Plakinamines C and D and Three Other New Steroidal Alkaloids from the Sponge *Corticium* sp.

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Dedicated to the memory of Professor Luigi Minale, deceased May 11th, 1997

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Five new steroidal alkaloids, plakinamines C (**1**) and D (**2**), and the related compounds **3**–**5** have been isolated from the Vanuatu sponge *Corticium* sp. Their structures have been elucidated by a detailed spectroscopic analysis, including 2D-HMBC and ROESY correlation experiments. The new compounds show significant in vitro cytotoxicity against human bronchopulmonary non-small-cell lung carcinoma cells (NSCLC-N6) with IC_{50} values of < 3.3–5.7 μg/mL. When tested against T leukemia virus type one (HTLV-I), compounds **1**, **4** and **5** were found to exhibit slight anti-HIV activity.

Introduction

Steroidal alkaloids are well-known metabolites of certain terrestrial plants,[1] but only a few examples have been reported from marine organisms.[2,3]

As part of a systematic screening of the bioactive compounds from marine organisms, we have examined the marine sponge *Corticium* sp., collected off Porth Havannah, Vanuatu, South Pacific, the crude ethanolic extracts of which showed 100% cytotoxic activity towards KB cells at 10 μg/mL. The first paper on the sponge *Corticium* sp. dealt with the isolation of lokysterolamines A and B,[3] and recently we have described new modified steroidal alkaloids with seven-membered B rings obtained from the same sponge.[4]

Results and Discussion

In this paper we report the isolation and structure elucidation of five new steroidal alkaloids **1**−**5** following a bioassay-guided fractionation. The methanolic extract of the lyophilized sponge *Corticium* sp. was subjected to Kupchan's partitioning methodology.[5] In preliminary tests on the NSCLC-N6 cell line, the active $CHCl_3$ and *n*BuOH fractions showed IC_{50} values < 1.1 μg/mL and < 3.3 μg/mL, respectively. These fractions were chromatographed and the components were purified to afford pure compounds **1**−**5**. Plakinamines C (**1**) and D (**2**), and the related alkalosteroids **3**−**5** bear a skeletal relationship to the previously described plakinamine A.[2] The steroidal nature of **1**−**5** was suggested by comparison with literature data and by NMR experiments.

Plakinamine C (1)

Compound **1** showed a molecular ion peak (HREIMS) at m/z = 510.4229, corresponding to the molecular formula $C_{33}H_{54}N_2O_2$ (calcd. 510.4185). This was further supported by ^{13}C-NMR data, which, in combination with the COSY spectrum, clearly indicated the steroidal nature of **1**. The ^{1}H-NMR spectrum in CD_3OD exhibits two methyl group singlet signals at δ = 0.62 and δ = 0.74, and a methyl doublet at δ = 0.98 (J = 6.8 Hz), which were correlated by HMQC to carbon atom signals at δ = 12.6, 15.3 and 19.6 (C-18, C-19 and C-21, respectively). The ^{13}C-NMR spectrum also features a signal at δ = 211.8 attributable to a ketone function. The downfield shift of the C-19 signal is compatible with the ketone carbon atom C-4 of the steroid nucleus, and this was corroborated by the chemical shift of the 5α-H signal, which was found to be downfield shifted at δ = 2.40 by analysis of the COSY spectrum. When the COSY experiment was performed in [D_5]pyridine, the 3-H signal was observed as a doublet of doublets at δ = 3.14 (J = 12.0, 5.8 Hz). The ^{1}H-NMR spectrum (CD_3OD) also features a signal at δ = 2.37 (s), corresponding to 6 H, typical for two *N*-methyl groups. The downfield shift of the C-3 signal (δ = 72.0) suggests a β orientation of the dimethylamino group; in the corresponding 3α-aminosteroids the ^{13}C-NMR chemical signal is found at much higher field.[6] This observation is in agreement with data obtained from ROESY experiments in [D_5]pyridine, which show a mutual

