	21.6, CH_3	16.3, CH_3	16.4, CH_3	21.9, CH_3	21.9, CH_3
28	110.4, CH_2	110.9, CH_2	110.1, CH_2	140.6, CH	141.3, CH	120.4, CH	106.4, CH_2
29				114.5, CH_2	113.8, CH_2	13.8, CH_3	
OCO_2			153.1, C=O				

Carbon multiplicities were determined from DEPT-90 and -135 spectra

Table 2

^{1}H NMR (300 MHz, $CDCl_3$) spectroscopic data for compounds **14**–**18**, **21**, and **22** (600 MHz, $CDCl_3$)

C	**14**	*J* (Hz)[a]	**15**	*J* (Hz)	**16**	*J* (Hz)	**17**	*J* (Hz)	**18**	*J* (Hz)	**21**	*J* (Hz)	**22**	*J* (Hz)
1	1.99		1.99		1.97		1.98		1.98		1.99		2.01	
	1.63		1.69		1.64		1.65		1.68		1.67		1.67	*td*, 10.8, 3.8
2	2.45		2.36		2.35		2.36		2.34		2.37		2.41	
	2.33		2.26				2.24	*d*, 7.3	2.22		2.26		2.33	
4	5.71	*br s*	5.71	*br d*, 0.9	5.72,	*br s*	5.71	*br s*	5.70	*br s*	5.71	*br s*	5.71	*s*
6	2.40		2.40		2.40		2.39		2.36		2.35		2.39	
	2.28		2.26		1.57		2.32		2.24		2.27		2.26	
7	1.88		1.85		1.81		1.84		1.84		1.86		1.83	
	1.04		1.01		1.02		0.97		0.98		1.00		1.01	
8	1.61		1.60		1.61		1.58		1.60		1.61		1.66	
9	0.90		0.90		0.91		0.89		0.88		0.92		0.91	
11a	1.49		1.49		1.53		1.48		1.47		1.48		1.52	
11b	–		1.41		1.47		1.43		1.43		1.43		1.50	
12a	1.94	*d*, 11.0	2.06		1.90		1.94	*d*, 13.0	1.94		1.99		2.16	*dt*, 12.5, 3.3

12b	1.12		1.13		1.14		1.07		1.08		1.13		1.17	
14	0.86		0.90		0.98		0.89		0.88		0.99		0.89	
15a	2.21		2.22		2.27		2.20		2.20		2.20		2.25	
15b	1.27		1.22		1.54		1.22		1.24		1.26		1.33	*td*, 11.3, 4.5
16	4.33	*ddd*, 8.1, 6.8, 4.7	4.39	*ddd*, 8.1, 7.1, 5.1	4.87,	*m*	4.36	*ddd*, 7.8, 6.9, 4.7	4.34	*ddd*, 7.8, 6.9, 4.7	4.38	*ddd*, 7.9, 6.8, 4.7	4.63	*ddd*, 7.4, 7.4, 4.5
17	1.14		1.10		1.39		1.06		1.09		1.23		1.23	
18	0.96	*s*	0.95	*s*	0.90	*s*	0.94	*s*	0.94	*s*	0.96	s	1.19	s
19	1.18	*s*	1.18	*s*	1.18,	*s*	1.17	*s*	1.17	*s*	1.18	s	1.19	s
20	2.43		2.10		2.23		2.26		2.29		2.19		-	
21	1.01	*d*, 7.2	0.99	*d*, 7.1	1.02	*d*, 6.8	0.88	*d*, 6.9	0.96	*d*, 7.0	1.02	*d*, 6.8	1.30	
22	3.68	*ddd*, 11.1, 2.4, 2.4	3.58	*ddd*, 10.8, 7.0, 2.2	4.41	*ddd*, 9.0, 3.9, 3.9	3.80	*ddd*, 10.3, 2.3, 2.3	4.00	*ddd*, 10.1, 2.3, 2.3	3.68	*ddd*, 11.2, 2.3, 2.3	1.84	
23a	2.29		2.51	*dt*, 14.1, 1.6	2.30		1.75		1.83		2.49		2.11	
23b	2.08		1.94		1.64		1.54	*br d*, 10.3	1.56		2.03		2.03	
25	2.22		2.20		2.32	*m*	1.67		2.00		2.41		2.24	
26	1.03	*d*, 6.8	1.03	*d*, 6.8	1.03	*d*, 6.8	0.89	*d*, 6.9	0.87	*d*, 7.0	1.00	*d*, 6.8	1.02	*d*, 7.8

27	1.06	*d*, 6.8	1.06	*d*, 6.8	1.05	*d*, 6.8	0.91	*d*, 6.9	0.87	*d*, 7.0	1.03	*d*, 6.8	1.02	*d*, 7.8
28a	4.95	*t*, 1.2	4.98	*t*, 1.2	4.90	*br s*	5.73	*dd*, 17.4, 11.9	5.95	*dd*, 17.4, 10.9	5.54	*q*, 6.7	4.73	*br s*
28b	4.82	*br s*	4.83	*br s*	4.86	*br s*							4.69	*d*, 1.0
29*E*							5.28	*dd*, 11.9, 1.2	5.20	*dd*, 10.9, 1.1	1.65 (3H)	*d*, 6.7		
29*Z*							5.24	*dd*, 17.4, 1.2	5.26	*dd*, 17.4, 1.1				
OH													3.62	*br s*
OH													2.94	*br s*

[a] Where no entry has been made, the nature of the splitting pattern of the signal could not be discerned due to heavy overlapping from other signals in the ^{1}H NMR spectrum.

Table 3: Percentage (%) of cell growth inhibition in response to 25μM drug.

cell line	**HT29** *Colon*	**SW480** *Colon*	**MCF-7** *Breast*	**A2780** *Ovarian*	**H460** *Lung*	**A431** *Skin*	**Du145** *Prostate*	**BE2-C** *Neuroblastoma*	**SJ-G2** *Glioblastoma*	**SMA** *Glioblastoma (Murine)*	**U87** *Glioblastoma*	**MIA** *Pancreas*
replicates	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 3	n = 2	n = 3
Compound												
14	52 ± 4	<0	38 ± 1	51 ± 2	2 ± 4	7 ± 3	<0	20 ± 18	<0	5 ± 2	<0	15 ± 3
15	99 ± 2	<0	25 ± 20	45 ± 24	7 ± 8	30 ± 15	<0	32 ± 23	1 ± 20	11 ± 10	<0	17 ± 6
18	71 ± 2	56 ± 7	68 ± 6	72 ± 2	46 ± 2	27 ± 4	<0	77 ± 5	46 ± 1	52 ± 3	9 ± 3	47 ± 1
19 and **20** (2:1)	100 ± 1	72 ± 8	55 ± 6	53 ± 4	45 ± 3	31 ± 4	3 ± 10	5 ± 10	43 ± 4	42 ± 5	<0	23 ± 2
21	65 ± 40	11 ± 11	76 ± 40	58 ± 29	33 ± 16	42 ± 20	22 ± 5	69 ± 35	33 ± 26	38 ± 20	<0	49 ± 22

Table 4: The GI_{50} (μM)* for compounds **15**, **19** and **20**, **21** and Irinotecan.

