Structure and Synthesis of a Progesterone Homologue from the Skin of the Dorid Nudibranch *Aldisa smaragdina*

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Dedicated to the memory of Prof. G. Sodano

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The short-side-chain steroid 24-norchol-4-ene-3,22-dione (**6**) has been found to be the main metabolite of both skin and mucus of the mollusc *Aldisa smaragdina*. This compound, previously reported as a microbial degradation product of cholesterol, has never previously been isolated from natural sources. The absolute stereochemistry was determined by synthesis from commercial stigmasterol, and a full NMR characterisation is also reported.

Introduction

A large variety of unusual steroids have been isolated from marine organisms, and especially sponges.[1,2] Among the most remarkable features encountered, extensive oxygenation and alkylation of the side chain and oxidative and structural modification of the polycyclic core are especially relevant. Several biosynthesis studies of steroids in sponges have also been conducted.[3]

In contrast, only a few steroid compounds from marine molluscs, and in particular from nudibranchs (Figure 1), have been reported. Some examples are the oxygenated sterols **1**, from the aeolidacean species *Cratena peregrina*, *Flabellina affinis* and *Coryphella lineata*,[4] **2**, from *Diaulula sandiegensis*,[5] and **3**, from *Adalaria* sp.[6] Nudibranchs are naked molluscs that, even though unprotected by the physical shield of a shell, appear to be free of predation. Their survival is in fact assured by defensive strategies that include the use of chemicals, usually selectively located on the skin and in the defensive mucous secretion.[7,8]

In continuation of our research of the chemical ecology of marine molluscs,[9] we have examined the skin metabolites of the doridacean nudibranch *Aldisa smaragdina* Ortea, Pérez & Llera, 1982. This species is known from Asturias (NW Spain) to Morocco, in the Azores, Madeira and the Canary Islands, and also occurs in the Alboran Sea (SW Mediterranean). In a remarkable example of colour camouflage, this nudibranch is quite invisible in the field, due to its red colour, the same as the sponge on which it lives. A previous study on the related Pacific species *Aldisa cooperi*[10] resulted in the finding of two antifeedant steroids (**4** and **5**) featuring cholic acid side chains. In this paper, we

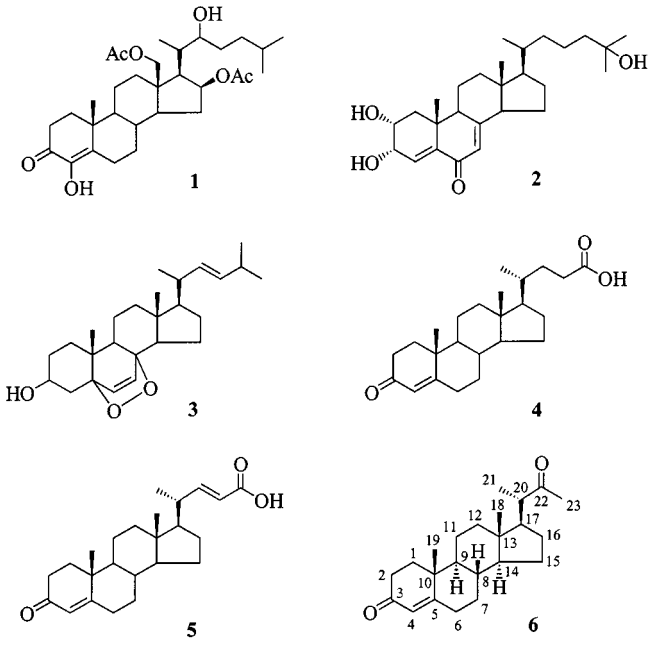

Figure 1. Structures of steroidal compounds from nudibranchs

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report on the isolation and the synthesis of the unusual steroid: 24-norchol-4-ene-3,22-dione (**6**), previously obtained by chemical synthesis[11] and by microbial conversion of cholesterol,[12−14] but never before reported as a natural product.