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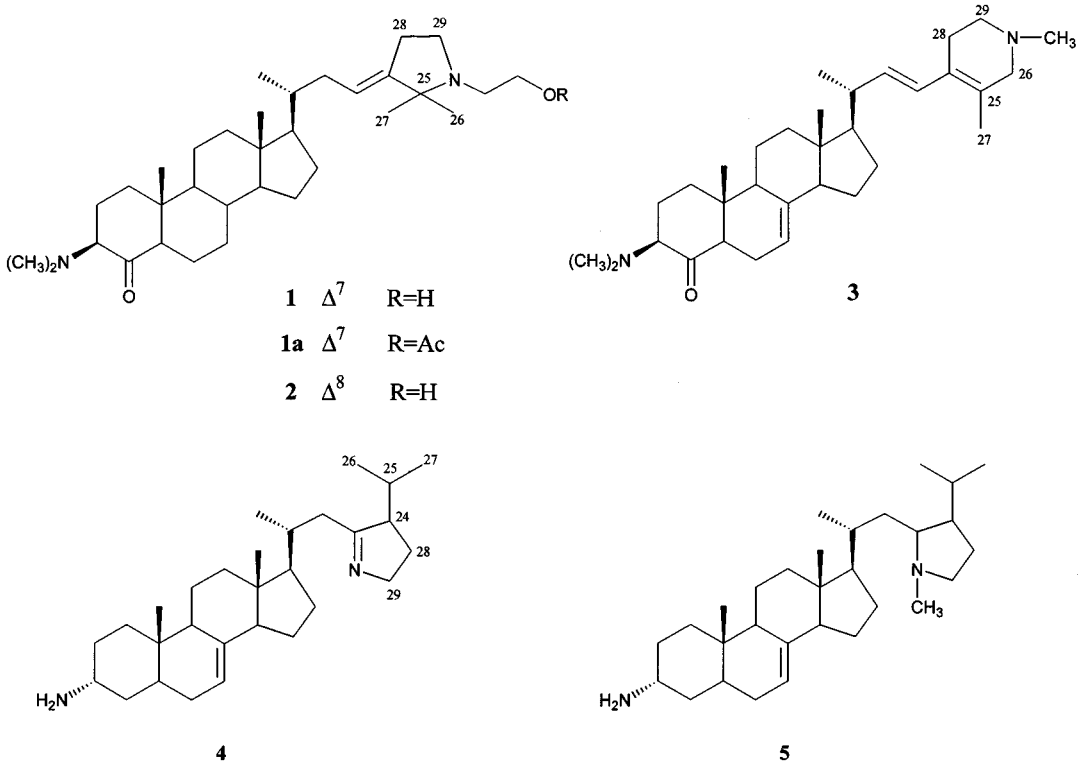

Scheme 1. New steroidal alkaloids from the sponge *Iortieium* sp.

correlation of 3α-H (δ = 3.14) with 5α-H at δ = 2.40, further corroborating the A/B *trans* junction. The configuration of the substituent at C-3 in **1** is epimeric to that reported for plakinamine A, which was established by comparison of the relevant ^{13}C-NMR shifts with those of synthetic 3α-amino-5α-ergosta-7,22-diene. The ^{13}C-NMR spectrum of **1** also features four low-field signals. Those at δ = 117.5 and δ = 140.0 were assigned to a Δ^7 double bond, in close analogy with plakinamine A.[2]

The substitution pattern of the side chain was elucidated by interpretation of ^{1}H-^{1}H coupling constants, 2D-COSY and HMBC data (Table 2). The ^{1}H-NMR spectrum features signals for two methyl groups (δ = 1.11 and δ = 1.12, 2 s), an oxygenated methylene (δ = 3.70, t, *J* = 6.2 Hz) and an olefinic proton (δ = 5.29). By means of an HMQC experiment, these proton signals could be correlated with the corresponding carbon signals at δ = 23.5, 23.6, 61.5 and 119.3, respectively. The presence of two methylene triplets at δ = 2.87 (*J* = 6.8 Hz) and δ = 2.65 (*J* = 6.4 Hz), showing correlation by HMQC with carbon signals at δ = 50.4 and δ = 51.9, suggests nitrogen substitution in the side chain. Comparison of the ^{13}C-NMR data with those of plakinamine A[2] allowed us to establish the stereochemistry at C-20, and also revealed the presence of two quaternary carbon atoms with signals at δ = 149.7 and δ = 64.6.

Interpretation of the COSY spectrum led to three partial structures: C-21 to C-23, C-28 to C-29, and $-$N*CH*$_2$-CH$_2$OH to $-$NCH$_2$*CH*$_2$OH. On the basis of HMBC data (Table 2), it could be established that these structural units are connected through non-protonated carbon atoms, including Me-26/C-24 and C-25; 28-H$_2$/C-23, C-24, C-29; and 29-H$_2$/C-24, C-25, C-28, $-$N*CH*$_2$CH$_2$OH, implying the presence of a pyrrolidine ring. HMBC cross-peaks 29-H$_2$/$-$N*CH*$_2$CH$_2$OH and $-$N*CH*$_2$CH$_2$OH/C-25, C-29 imply that the ethanolamine residue is bonded to the pyrrolidine ring through the nitrogen atom.