Cell line **Compound**	**HT29** *Colon*	**SW480** *Colon*	**MCF-7** *Breast*	**A2780** *Ovarian*	**H460** *Lung*	**A431** *Skin*	**Du145** *Prostate*	**BE2-C** *Neuroblastoma*	**SJ-G2** *Glioblastoma*	**SMA** *Glioblastoma (Murine)*	**U87** *Glioblastoma*	**MIA** *Pancreas*
15	18 ± 0 (n = 2)	>50 (n =2)	21 ± 6 (n = 2)	6.2 ± 0.1 (n = 2)	34 ± 1 (n = 2)	28 ± 0 (n = 2)	>50 (n = 2)	33 ± 1 (n = 2)	40 ± 1 (n = 2)	31 ± 1 (n = 2)	>50 (n = 2)	35 ± 2 (n = 2)
19 and **20** (2:1)	15 ± 1 (n = 3)	21 ± 3 (n = 3)	28 ± 0 (n = 3)	14 ± 8 (n = 3)	23 ± 3 (n = 3)	30 ± 1 (n = 3)	34 ± 2 (n = 3)	32 ± 1 (n = 3)	28 ± 0 (n= 3)	26 ± 3 (n = 3)	41 ± 1 (n = 3)	33 ± 2 (n = 3)
21	8.7 ± 0.7 (n = 2)	36 ± 2 (n = 2)	5.6 ± 0.8 (n = 2)	4.5 ± 0.2 (n = 2)	29 ± 1 (n = 2)	22.0 ± 0.5 (n = 2)	34 ± 2 (n = 2)	25.0 ± 2.5 (n = 2)	30.0 ± 1.5 (n = 2)	20.0 ± 0.5 (n = 2)	20 ± 5 (n = 2)	18.0 ± 0.5 (n = 2)
Irinotecan	9.3 ± 0.4 (n = 3)	18 ± 3 (n = 3)	5.0 ± 0.0 (n = 3)	1.0 ± 0.0 (n = 3)	3.3 ± 0.9 (n = 3)	3.2 ± 0.4 (n = 3)	1.5 ± 0.2 (n = 3)	1.5 ± 0.1 (n = 3)	1.5 ± 0.0 (n = 3)	2.9 ± 0.4 (n = 3)	15 ± 3 (n = 3)	9.2 ± 0.4 (n = 3)

* lowest concentration that inhibits cell growth by 50 %

Supplementary Data

Bioactive α,β-conjugated 3-keto-steroids from the Australian brown alga *Cystophora xiphocarpa*

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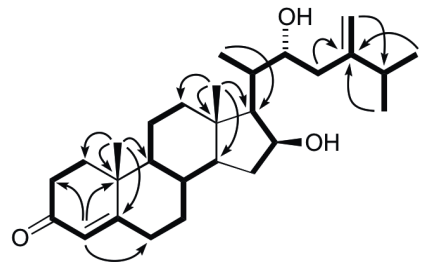


Fig. S1. Key $^{1}H,^{1}H$ COSY (—) and HMBC (H→C) correlations found for **15**.

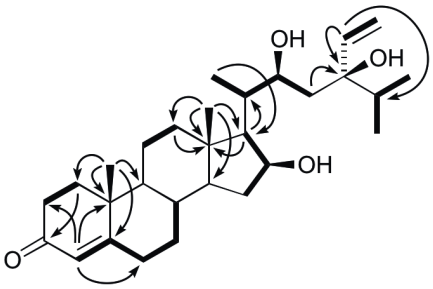


Fig. S2. Key $^{1}H,^{1}H$ COSY (—) and HMBC (H→C) correlations found for **17**

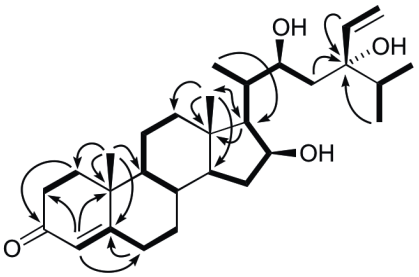


Fig. S3. Key $^{1}H,^{1}H$ COSY (—) and HMBC (H→C) correlations found for **18**

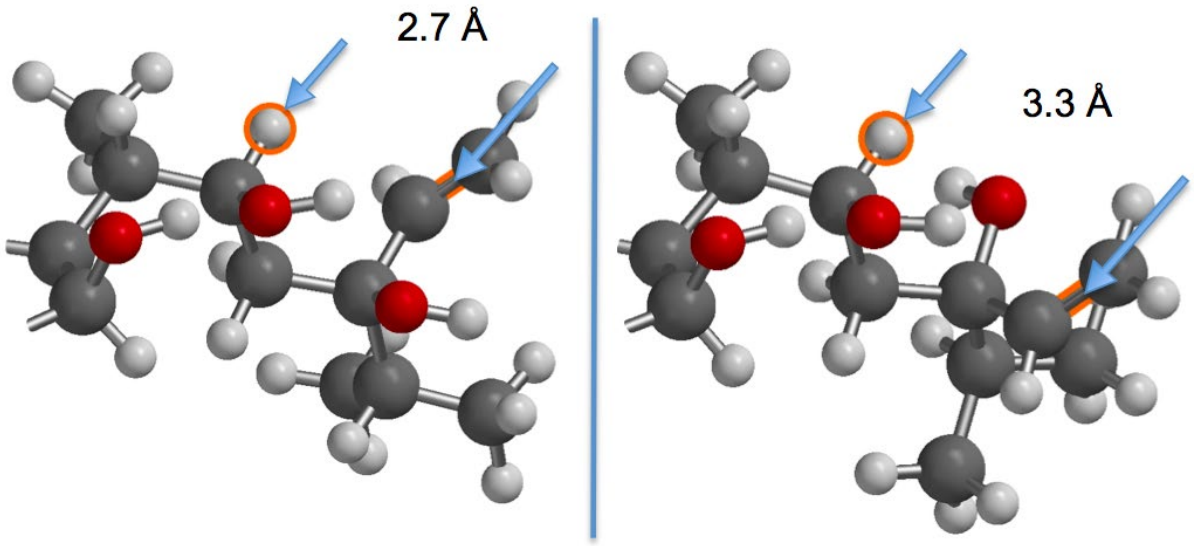


Fig. S4. Lowest energy conformations obtained from molecular modelling of compounds **17**, C-24*R* (left), and **18,** 24*S* (right). The figure shows partial structures C-16, C-17, C-20–C29.

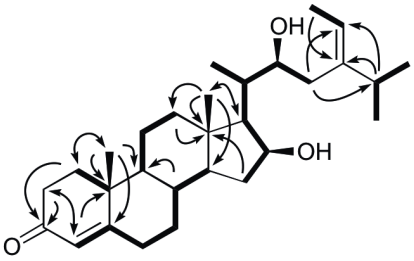


Fig. S5. Key $^{1}H,^{1}H$ COSY (—) and HMBC (H→C) correlations for **21**

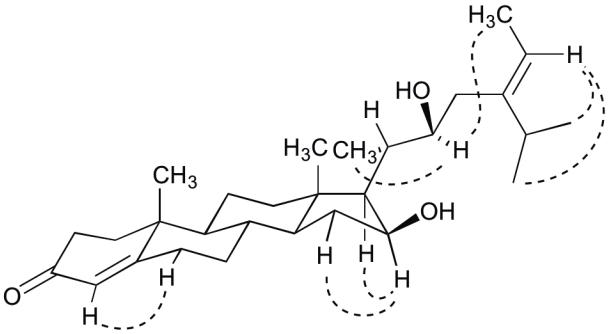


Fig. S6. Key NOESY correlations observed for compound **21**

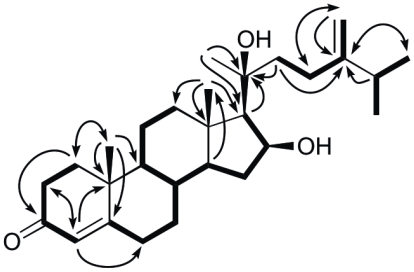


Fig. S7. Key $^{1}H,^{1}H$ COSY (—) and HMBC (H→C) correlations for **22**

Figure S8. Molecular models of compound **16**, carbonate derivatives of **14** and **15**-— Extract from PhD Thesis (Holland, I.P., 2011, 1. Dynamin-I Inhibitors from Sessile Marine Invertebrates; 2. Chemotaxonomy of *Cystophora* spp. The University of Newcastle, Australia. pp. 156–158)

"3.6.5.2 Stereochemistry at C-22

Upon inspection of the molecular modelling results of compound [the C-22*R* epimer] it was immediate apparent that significant energy differences between the conformational families of the 16*S*,22*R* diastereomer do occur, with a difference of 27.7 kJ/mol between the most stable and next most stable conformations. In this case the molecule would exist in its lowest energy conformation at > 99 %, thus only the lowest energy conformation would be responsible for the observed correlations. The molecular model, [below], indicated that a positive NOE between H_3-18 and H-22 was highly unlikely to exist as the predicted distance is > 5.1 Å and the relaxation pathway would be obstructed by H-20.