Results and Discussion

Two specimens (sizes 0.8 and 1.5 cm in length) were caught by scuba divers off Cabo Cope (Murcia, SE Spain) during November 1997 and immediately frozen at −20 °C. A small quantity of mucus secreted by the animals was recovered and also frozen separately. A fragment of the red encrusting demosponge on which the animals were found, identified as *Phorbas fictitius*, was also collected and frozen. A further small specimen (0.5 cm in length) was collected off the island of Alboran (SW Mediterranean) during July 1996, within the framework of the Spanish oceanographic campaign "Fauna IV". Later, all this material was transferred to ICMIB in Naples and chemically analysed. The largest animal was carefully dissected into the mantle and the digestive gland, which were separately extracted with acetone. Because of their small size, the remaining two animals were not dissected, but first immersed in acetone and submitted to ultrasound vibration for 1 min. Only the metabolites present in the skin (external part) were extracted by this procedure. The solvent was removed and the animals were homogenised with a pestle and again treated with acetone, so as to extract the digestive gland content (internal part). The mucous secretion was directly treated with diethyl ether. The sponge sample was also extracted with acetone. The ether-soluble portions of the mantle and digestive gland acetone extracts, obtained in the two different ways, were analysed by TLC, together with mucus extract, and compared to the ether-soluble portion of the sponge acetone extract. One main UV-sensitive metabolite at $R_f = 0.35$ (light petroleum ether/diethyl ether, 1:1) characterised both the skin and the mucus, but was absent in the digestive gland of the mollusc and in the sponge. The two external part and mucus extracts were combined (11.2 mg) and fractionated by silica gel chromatography (petroleum ether/diethyl ether gradient). Pure compound **6** (1.5 mg) was obtained from the fractions eluted with light petroleum ether/diethyl ether (8:2).

The molecular formula of $C_{23}H_{34}O_2$, deduced from HREIMS on the molecular ion at $m/z = 342$, indicated seven degrees of unsaturation. The presence of two oxo functions, one of which was α,β-unsaturated, was suggested by two intense infrared bands at 1708 and 1650 cm^{-1} and confirmed by two signals in the ^{13}C NMR spectrum at δ = 212.5 and 199.4. A conjugated trisubstituted double bond (δ = 5.73 in ^1H NMR spectrum and δ = 123.9 and 171.1 in ^{13}C NMR spectrum) accounted for another degree of unsaturation, the remainder being due to four rings. ^1H and ^{13}C NMR spectra showed signals supporting a steroid tetracyclic structure featuring three tertiary methyl groups (singlets at δ = 0.74, 1.18 and 2.10) and one secondary methyl group (doublet at δ = 1.12, $J = 7.0$ Hz). In particular, a structure derived from the 4-cholest-3-one skeleton with a short side chain was clearly indicated by detailed analysis of 2D NMR spectra (^1H-^1H COSY and HMQC experiments). The position of the saturated carbonyl group at C-22 was confirmed by diagnostic correlations of this carbon atom (δ = 212.5) with 23-H_3 (δ = 2.10), 20-H (δ = 2.50) and 21-H_3 (δ = 1.12) in the HMBC spectrum. All proton and carbon resonances, assigned as shown in Table 1, were consistent with the structure **6** (Figure 1), a 22-oxosteroid synthesised in 1945[11] and subsequently obtained from cholesterol by microbial degradation.[12−14] No spectral characterisation was reported in previous papers.