Acetylation of plakinamine C (**1**) with acetic anhydride/pyridine gave the amorphous monoacetate **1a**, the ^{1}H-NMR spectrum of which showed the signals corresponding to the hydroxy methylene protons of the ethanolamine residue downfield shifted at δ = 4.30.

Plakinamine D (2)

The HREIMS of **2** showed a molecular ion peak at *m/z* = 510.4229, in agreement with the molecular formula $C_{33}H_{54}N_2O_2$ (calcd. 510.4185), indicating this component to be an isomer of compound **1**. The ^{1}H- and ^{13}C-NMR data of **2** are superimposable on those of **1** as far as the side chain from C-22 to $-$NCH$_2$*CH*$_2$OH is concerned, but significantly different NMR shifts are seen for the tetracyclic nucleus. The ^{13}C-NMR spectrum features a signal at δ = 212.4, attributable to the ketone carbon atom C-4, and signals for two quaternary carbon atoms at δ = 130.1 and δ = 135.3. These spectral features indicate that **2** possesses a Δ^8 or $\Delta^{8(14)}$ steroidal nucleus. The former was favored owing to the fact that the observed chemical shift of Me-18 at δ = 0.69 is in excellent agreement with that reported for Δ^8 sterols, but is significantly different from that reported for $\Delta^{8(14)}$ sterols.[7] Two-dimensional COSY, HMQC, and HMBC correlations allowed assignment of all the proton

Table 1. ^{1}H- (500 MHz) and ^{13}C- (125 MHz) -NMR assignments (CD_3OD) of the steroid nucleus in compounds **1**−**5**

	1 and **3**[a]		**2**		**4** and **5**[d]	
C-	δ_H[b]	δ_C	δ_H	δ_C	δ_H	δ_C
1	2.12, 1.73	38.0	2.11, 1.79	36.5	1.66, 1.43	32.8
2	1.90	22.5	1.93	24.0	1.76, 1.33	30.7
3		72.0		71.6	3.28 br. s	47.6
4		211.8		212.4	1.70, 1.47	35.2
5	2.40 dd[c]	55.0	2.49 br. t	56.0	1.68	35.6
6	2.18, 1.92	26.0	1.76, 1.72	18.3	2.03, 1.33	29.5
7	5.25 br. s	117.5	2.26, 1.81	26.1	5.24 br. s	118.8
8		140.0		130.1		140.5
9	2.07	50.7		135.3	1.86	50.7
10		42.6		43.9		35.9
11	1.74, 1.59	23.2	2.12	27.2	1.54, 1.68	22.3
12	2.10, 1.78	40.6	2.09, 1.50	38.2	2.13, 1.33	40.9
13		44.6		43.4		44.6
14	1.92	56.2	2.17	53.1	1.93	56.4
15	1.60, 1.50	23.8	1.68, 1.42	24.8	1.49, 1.60	23.9
16	1.74	29.0	2.01, 1.44	29.9	1.84, 1.61	28.4
17	1.39	57.3	1.28	56.3	1.29	58.3
18	0.62 s	12.6	0.69 s	11.7	0.62 s	12.3
19	0.74 s	15.3	0.92 s	19.6	0.86 s	12.6
$N(CH_3)_2$	2.37 s	42.0				

[a] Data obtained from plakinamine C (**1**). − [b] ^{1}H assignments aided by COSY experiments. − [c] Overlapped with other signals. − [d] Data obtained from compound **5**.

Table 2. ^{1}H- (500 MHz) and ^{13}C- (125 MHz) -NMR assignments and HMBC correlations of the side chains in compounds **1**−**3** (CD_3OD)

	1 and **2**[a]			**3**		
C-	δ_H[b]	δ_C	HMBC[c]	δ_H	δ_C	HMBC
20	1.53	38.5		2.20	42.3	
21	0.98 (6.8)	19.6	C-17,C-20,C-22	1.11 d (6.6)	21.5	C-17,C-20,C-22
22	2.13, 1.76	36.5	C-20,C-21,C-23,C-24	5.52 dd (15.4, 8.8)	136.0	C-17,C-20,C-21
23	5.29[d]	119.3	C-28	6.42 d (15.4)	126.0	C-20,C-24,C-28
24		149.7			128.0	
25		64.6			127.3	
26	1.11 s	23.5	C-24,C-25	2.93 br. s	60.8	C-24,C-27,C-29,NCH_3
27	1.12 s	23.6		1.76 s	16.4	C-25,C-26
28	2.48 br. t	28.0	C-23,C-24,C-29	2.30 br. t	27.0	
29	2.87 t (6.8)	50.4	C-24,C-25,C28, NCH_2CH_2OH	2.60 t (5.9)	53.2	C-26,C-28,NCH_3
NCH_2CH_2OH	2.65 t (6.4)	51.9	C-25,C-29, NCH_2CH_2OH			
NCH_2CH_2OH	3.70 t (6.2)	61.5	NCH_2CH_2OH			
NCH_3				2.36 s	45.5	C-26,C-29