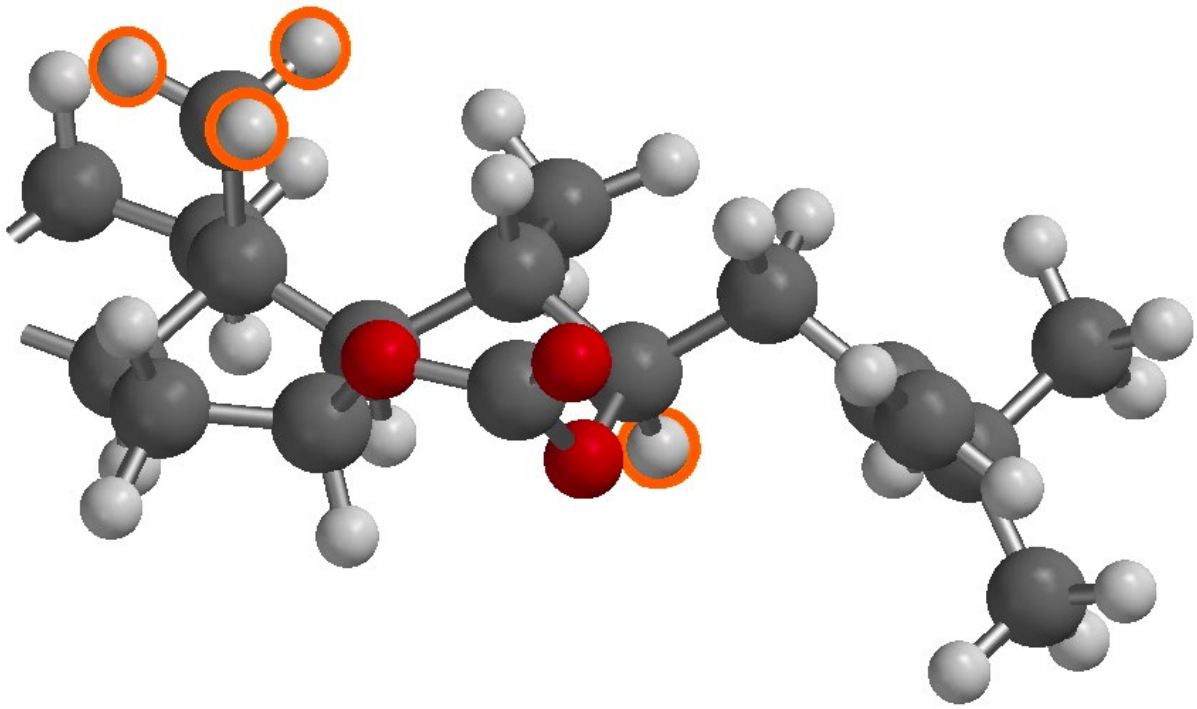


Figure …: Molecular modelling structure of the D- and E-rings of the lowest energy configuration of the 16*S*,22*R* diastereomer of compound [the C-22*R* epimer]. An NOE between H_3 -18 and H-22 (both highlighted in orange) is unlikely to exist due to the large distance (> 5.1 Å) and obscured NOE relaxation pathway.

The relative energy difference between the energies of the lowest two conformational families of the alternative diastereomer 16*S*,22*S* was only 1.7 kJ/mol as its E-ring conformation changed from chair to boat. The energy level difference to the third lowest energy conformational family was large, hence all observed data was considered from these two conformational families. As this diastereomer would only exist in its two lowest energy conformation, E-ring chair, for approximately 60 % of the time with the E-ring boat conformation accounting for the majority of the remainder (Figure [below]), the ensuing discussion of stereochemistry took both conformational families into account. This 60:40 split in conformation distribution explained the observed NOESY correlations.

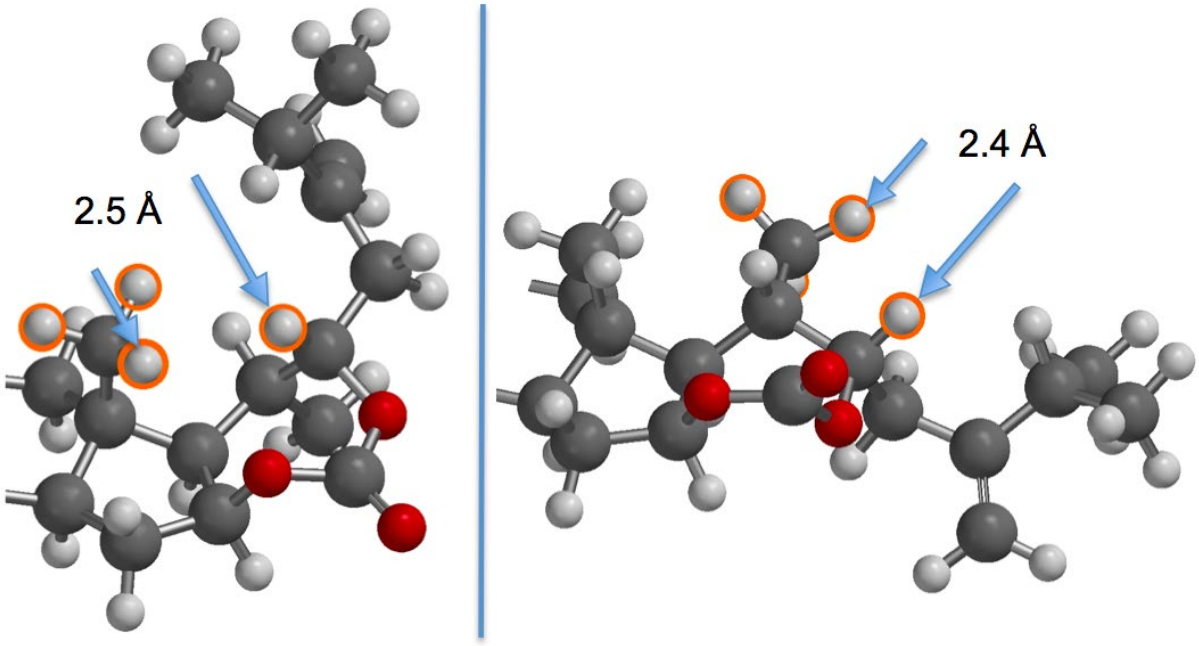


Figure …: The D- and E-rings and orientation of the steroidal side chain of compound [the C-22*S* epimer] in the compound's two lowest conformations are shown. The chair conformation of the E-ring (left) has a lower relative energy than the boat conformation (right) by 1.7 kJ/mol. The protons highlighted in orange show the observed NOESY correlations, H-22 – H_3 -18 on the left and H-22 – H_3 -21 on the right.