Table 1. NMR spectroscopic data[a] of steroid **6**

C	δ ^{13}C[b]	m[c]	δ ^1H[d]	m, (J [Hz])	HMBC correlations[e]
1	35.7	t	1.70	m	H_3-19
			2.02	m	
2	34.0	t	2.35	m	H-1a, H-4, H_3-19
			2.40	m	
3	199.4	s	–	–	H-1b, H-2a
4	123.9	d	5.73	s	H-6a
5	171.1	s	–	---	H-1b, H-6a, H_3-19
6	32.8	t	2.28	m	H-5
			2.35	m	
7	32.0	t	1.05	m	H_2-6
			1.82	m	
8	35.6	d	1.52	m	H-9, H-14
9	53.7	d	0.95	ddd (11, 12, 4)	H_2-11, H-12a, H_3-19
10	38.6	s	–	–	H_2-1,H-4, H_2-6,H-9, H_3-19
11	21.0	t	1.45	ddt (4, 14, 13)	H-12a
			1.55	m	
12	39.6	t	1.28	m	H_3-18
			1.97	m	
13	42.6	s	–	–	H-14, H_2-15, H_2-16, H_3-18
14	55.3	d	1.05	m	H-12a, H_3-18
15	24.4	t	1.20	m	H-14
			1.70	m	
16	27.4	t	1.65	m	H_2-15
17	52.0	d	1.55	m	H_3-18, H-20, H_3-21
18	12.2	q	0.74	s	H-12b, H-14, H-17
19	17.4	q	1.18	s	H_2-1, H-9
20	50.3	d	2.50	dq (11, 7)	H-17, H_3-21, H_3-23
21	16.3	q	1.12	d (7)	H-20
22	212.5	s	–	–	H-20, H_3-21, H_3-23
23	28.0	q	2.10	s	

[a] Bruker 500 MHz; $CDCl_3$; chemical shifts (ppm) referred to $CHCl_3$ (δ = 7.26) for proton and to $CDCl_3$ (δ = 77.0) for carbon atom. [b] Assignments by HMQC and HMBC experiments. [c] By DEPT sequence. [d] Assignments by ^1H-^1H COSY experiment. [e] $J = 10.0$ Hz.

The configuration of the stereogenic centres in the polycyclic core, believed to be the same as in the 4-cholest-3-one skeleton, was confirmed by diagnostic NOEs (Figure 2). In

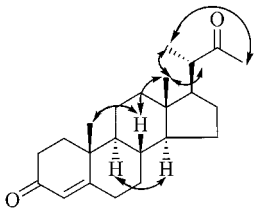
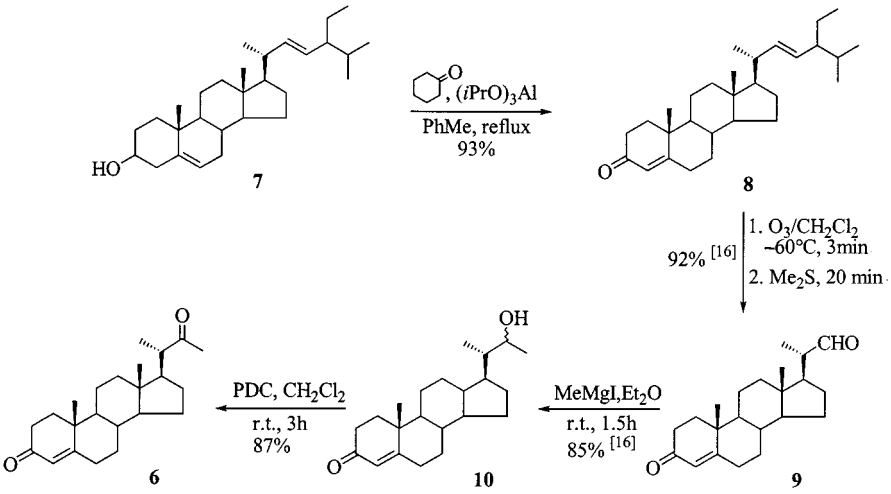


Figure 2. Selected NOEs in steroid **6**



Scheme 1. Synthesis of steroid **6** from stigmasterol (**7**)

particular, cross-peaks in the NOESY spectrum between 8-H and both 19-H_3 and 18-H_3, as well as 18-H_3 and 20-H, indicated their β-orientation. A significant NOE was also observed between 9-H and 14-H, which were α-oriented.