The molecular modelling demonstrated that when the seven-membered ring was in the lowest energy (chair) conformation then protons H_3-18 and H-22 could actually be as close as 2.5 Å (Figure [above]), a distance small enough for an NOE should this be a relaxation pathway. In this conformation the observed NOE between H-22 and H_3-21 would not be possible as the protons are *trans*-diaxial with respect to each other. Only when the seven-membered ring is in a boat conformation (Figure [above]) is the NOE correlation between H-22 to H_3-21 likely to exist. These key NOE correlations in conjunction with the molecular modelling of compound [**16**] strongly suggested 22*S* stereochemistry."

Table S1. ^{1}H and ^{13}C NMR data of compound **14** with supporting 2D correlation information.

position	^{13}C	mult.	^{1}H	*mult., J* (Hz)	COSY	gHMBC
1a	35.7	CH_2	1.99			5
1b			1.63		2b	
2a	34.0	CH_2	2.45		4	
2b			2.33		1b, 4	
3	199.6	qC				
4	123.8	CH	5.71	*br s*	2a, 2b, 6a, 6b	6, 10
5	171.4	qC				
6a	32.9	CH_2	2.40		4, 7a	
6b			2.28		4	
7a	32.0	CH_2	1.88		6a, 8	
7b			1.04			
8	35.3	CH	1.61		7a, 14	
9	53.8	CH	0.90		11	11
10	38.6	qC				
11	20.8	CH_2	1.49		9, 12a, 12b	
12a	40.0	CH_2	1.94	*d*, 11.0	11	
12b			1.12		11	
13	42.7	qC				
14	53.3	CH	0.86		8, 15a, 15b	7 (w)
15a	35.1	CH_2	2.21		14, 16	
15b			1.27		14, 16	
16	71.7	O–CH	4.33	*ddd*, 8.1, 6.8, 4.7	15a, 15b, 17	
17	57.1	CH	1.14		16, 20	
18	12.9	CH_3	0.96	*s*		12, 13, 14, 17
19	17.4	CH_3	1.18	*s*		1, 5, 9, 10
20	33.4	CH	2.43		17, 21, 22	
21	17.0	CH_3	1.01	*d*, 7.2	20	17, 22
22	74.6	O–CH	3.68	*ddd*, 11.1, 2.4, 2.4	20, 23a, 23b	
23a	37.1	CH_2	2.29		22, 28b	
23b			2.08		22, 28b	
24	153.2	qC				
25	33.2	CH	2.22		26, 27, 28a	
26	22.6	CH_3	1.03	*d*, 6.8	25	24, 25, 27
27	21.5	CH_3	1.06	*d*, 6.8	25	24, 25, 26
28a	110.4	CH_2	4.95	*t*, 1.2	25	23, 25
28b			4.82	br s	23a, 23b	25

Spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$; (w) = weak.

Table S2. ^{1}H and ^{13}C NMR Spectroscopic Data of Compound **15** with supporting 2D correlation information.

position ^{13}C		mult.	^{1}H	*mult., J* (Hz)	COSY-45	gHMBC
1a	35.7	CH_2	1.99		1b, 2a	3, 10
1b			1.69		1a	10
2a	34.0	CH_2	2.36		1a, 4	3
2b			2.26			
3	199.6	qC				
4	123.8	CH	5.71	*br s*	6a, 6b	2, 6, 10
5	171.3	qC				
6a	32.9	CH_2	2.40		4, 6b, 7a	4, 10
6b			2.26		4, 6a, 7a	
7a	32.0	CH_2	1.85		6a, 6b, 7b, 8	
7b			1.01		7a	
8	35.2	CH	1.60		7a, 9, 14	
9	53.7	CH	0.90		8, 11a	
10	38.6	qC				
11a	20.8	CH_2	1.49		9, 12a	
11b			1.41			
12a	40.2	CH_2	2.06		11a, 12b	13
12b			1.13		12a	
13	43.0	qC				
14	53.6	CH	0.90		8	
15a	35.3	CH_2	2.22		15b, 16	13
15b			1.22		15a, 16	16
16	72.5	O–CH	4.39	*ddd*, 8.1, 7.1, 5.1	15a, 15b, 17	
17	61.1	CH	1.10		16, 20	13, 16, 18, 20, 22
18	13.0	CH_3	0.95	*s*		12, 13, 14, 17
19	17.4	CH_3	1.18	*s*		1, 5, 9, 10
20	35.9	CH	2.10		17, 21, 22	22
21	14.7	CH_3	0.99	*d*, 7.1	20	17, 20, 22
22	73.1	O–CH	3.58	*ddd*, 10.8, 7.0, 2.2	20, 23a, 23b	
23a	40.4	CH_2	2.51	*dt*, 14.1, 1.6	22, 23b, 28a, 28b	22, 24, 28
23b			1.94		22, 23a, 28b	22, 24, 28
24	153.3	qC				
25	33.2	CH	2.20		26, 27, 28a, 28b	
26	22.3	CH_3	1.03	*d*, 6.8	25	24, 25, 27
27	21.5	CH_3	1.06	*d*, 6.8	25	24, 25, 26
28a	110.9	CH_2	4.98	*br s*	23a, 25, 28	23, 25
28b			4.83	br s	23a, 23b, 25, 28a	23, 25

Spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$.

Table S3. Comparison of the NMR spectroscopic data of the compound **16** in $CDCl_3$ and C_6H_6.

position	δ_C, mult. ($CDCl_3$)	δ_H, *J* (Hz) ($CDCl_3$)	δ_C, mult. (C_6D_6)	δ_H, *J* (Hz) (C_6D_6)	$^1H,^1H$ COSY ($CDCl_3$)	$^1H,^1H$ COSY (C_6D_6)	HMBC ($CDCl_3$)	HMBC (C_6D_6)	NOE (C_6D_6)
1a	35.7, CH_2	1.97	36.3, CH_2	1.49	2		2, 10		1b
1b		1.64		1.26					1a
2a	33.9, CH_2	2.35	34.6, CH_2	2.32	1a				
2b				2.22					
3	199.3, C=O		197.3, C=O						
4	124.1, CH	5.72, *br s*	125.0, CH	5.84, *br s*				2, 6, 10	
5	170.4, qC		168.4, qC						
6a	32.6, CH_2	2.40	32.9, CH_2	1.70					
6b		1.57		1.26				8	
7a	31.9, CH_2	1.81	32.3, CH_2	1.29		8, 14			
7b		1.02		0.56					
8	34.8, CH	1.61	35.1, CH	1.11		7a, 14			18, 19
9	53.8, CH	0.91	54.2, CH	0.45	11a, 12b, 19	11a			14
10	38.6, qC		38.7, qC						
11β	20.5, CH_2	1.53	20.9, CH_2	1.07	9, 12a	9, 12a, 12b			12β, 18, 19
11α		1.47		0.98	12a	12a, 12b		9	
12β	39.1, CH_2	1.90	39.5, CH_2	1.48	11a, 11b, 12b	11a, 11b			11β, 18
12α		1.14		0.64	9, 12a	11a, 11b			17
13	42.1, qC		42.3, qC						
14	53.3, CH	0.98	53.3, CH	0.32, *ddd*, 13.6, 10.7, 7.0		7a, 8, 15a, 15b			9
15β	32.6, CH_2	2.27	32.8, CH_2	1.72, *ddd*, 13.6, 8.1, 7.4	16	14, 16	16	14, 16	15α
15α		1.54		1.26	16	14, 16			14, 15β, 16
16	80.2, O–CH	4.87, *m*	79.9, O–CH	4.49, *ddd*, 8.4, 7.3, 4.8	15a, 15b, 17	15a, 15b, 17			15α, 17

Table S3 is continued on the following page.

Table S3 continued

17	54.6, CH	1.39	55.0, CH	0.89, *dd*, 10.2, 7.2	16	16, 20	13	13	12α, 16, 21
18	13.1, CH_3	0.90, *s*	13.4, CH_3	0.63, *s*			12, 13, 14, 17	12, 13, 14, 17	8, 11β, 12β, 20, 22
19	17.4, CH_3	1.18, *s*	17.3, CH_3	0.70, *s*	9		1, 5, 9, 10	1, 5, 9, 10	8, 11β,
20	34.9, CH	2.23	32.8, CH	2.03	21, 22	17, 21, 22			18, 21, 22, 23a
21	15.8, CH_3	1.02, *d*, 6.8	15.9, CH_3	0.57, *d*, 6.7	20	20	17, 22	10, 20, 22	17, 20, 22
22	83.4, O–CH	4.41, *ddd*, 9.0, 3.9, 3.9	82.9, O–CH	4.21, *ddd*, 8.5, 4.0, 4.0	20, 23a	20, 23a, 23b			18, 20, 21, 23a, 23b
23a	34.6, CH_2	2.30	35.4, CH_2	2.14	22	22, 28_Z, 28_Z	22, 24, $28_{E/Z}$	22, 24, 25, $28_{E/Z}$	20, 22, 28_Z, 26, 27
23b		1.64		2.07		22			22
24	150.8, qC		151.8, qC						
25	32.3, CH	2.32, *m*	33.9, CH	2.27	26, 27	26, 27, 28_Z		26, 27	28a, 26, 27
26	21.8, CH_3	1.03, *d*, 6.8	22.33, CH_3	1.03, *d*, 6.9	25, 27	25	24	24, 25, 27	23a, 25, 28_E
27	21.6, CH_3	1.05, *d*, 6.8	22.28, CH_3	1.08, *d*, 6.9	25, 26	25	24	24, 25, 26	23a, 25, 28_E
28_Z	110.1, CH_2	4.90, *br s*	110.7, CH_2	5.01, *br s*	28_E	25	23a	24, 25	25, 26, 27
28_E		4.86, *br s*		4.99, *br s*	28_Z	23a	23a	24, 25	23a
29	153.1, C=O		152.7, C=O						

Unassigned diastereotopic hydrogen atoms are distinguished by 'a' further downfield than 'b'.

Table S4. ^{1}H and ^{13}C NMR Spectroscopic Data of Compound 17 with supporting 2D correlation information.

position	^{13}C	mult.	^{1}H	*mult., J* (Hz)	COSY-45	gHMBC
1a	35.7	CH_2	1.98		1b, 2a	3, 10
1b			1.65		1a, 2a, 19	3
2a	32.9	CH_2	2.36		1a, 1b	3
2b			2.24	*d*, 7.3		
3	199.6	qC				
4	123.8	CH	5.71	*br s*	6a	2a, 6a, 10
5	171.5	qC				
6a	34.0	CH_2	2.39		4, 7a	7(w)
6b			2.32			
7a	32.0	CH_2	1.84		6a, 8	
7b			0.97			
8	35.3	CH	1.58		7a	9 or 14
9	53.9	CH	0.89			
10	38.6	qC				
11a	20.8	CH_2	1.48		12a	
11b			1.43			
12a	40.0	CH_2	1.94	*d*, 13.0	11a	11, 13
12b			1.07			13
13	42.7	qC				
14	53.8	CH	0.89			
15a	35.1	CH_2	2.20		15b, 16	
15b			1.22		15a, 16	16
16	71.6	O–CH	4.36	*ddd*, 7.8, 6.9, 4.7	15a, 15b, 17	
17	57.6	CH	1.06		16	12, 13, 16, 20
18	13.0	CH_3	0.94	*s*	12, 13, 14, 17	
19	17.3	CH_3	1.17	*s*	1b	1, 5, 9, 10
20	34.3	CH	2.26		21, 22	
21	17.4	CH_3	0.88	*d*, 6.9	20	17, 20, 22
22	75.1	O–CH	3.80	*ddd*, 10.3, 2.3, 2.3	20, 23a, 23b	
23a	36.7	CH_2	1.75	*d*, 10.3	22	22, 24, 28
23b			1.54		22	22, 24, 28
24	79.8	O–qC				
25	38.4	CH	1.67		26, 27	26(w), 27(w)
26	17.4	CH_3	0.89	*d*, 6.9	25	25, 27
27	16.3	CH_3	0.91	*d*, 6.6	25	25, 26
28	140.6	CH	5.73	*dd*, 17.4, 11.9	29a, 29b	24, 25
29_E	114.5	CH_2	5.28	*dd*, 11.9, 1.2	28, 29b	24, 28
29_Z			5.24	*dd*, 17.4, 1.2	28, 29a	24, 28

Spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$. C-26 and C-27 may be interchanged; (w) for weak correlations.

Table S5. ^{1}H and ^{13}C NMR Spectroscopic Data of Compound 18 with supporting 2D correlation information.

position	^{13}C	mult.	^{1}H	*mult., J (*Hz)	COSY-45	gHMBC
1a	35.7	CH_2	1.98		1b, 2a	3
1b			1.68		1a, 2a	
2a	34.0	CH_2	2.34		1a, 1b	3
2b			2.22			
3	199.6	C=O				
4	123.8	CH	5.70	*br s*	6a	2a, 6a, 10
5	171.5	qC				
6a	32.9	CH_2	2.36		4, 7a	5
6b			2.24			
7a	32.0	CH_2	1.84		6a, 7b	
7b			0.98		7a	
8	34.7	CH	1.60		9	
9	53.8	CH	0.88		8, 11	
10	38.6	qC				
11a	20.8	CH_2	1.47		9, 12a	
11b			1.43		9, 12b	
12a	40.0	CH_2	1.94		11, 12b	
12b			1.08		11, 12a	
13	42.6	qC				
14	53.8	CH	0.88		15b	
15a	35.2	CH_2	2.20		15b, 16	16
15b			1.24		14, 15a, 16	16
16	71.6	O–CH	4.34	*ddd*, 7.8, 6.9, 4.7	15a,b, 17	
17	57.2	CH	1.09		16, 21	13, 18
18	13.0	CH_3	0.94	*s*		12, 13, 14, 17
19	17.4	CH_3	1.17	*s*		1, 5, 9, 10
20	34.7	CH	2.29		21, 22	
21	16.8	CH_3	0.96	*d*, 7.0	17, 20	17, 20, 22
22	73.8	O–CH	4.00	*ddd*, 10.1, 2.3, 2.3	20, 23a,b	21
23a	38.7	CH_2	1.83		22, 23b	
23b			1.56		22, 23a	20, 22, 24, 25
24	78.8	O–qC				
25	34.5	CH	2.00		26, 27	
26	17.8	CH_3	0.87	*d*, 7.0	25	24, 25
27	16.4	CH_3	0.87	*d*, 7.0	25	24, 25
28	141.3	CH	5.95	*dd*, 17.4, 10.9	$29_{E,Z}$	24
29_E	113.8	CH_2	5.20	*dd*, 10.9, 1.1	28, 29_Z	24, 28
29_Z			5.26	*dd*, 17.4, 1.1	28, 29_E	24, 28

Spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$. C-26 and C-27 may be interchanged.