The configuration at C-20 was not determined at this point: the common (20*R*) stereochemistry should be favoured even though the opposite configuration has also been reported for marine sterols.[15] In order to confirm this, however, the partial synthesis of **6** from commercial stigmasterol (**7**) was carried out in four steps (Scheme 1). Stigmasterol (**7**) was oxidised by Oppenauer reaction (cyclohexanone, aluminium isopropoxide) to α,β-unsaturated ketone **8** (93% yield), which was subsequently subjected to ozonolysis to give the oxo aldehyde **9** (92% yield[16]).[17] A Grignard reaction between **9** and MeMgI in Et_2O gave a mixture of epimeric alcohols **10** (85% yield[16]), which were transformed by treatment with PDC into the corresponding ketone (87% yield), the spectroscopic data (including the CD profile) of which were identical with those of **6**, clearly indicating a (20*R*) stereochemistry for the natural compound.

A defensive role is strongly suspected for steroid **6** in the nudibranch, due to its selective presence in the skin and in the mucous secretion of the animal. The absence of **6** in the digestive gland of the mollusc and in the sponge *Phorbas fictitius*, on which the animal lives, should rule out a dietary origin for this compound. Analogously with *A. cooperi*,[10] *A. smaragdina* might obtain compound **6** from a dietary steroid precursor by degradation of the side chain, or might biosynthesise it de novo. This latter hypothesis seems to be favoured; no steroidal precursors of **6** exhibiting an oxidised A-ring were in fact detected in the preyed-upon sponge by chemical analysis. However, it is worth noting that compound **6** is biogenetically related to steroid **4**, from which it might derive through β-oxidation and subsequent decarboxylation.

In addition to the ecological aspect, the discovery of a 3,22-dioxosteroid from a natural source is very intriguing, due to the structural feature that this molecule, with the α,β-unsaturated double bond in the A-ring and the oxo group at C-22, is an interesting homologue of progesterone.

Experimental Section

General: Silica gel chromatography was performed with precoated Merck F_{254} plates and Merck Kieselgel 60 powder. Optical rotations were measured with a Jasco DIP 370 digital polarimeter, and CD curves were recorded on a Jasco 710 spectropolarimeter. The IR spectra were taken with a Bio-Rad FTS 7 spectrophotometer. The UV spectra were obtained with a Varian DMS 90 spectrophotometer. ^1H and ^{13}C NMR spectra were recorded with WM 500 and DPX 300 MHz Bruker spectrometers in $CDCl_3$; chemical shifts are reported in ppm referred to $CHCl_3$ as internal standard (δ = 7.26 for ^1H and δ = 77.0 for ^{13}C). EIMS and HREIMS spectra were measured with TRIO 2000 VG Carlo Erba and on Kratos MS50 instruments, respectively. Stigmasterol (**7**) was purchased from Aldrich Chemical Co. (m.p., $[\alpha]_D$ and ^1H NMR spectroscopic data were identical to those reported in the literature).[18,19]

Biological Material: *Aldisa smaragdina* (two specimens, 0.8 and 1.5 cm long) was collected off Cabo Cope (SE Spain) by hand (scuba), during November 1997 at a depth of 2−3 m and frozen at −20 °C. A small quantity of mucus secreted by the animals was recovered and separately frozen at −20 °C. A sample of the sponge *P. fictitius* was also collected and frozen at −20 °C. The other small specimen of *A. smaragdina* (0.5 cm long) was collected off the island of Alboran, at a depth of 9 m, on July 27, 1996, during the Spanish oceanographic campaign "Fauna IV". All the material was transferred to ICMIB. The nudibranch and the sponge were identified by one of us (J. T.).