Table S6. ^{1}H and ^{13}C NMR Spectroscopic Data of compound **21** with supporting 2D correlation information.

position	^{13}C	mult.	^{1}H	*mult., J (*Hz)	COSY-45	gHMBC
1a	35.7	CH_2	1.99			3, 9, 10, 19
1b			1.67		19	9, 10, 19
2a	34.0	CH_2	2.37			3, 4, 10
2b			2.26			
3	199.5	C=O				
4	123.8	CH	5.71	*br s*	6a	2a, 6a, 10
5	171.9	qC				
6a	32.9	CH_2	2.35		4, 6b, 7a	5
6b			2.27		6a	5
7a	32.0	CH_2	1.86		6a, 7b, 8	
7b			1.00		7a	
8	35.3	CH	1.61		7a, 14	9, 14
9	53.9	CH	0.92		11a	
10	38.6	qC				
11a	20.8	CH_2	1.48		9, 12a, 12b	12, 13
11b			1.43			
12a	40.0	CH_2	1.99		11a, 12b	13
12b			1.13		11a, 12a	
13	42.8	qC				
14	53.8	CH	0.99		8	17
15α	35.1	CH_2	2.20		16	13, 16, 17
15β			1.26		16	16
16	71.9	O–CH	4.38	*ddd*, 7.9, 6.8, 4.7	15a, 15b, 17	
17	57.2	CH	1.23		16, 20	13, 16, 18, 20, 22
18	12.9	CH_3	0.96	*s*		12, 13, 14, 17
19	17.4	CH_3	1.18	*s*	1b	1, 5, 9, 10
20	33.5	CH	2.19		17, 21, 22	
21	17.1	CH_3	1.02	*d*, 6.8	20	17, 20, 22
22	75.9	CH	3.68	*ddd*, 11.2, 2.3, 2.3	20, 23a, 23b	
23a	30.8	CH_2	2.49		22	20, 22, 25, 28
23b			2.03		22	20, 25, 28
24	143.8	qC				
25	33.6	CH	2.41		26, 27	24, 26, 27, 28
26	22.9	CH_3	1.00	*d*, 6.8	25	24, 25, 27
27	21.9	CH_3	1.03	*d*, 6.8	25	24, 25, 26
28	120.4	CH	5.54	*q*, 6.7	29	23, 25, 29
29	13.8	CH_3	1.65	*d*, 6.7	28	24, 28

Spectra were recorded at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$.

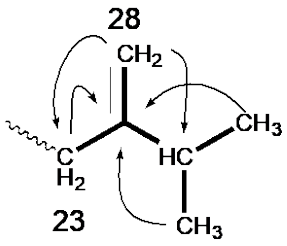
Table S7. ^{1}H and ^{13}C NMR Spectroscopic Data of compound **22** with supporting 2D correlation information.

position	^{13}C	mult.	1H	*mult., J* (Hz)	COSY	gHMBC
1a	35.6	CH_2	2.01		1b, 2a, 2b	3
1b			1.67	*td*, 10.8, 3.8	1a, 2a, 2b	3, 19
2a	34.0	CH_2	2.41		1a, 1b, 2b, 4	3, 5
2b			2.33		1a, 1b, 2a	4
3	199.6	C=O				
4	123.9	=CH	5.71	*s*	2a	2, 6, 10
5	171.1	=C				
6a	32.8	CH_2	2.39		6b, 7a, 7b	7
6b			2.26		6a	4, 5
7a	31.8	CH_2	1.83		6a, 7b	5
7b			1.01		6a, 7a, 8	
8	34.6	CH	1.66		7b, 9, 14	
9	53.8	CH	0.91		8, 11a, 11b	
10	38.6	qC				
11a	20.7	CH_2	1.52		9, 12a, 12b	
11b			1.50		9	
12a	40.2	CH_2	2.16	*dt*, 12.5, 3.3	11a, 12b	
12b			1.17		11a, 12a	
13	43.0	qC				
14	53.9	CH	0.89		8, 15a, 15b	18
15a	37.3	CH_2	2.25		14, 15b, 16	9, 13, 17
15b			1.33	*td*, 11.3, 4.5	14, 15a, 16	9, 16
16	74.0	O–CH	4.63	*ddd*, 7.4, 7.4, 4.5	15a, 15b, 17	13
17	60.4	CH	1.23		16	13, 16, 18, 20
18	14.9	CH_3	1.19	*s*		12, 13, 17
19	17.3	CH_3	1.19	*s*		1, 5, 9, 10
20	76.6	O–qC				
21	26.6	CH_3	1.30	*s*		17, 20
22	42.4	CH_2	1.84		23a, 23b	20, 24
23a	29.3	CH_2	2.11		22, 23b, 28a, 28b	24, 28
23b			2.03		22, 23a, 28a, 28b	24, 28
24	156.3	=C				
25	33.9	CH	2.24		26, 27	24, 26, 27, 28
26	21.9	CH_3	1.02	*d*, 7.8	25	25, 27
27	21.9	CH_3	1.02	*d*, 7.8	25	25, 26
28a	106.4	$=CH_2$	4.73	*br s*	23a, 23b	23, 24, 24
28b			4.69	*d*, 1.0	23a, 23b	23, 24, 25
OH			3.62, 3.56	*br s, br s*		
OH			2.94	*br s*		

Spectra were recorded at 600 MHz (^{1}H) and at 150 MHz (^{13}C) in $CDCl_3$.

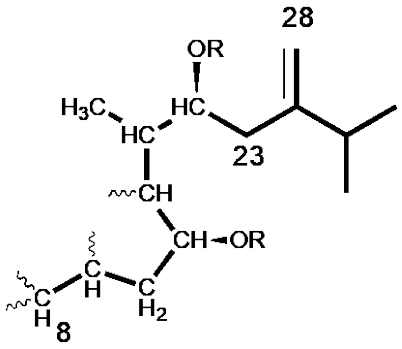
Structure Elucidation of Compound 14

The molecular formula of compound **14** was determined to be $C_{28}H_{44}O_3$ by high-resolution mass spectrometry, which showed a quasi-molecular ion $[M + H]^+$ at *m/z* 429.3361 (calc. for $C_{28}H_{45}O_3$ 429.3369), implying seven units of unsaturation. The infrared (IR) spectrum of **14** showed a broad absorbance at 3395 cm^{-1} indicating the presence of at least one hydroxy group and an absorbance at 3081 cm^{-1} (=C—H stretch) indicating the presence of at least one carbon-carbon double bond. Also visible in the IR spectrum are distinct absorbances at 1667 cm^{-1} (s, C=O) and 1614 cm^{-1} (w, C=C) consistent with the presence of an α,β-unsaturated ketone group. The ^{13}C NMR (Table 1) and DEPT spectra of compound **14** showed 28 signals: five methyl groups, eight sp^3 and one sp^2 methylene group, six sp^3 and one sp^2 methine group, two oxymethine groups and two sp^3 and three sp^2 quaternary carbons. The ^1H (Table 2) and ^{13}C NMR spectroscopic data obtained from compound **14** revealed the presence of a terminal vinyl group with signals δ_C 110.4 (CH_2, C-28), δ_H 4.95 (1H, *t*, *J* = 1.2 Hz, H-28a) and δ_H 4.82 (1H, *br s*, H-28b). The very small coupling constant of these two protons indicates that the attached sp^2 carbon is quaternary. HMBC correlations (Fig. 3, supplementary data Table S1) were observed from H-28a and H-28b to the methylene carbon at δ_C 37.1 (CH_2, C-23) and to the methine carbon at δ_C 33.2 (CH, C-25). $^1H,^1H$-COSY correlations were observed between H-25 and the protons of two methyl groups at δ_H 1.03 (3H, *d*, *J* = 6.8 Hz, H-26) and δ_H 1.06 (3H, *d*, *J* = 6.8 Hz, H-27). Strong HMBC correlations were observed from both H_3-26 and H_3-27 to a quaternary sp^2 carbon δ_C 153.2 (qC, C-24). The first unit of unsaturation is now accounted for as a carbon-carbon double bond, $\Delta^{24(28)}$.

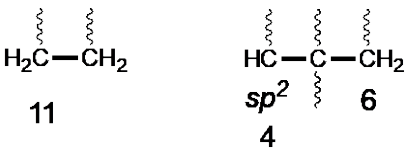


A long spin system was elucidated by COSY from this point extending around to C-8 (Fig. 3). Strong correlations were observed between H-23b and the oxymethine proton δ_H 3.68 (1H, *ddd*, *J* = 11.1, 2.4, 2.4 Hz, H-22) and between H-22 and the methine proton at δ_H 2.43 (1H, *m*, H-20). Two additional correlations were noted from H-20, the first to the methyl protons at δ_H

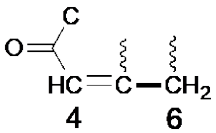
1.01 (3H, *d*, *J* = 7.2, H-21) and the second to the methine proton at δ_H 1.14 (1H, *m*, H-17). Further COSY correlations were observed between H-17 and the oxymethine proton at δ_H 4.33 (1H, *ddd*, *J* = 8.1, 6.8, 4.7 Hz, H-16), which in turn shows correlations with two sp^3 methylene protons at δ_H 2.21 (1H, *m*, H-15a) and at δ_H 1.27 (1H, *m*, H-15b). Both H-15a and H-15b are correlated to a methine group at δ_H 0.86 (1H, *m*, H-14). The final COSY correlation to complete the spin system observed was between H-14 and the methine proton δ_H 1.61 (1H, *m*, H-8).



The only remaining discernible COSY correlations were between the sp^3 methylene groups whose protons appear at δ_H 1.49 (2H, *m*, H-11) and one of the vicinal protons at δ_H 1.12 (1H, *m*, H-12b) and long range correlations between the vinylic proton at δ_H 5.71(1H, *br s*, H-4) and the methylene protons at δ_H 2.40 and 2.28 (H-6a, H-6b).

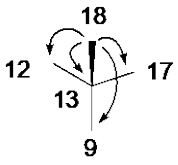


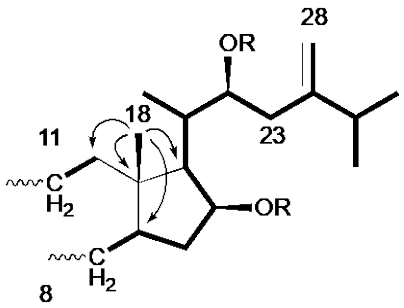
A downfield signal in the ^{13}C NMR spectrum at δ_C 199.6 (qC, C-3) is consistent with the presence of a ketone group, accounting for a second unit of unsaturation. Since the NMR signals of an sp^2 methine at δ_C 123.8 (CH, C-4) and δ_H 5.71 (1H, *br s*, H-4) and the quaternary sp^2 carbon at δ_C 171.4 (qC, C-5) are the only remaining signals consistent with the chemical shift of an alkene. The significant downfield shift of C-5 at δ_C 171.4 can only be explained if it is the β-carbon of an α,β-unsaturated ketone in compound **14**, as suggested by the low frequency of the carbonyl absorption in the IR spectrum of **14**.



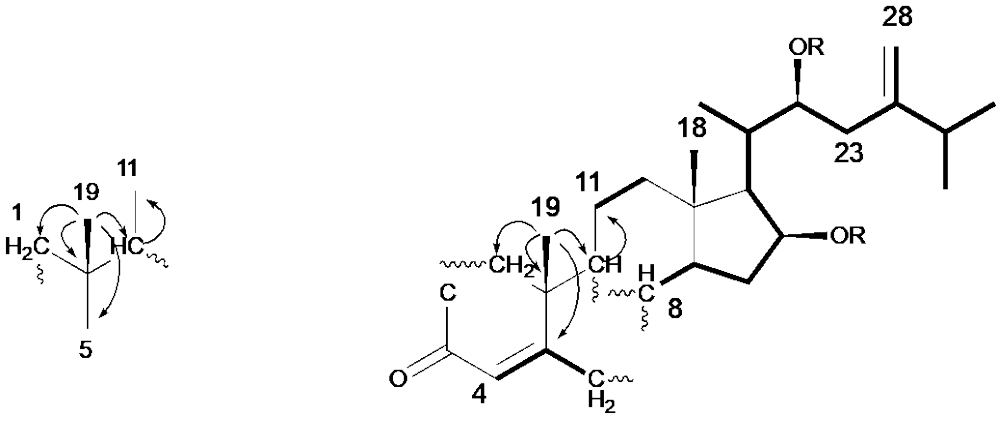
The downfield shifts of H_2-6 (δ 2.40 and 2.28) are consistent with C-6 being allylic to Δ^4.

As there are no unaccounted for unsaturated carbon atoms visible in the NMR data, the remaining four units of unsaturation have been attributed to the presence of four rings. Several strong correlations were observed in the HMBC spectrum (Fig. 3) from the methyl singlet at δ_H 0.96 (3H, *s*, H-18). Correlations were observed to carbons at δ_C 40.0 (CH_2, C-12), δ_C 42.7 (qC, C-13), δ_C 53.3 (CH, C-14) and δ_C 57.1 (CH, C-17). Since the methyl group, C-18, is a singlet, the quaternary carbon, C-13, must be at the centre of this partial structure, as shown. The observed correlations to C-14 and C-17 suggests that the large spin system (see above) closes to a ring at this point through C-13.

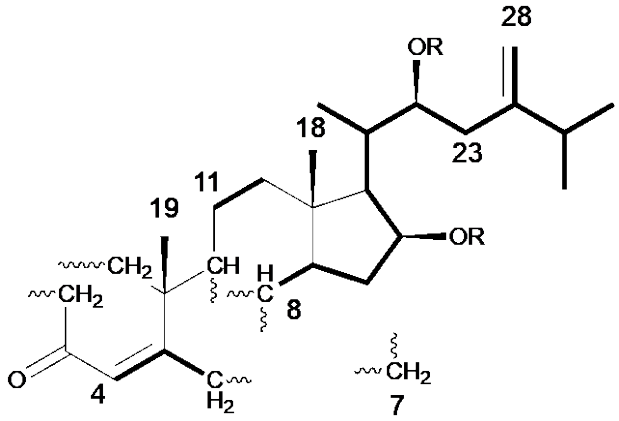




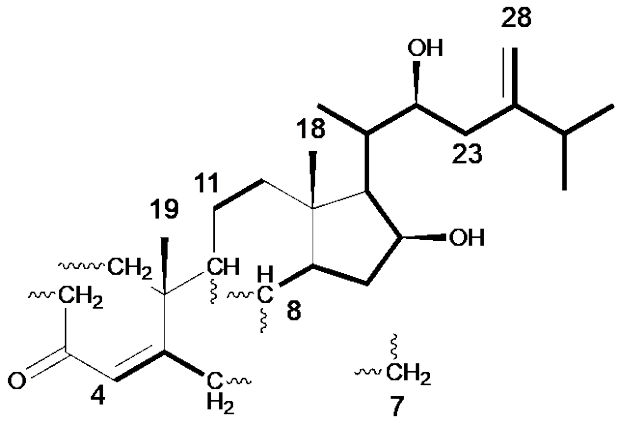
In the same way strong HMBC correlations observed from a second methyl group singlet at δ_H 1.18 (3H, *s*, H-19) to δ_C 35.7 (CH_2, C-1), δ_C 171.4 (qC, C-5), δ_C 38.6 (qC, C-10), δ_C 53.8 (CH, C-9) led to a similar partial structure centred around the quaternary carbon C-10. Crucially, this part of the structure was shown to be connected to the established partial structure by the observation of a HMBC correlation from the methine proton at δ_H 0.90 (1H, *m*, H-9) to a carbon atom at δ_C 20.8 (CH_2, C-11).