Extraction and Purification Procedure: The largest individual of *A. smaragdina* was dissected into the mantle and the digestive gland, which were extracted separately with acetone (each 3 mL × 3). The two other individuals were immersed in acetone (5 mL) and extracted by use of ultrasound vibration for 1 min. Only the metabolites present in the external part were extracted by this procedure. The solvent was removed, and the animals were homogenised with a pestle and again treated with acetone (5 mL × 3). The sample of the sponge *P. fictitius* was also extracted with acetone (10 mL × 3). The small quantity of mucus secreted by the mollusc, recovered

during collection, was directly extracted with diethyl ether (10 mL × 3). The extract obtained was compared by TLC chromatography (silica gel and light petroleum ether/diethyl ether in different ratios as eluent) to the ether-soluble portion of the sponge acetone extract and to the ether-soluble portions of both the mollusc external and the mollusc internal part acetone extracts, obtained either by treatment of dissected sections or by ultrasound extraction. The external part and mucus extracts were combined (11.2 mg) and submitted to silica gel column chromatography (petroleum ether/diethyl ether gradient). The fractions eluted by light petroleum ether/diethyl ether (8:2) were combined to give pure compound **6** (1.5 mg).

Spectroscopic Data for Natural 6: R_f (light petroleum ether/diethyl ether, 1:1) = 0.35. IR (liquid film): $\tilde{\nu}$ = 1707, 1645 cm^{-1}. UV (MeOH): λ_{max} (lg ε) = 238 nm (3.48). CD (EtOH): $[\theta]_{217}$ = 6200. ^1H and ^{13}C NMR: see Table 1. EIMS: *m*/*z* (%) = 342 (30) [M^+], 300 (12) [$M^+ - CH_2CO$], 271 (18) [M^+ − side chain], 229 (24), 147 (35), 124 (100). HREIMS: calcd. for $C_{23}H_{34}O_2$ 342.2559, found 342.2552.

Compound 8: Oppenauer oxidation of stigmasterol (**7**) was conducted under the conditions reported in the literature.[20] Freshly distilled cyclohexanone (14.0 mL, 135.1 mmol) was added to a solution of stigmasterol (**7**, 500.0 mg, 1.2 mmol) in anhydrous toluene (125.0 mL) under argon. The solution was then distilled until toluene (18.0 mL) had been removed. Aluminium isopropoxide (1.12 g, 5.5 mmol) in toluene (42.0 mL) was added dropwise (over 30 min) to the residue and the solution was again distilled to remove toluene. After 45 min, the reaction mixture was cooled, washed with water and dried with Na_2SO_4. The solvent was evaporated and the residue was purified by flash chromatography (silica gel, light petroleum ether/diethyl ether, 95:5) to give crystalline compound **8** (462.1 mg, 93%). R_f (light petroleum ether/ethyl acetate, 7:3) = 0.62. M.p. 128−129 °C (light petroleum ether). $[\alpha]_D$ = +40.5 (*c* = 1.25, $CHCl_3$). IR (liquid film): $\tilde{\nu}$ = 1685 cm^{-1}. UV (MeOH): λ_{max} (lg ε) = 238 nm (3.32). ^1H NMR (300 MHz): δ = 5.72 (s, 1 H, 4-H), 5.14 (dd, *J* = 8.4, 15.5 Hz, 1 H, 22-H), 5.01 (dd, *J* = 8.4, 14.7 Hz, 1 H, 23-H), 1.18 (s, 3 H, 19-H_3), 1.01 (d, *J* = 6.7 Hz, 3 H, 21-H_3), 0.84 (d, *J* = 6.4 Hz, 3 H, 26-H_3), 0.80 (t, *J* = 7.3 Hz, 3 H, H_3-29), 0.80 (d, *J* = 7.1 Hz, 3 H, 27-H_3), 0.72 (s, 3 H, 18-H_3). ^{13}C NMR (75.5 MHz): δ = 199.7 (C-3), 171.8 (C-5), 138.1 (C-22), 129.4 (C-23), 123.7 (C-4), 55.9 (C-17 or C-14), 55.8 (C-14 or C-17), 53.8 (C-9), 51.2 (C-24), 42.2 (C-13), 40.5 (C-20), 39.5 (C-12), 38.6 (C-10), 35.64 (C-1 or C-8), 35.56 (C-8 or C-1), 34.0 (C-2), 32.9 (C-6), 32.0 (C-7), 31.8 (C-25), 28.9 (C-16), 25.4 (C-28), 24.2 (C-15), 21.13 (C-26), 21.10 (C-21), 21.0 (C-11), 18.9 (C-27), 17.3 (C-19), 12.2 (C-29), 12.1 (C-18). EIMS: *m*/*z* (%) = 410 (100) [M^+], 367 (60) [M^+ − *i*Pr], 298 (50), 271 (75) [M^+ − side chain], 245 (42). HREIMS: calcd. for $C_{29}H_{46}O$ 410.3548, found 410.3540.

Compound 9: An O_3/O_2 mixture was slowly bubbled, at −60 °C for 3 min, through a solution of ketone **8** (136.0 mg, 0.33 mmol) in anhydrous CH_2Cl_2 (7.2 mL). Me_2S (0.1 mL) was then added to the reaction mixture, and the solution was stirred for 20 min. The solvent was evaporated and the residue (108.2 mg) was chromatographed on an silica gel column (light petroleum ether/diethyl ether, from 99:1 to 95:5), to give starting compound **8** (24.1 mg, 18%) and oxo aldehyde **9** (82.0 mg, 92%[16]). R_f (light petroleum ether/ethyl acetate, 7:3) = 0.33. M.p. 175−176 °C (light petroleum ether/diethyl ether, 1:1). $[\alpha]_D$ = +56.5 (*c* = 0.96, $CHCl_3$). IR (liquid film): $\tilde{\nu}$ = 1672 cm^{-1}, 1720. UV (MeOH): λ_{max} (lg ε) = 239 nm (3.23). ^1H NMR (300 MHz): δ = 9.56 (d, *J* = 3.2 Hz, 1 H, 22-H), 5.73 (s, 1 H, 4-H), 1.18 (s, 3 H, 19-H_3), 1.12 (d, *J* = 6.9 Hz, 3 H, 21-H_3), 0.75 (s, 3 H, 18-H_3). ^{13}C NMR (75.5 MHz): δ = 205.0 (C-22), 199.8 (C-3), 171.3 (C-5), 123.8 (C-4), 55.1 (C-14), 53.7 (C-9), 50.8 (C-20 or C-17), 49.4 (C-17 or C-20), 43.0 (C-13), 39.2 (C-12), 38.5 (C-10), 35.6 (C-1 or C-8), 35.5 (C-8 or C-1), 33.9 (C-2), 32.8 (C-6), 31.9 (C-7), 27.0 (C-16), 24.5 (C-15), 20.9 (C-11), 17.3 (C-19), 13.4 (C-21), 12.3 (C-18). EIMS: *m*/*z* (%) = 328 (73) [M^+], 286 (30), [$M^+ - CH_2CO$], 271 (20) [M^+ − side chain], 229 (32), 124 (100). HREIMS: calcd. for $C_{22}H_{32}O_2$ 328.2402, found 328.2394.