At this point there are only 2 carbon atoms which remain unassigned, namely, CH_2-2 (δ_H 2.45 and 2.33) and CH_2-7 (δ_H 1.88 and 1.04). The downfield shifts of the protons on C-2 place it adjacent to the carbonyl group.



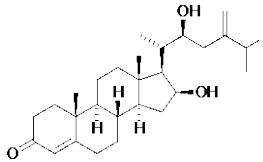
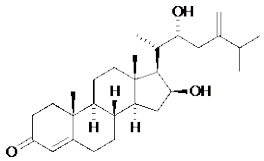
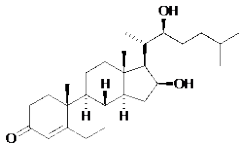
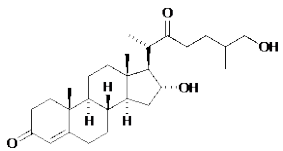
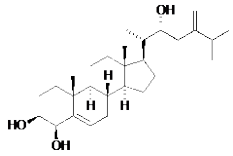
The molecular formula of compound **14** indicates the presence of three oxygen atoms in the structure, one of which has been attributed to the presence of the ketone group. As the IR spectrum indicates the presence of at least one hydroxy group and since there are only two sp^3 carbon atoms bonded to oxygen visible in the ^{13}C NMR spectrum, the third oxygen atom must be part of a second hydroxy group.



While COSY correlations could theoretically be used to complete compound **14**'s structure, there was insufficient resolution in the COSY spectrum to unambiguously distinguish any further bonding interactions. At this stage it was evident that compound **14** is most likely a steroid and the literature was searched for steroids with similar structures. Comparison of NMR data from compound **14** to data from a steroid nucleus and a steroidal side chain reported in previous reports showed good agreement (Achenbach et al., 1996; Harding et al., 2001; Saez et al., 1998, Supplementary Data Table S8) with the values obtained from compound **14** for the steroidal nucleus ($\Delta\delta_C$ < 4ppm, $\Delta\delta_H \leq 0.06$ ppm) and allowed the planar structure of compound **14** to be confidently assigned.

Table S8

^{13}C NMR Spectroscopic Data for Compounds **14** and **15** Compared to Published Compounds.

					
	14 in $CDCl_3$	**15** in $CDCl_3$	in CD_3OD	in $CDCl_3$	in $CDCl_3$
C	δ, mult.	δ, mult.	Achenbach et al. 1996	Saez et al. 1998	Harding et al. 2001
1	35.7, CH_2	35.7, CH_2	36.7	35.2	36.9
2	34.0, CH_2	34.0, CH_2	34.0	33.5	25.1
3	199.6, qC	199.6, qC	202.3	199.4	72.5
4	123.8, CH	123.8, CH	124.1	123.4	77.3
5	171.4, qC	171.4, qC	175.1	171.2	142.8
6	32.9, CH_2	32.9, CH_2	34.7	32.4	128.7
7	32.0, CH_2	32.0, CH_2	33.3	31.5	32.1
8	35.3, CH	35.2, CH	36.4	34.5	31.8
9	53.8, CH	53.7, CH	55.1	53.1	50.2

10	38.6, qC	38.6, qC	40.0	38.2	36.0
11	20.8, CH_2	20.8, CH_2	21.9	20.3	20.5
12	40.0, CH_2	40.2, CH_2	41.3	39.2	39.7
13	42.7, qC	43.0, qC	43.5	43.6	42.7
14	53.8, CH	53.6, CH	55.3	52.1	56.5
15	37.1, CH_2	35.3, CH_2	37.2	35.1	24.4
16	71.7, CH	72.5, CH	72.5	76.0	27.4
17	57.1, CH	61.1, CH	58.9	62.1	52.9
18	12.9, CH_3	13.0, CH_3	14.4	13.3	11.9
19	17.4, CH_3	17.4, CH_3	17.7	17.0	21.0
20	33.4, CH	35.9, CH	36.6	48.3	40.5
21	17.0, CH_3	14.7, CH_3	13.6	16.5	12.5
22	74.6, CH	73.1, CH	76.0	216.9	69.9
23	37.1, CH_2	40.4, CH_2	32.6	38.4	35.9
24	153.2, qC	153.3, qC	37.2	26.6	153.5

25	33.2, CH	33.2, CH	29.3	34.7	33.1
26	22.3, CH_3	22.3, CH_3	23.0	66.9	21.6
27	21.5, CH_3	21.5, CH_3	23.1	16.3	22.4
28	110.4, CH_2	110.9, CH_2			109.8

^{1}H NMR Spectroscopic Data for Compounds **14** and **15** Compared to Published Compounds.

C	**14**	*J* (Hz)	**15**	*J* (Hz)	Achenbach et al. 1996 ($CDCl_3$)		Saez et al. 1998		Harding et al. 2001	
1	1.99		1.99		~2.01		1.88		1.83	
	1.63		1.69				1.50		1.09	
2	2.45		2.36		2.20–2.48		2.22		1.92	
	2.33		2.26						1.66	
4	5.71	*br s*	5.71	*br d*, 0.9	5.73	*br s*	5.52	*s*	4.14	*d*, 3.3
5									5.68	*dd*, 5.0, 2.0
6	2.40		2.40		2.20–2.48		2.30		–	
	2.28		2.26				2.13			

7	1.88		1.85	~1.87	1.69	2.11	
	1.04		1.01		0.96	1.57	
8	1.61		1.60		1.42	1.56	
9	0.90		0.90		0.88	0.90	*dd*, 7.5, 1.1
11a	1.49		1.49		1.43	1.48	
11b	–		1.41		1.24		
12a	1.94	*d*, 11.0	2.06	~2.01	1.78	2.04	
12b	1.12		1.13		1.27	1.18	
14	0.86		0.90		1.33	1.02	
15a	2.21		2.22	2.20–2.48	1.80	1.62	
15b	1.27		1.22		1.45	1.15	

16	4.33	*ddd*, 8.1, 6.8, 4.7	4.39	*ddd*, 8.1, 7.1, 5.1	4.35	*ddd*, 8, 7, 4.5	3.65	*dd*, 14, 7	1.74, 1.42	
17	1.14		1.10				1.48		1.12	*td*, 12.1, 3.0
18	0.96	*s*	0.95	*s*	0.96	*s*	0.62	*s*	*0.72*	*s*
19	1.18	*s*	1.18	*s*	1.20	*s*	1.05	*s*	*1.19*	*s*
20	2.43		2.10		2.20–2.48		2.58		1.86	
21	1.01	*d*, 7.2	0.99	*d*, 7.1	0.98	*d*, 7	1.02	*d*, 7	0.96	*d*, 6.7
22	3.68	*ddd*, 11.1, 2.4, 2.4	3.58	*ddd*, 10.8, 7.0, 2.2	3.63	*ddd*, 9.5, 2.5, 2.5	–		3.75	*br d*, 10.9
23a	2.29		2.51	*dt*, 14.1, 1.6			2.53		2.20	
23b	2.08		1.94				2.39		1.93	

24							1.55, 1.32			
25	2.22		2.20				1.46		2.22	
26	1.03	*d*, 6.8	1.03	*d*, 6.8	0.91	*d*, 6.5	3.2		1.08	*d*, 6.8
27	1.06	*d*, 6.8	1.06	*d*, 6.8	0.90	*d*, 6.5	0.77	*d*, 7	1.05	*d*, 6.8
28a	4.95	*t*, 1.2	4.98	*t*, 1.2					4.94	*br t*, 1.1
28b	4.82	*br s*	4.83	*br s*					4.83	*br s*