Compound 10: MeMgI in Et_2O (3 M, 50 μL) was added, at room temperature and under Ar, to a stirred solution of aldehyde **9** (41.1 mg, 0.12 mmol) in 1.0 mL of anhydrous Et_2O. The reaction mixture was stirred at room temp. for 1.5 h, and at the end of this period poured into 10% H_2SO_4 solution (5 mL) and extracted with Et_2O (5 mL × 3). The combined ethereal extract was washed with H_2O, a saturated solution of $NaHCO_3$ and again H_2O, and was finally dried with Na_2SO_4. After evaporation of the solvent, the residue was chromatographed on a silica gel column (0.8 g, light petroleum ether/diethyl ether, from 100:0 to 50:50), to give starting compound **9** (9.3 mg, 23%) and alcohol **10** (mixture of the two epimers at C-22) (28.2 mg, 85%[16]). R_f (light petroleum ether/ethyl acetate, 7:3) = 0.20. IR (liquid film): $\tilde{\nu}$ = 1660 cm^{-1}. UV (MeOH): λ_{max} (lg ε) = 239 nm (3.29). ^1H NMR (300 MHz, signals of the predominant epimer): δ = 5.72 (s, 1 H, 4-H), 3.94 (m, 1 H, 22-H), 1.19 (s, 3 H, 19-H_3), 1.16 (d, *J* = 6.5 Hz, 3 H, 21-H_3), 0.92 (d, *J* = 6.3 Hz, 3 H, 23-H_3), 0.74 (s, 3 H, 18-H_3). ^{13}C NMR (75.5 MHz, signals of the predominant epimer): δ =199.8 (C-3), 171.8 (C-5), 123.8 (C-4), 69.3 (C-22), 55.8 (C-14), 53.8 (C-9), 52.8 (C-17), 42.1 (C-13), 41.8 (C-20), 39.7 (C-12), 38.6 (C-10), 35.7 (2 C, C-1 and C-8), 34.0 (C-2), 33.0 (C-6), 32.0 (C-7), 27.7 (C-16), 24.2 (C-15), 21.5 (C-21), 21.1 (C-11), 17.4 (C-19), 12.0 (C-18), 11.3 (C-23). EIMS: *m*/*z* (%) = 344 (95) [M^+], 326 (75) [$M^+ - H_2O$], 281 (44), 269 (35) [M^+ − side chain], 229 (65), 207 (90), 124 (100). HREIMS: calcd. for $C_{23}H_{36}O_2$ 344.2715, found 344.2788.

Compound 6: PDC (30 mg) was added to a stirred solution of compound **10** (20.0 mg, 0.06 mmol) in anhydrous CH_2Cl_2 (1.0 mL) under Ar. The reaction mixture was stirred at room temp. for 3 h and was then filtered through SiO_2. The solvent was evaporated and the residue (20.7 mg) was subjected to chromatography on a silica gel column (0.4 g, light petroleum ether/diethyl ether, from 100:0 to 80:20), to give crystalline diketone **6** (17.4 mg, 87%). R_f (light petroleum ether/ethyl acetate 7:3) = 0.32. M.p. 203−204 °C (light petroleum ether/diethyl ether, 1:1). $[\alpha]_D$ = +34.0 (*c* = 0.12, $CHCl_3$). IR (liquid film): $\tilde{\nu}$ = 1663 cm^{-1}, 1710. UV (MeOH): λ_{max} (lg ε) = 238 nm (3.50). CD (EtOH): $[\theta]_{217}$ = 21800. ^1H NMR (300 MHz): δ = 5.73 (s, 1 H, 4-H), 2.10 (s, 3 H, 23-H_3), 1.18 (s, 3 H, 19-H_3), 1.12 (d, *J* = 6.9 Hz, 3 H, 21-H_3), 0.73 (s, 3 H, 18-H_3). ^{13}C NMR (75.5 MHz): δ = 212.3 (C-22), 199.3 (C-3), 171.0 (C-5), 123.9 (C-4), 55.4 (C-14), 53.8 (C-9), 52.1 (C-17), 50.3 (C-20), 42.7 (C-13), 39.6 (C-12), 38.6 (C-10), 35.8 (C-8 or C-1), 35.7 (C-1 or C-8), 34.0 (C-2), 32.9 (C-6), 32.0 (C-7), 28.0 (C-23), 27.4 (C-16), 24.4 (C-15), 21.0 (C-11), 17.4 (C-19), 16.3 (C-21), 12.2 (C-18). EIMS: *m*/*z* (%) = 342 (80) [M^+], 300 (30) [$M^+ - CH_2CO$], 271 (55) [M^+ − side chain], 228 (34), 147 (55), 124 (100). HREIMS: calcd. for $C_{23}H_{34}O_2$ 342.2559, found 342.2570.

Acknowledgments

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