[a] Data obtained from plakinamine C (**1**). − [b] Coupling constants (in Hz) are given in parentheses; ^{1}H assignments aided by COSY experiments. − [c] HMBC optimized for $^{2,3}J_{CH}$ = 10 Hz. − [d] Overlapped with signal of 7-H.

and carbon resonances[8] and fully confirmed this hypothesis (Tables 1 and 2).

Other Components

The molecular composition of compound **3** was determined as $C_{32}H_{50}N_2O$ from the pseudomolecular ion peak at m/z = 479 $[M + H]^+$ in the positive-ion FAB mass spectrum, which is consistent with the ^{13}C-NMR data. Analysis of the ^{1}H-, ^{13}C-NMR and COSY spectra revealed the tetracyclic system of the steroid nucleus of **3** to be identical to that observed for plakinamines C (**1**) and D (**2**). A ROESY experiment showed mutual correlation between 3α-H and 5α-H, suggesting β orientation of the dimethylamino group at C-3. The substitution pattern of the side chain was elucidated by analysis of 2D-COSY, HMQC, and HMBC experiments. The spin sequence from C-20 to C-29 was identical to that reported for the side chain of plakinamine B,[2] with a tetrasubstituted olefinic bond (δ = 128.0 and 127.3). The UV absorption at λ_{max} = 242 nm (ε = 2900), together with the chemical shifts of the C-22 and C-23 signals, indicates the presence of a conjugated diene between C-22 and C-25. HMBC correlations (Table 2) allowed the structural elucidation of **3**, which as a result can be defined as *N*,*N*-dimethyl-4-oxo-3-*epi*-plakinamine B.

Steroidal alkaloid **4** is the 24,25-dihydro derivative of plakinamine A.[2] It shows a pseudomolecular ion peak at m/z = 425 $[M + H]^+$ in the positive-ion FAB mass spectrum, corresponding to the composition $C_{29}H_{48}N_2$, which is in accordance with ^{13}C-NMR data. Analysis of its ^{1}H-,

^{13}C-NMR and COSY spectra (Table 1) revealed the tetracyclic system of **4** to be identical to that of plakinamine A. The ^{13}C-NMR spectrum shows three low-field signals. The signals at δ = 118.8 and δ = 140.5 are in good agreement with literature values for C-7 and C-8 of Δ^7 sterols,[7][8] while the signal at δ = 183.0 can be assigned to an imine function. The most significant difference observed in the ^{1}H-NMR spectrum of **4** is the presence of two methyl signals at δ = 0.76 (d, J = 7.0 Hz) and δ = 1.04 (d, J = 7.0 Hz), attributable to the methyl groups Me-26 and Me-27. In the COSY spectrum, both of these signals show coupling with a methine proton at δ = 2.14, which in turn shows correlation with a signal at δ = 2.88. This last proton shows cross-peaks with signals at δ = 1.90 and δ = 1.79 (28-H_2), which in turn correlate with a triplet at δ = 3.71 (29-H_2). The latter can be assigned to the methylene group in a ring formed between the imine nitrogen atom and the isopropyl group. Further useful information was provided by HMBC experiments, which showed correlations between the proton signal at δ = 3.71 and the imine signal at δ = 183.0. Other correlations found in the HMBC spectrum are reported in Table 3.

Compound **5** is related to steroidal alkaloid **4**. It has the molecular formula $C_{30}H_{52}N_2$, as determined on the basis of ^{13}C-NMR data and from analysis of its positive-ion FAB mass spectrum, which shows a pseudomolecular ion peak at m/z = 441 $[M + H]^+$, i.e. 16 mass units more than **4**. The structure of the steroid nucleus (Table 1) was readily established by comparison of the ^{1}H-, ^{13}C-NMR and COSY spectral data with those of **4**, since the chemical shifts of the signals of carbon atoms 1 to 19 are virtually identical in the two compounds. The main difference is observed in the substitution pattern of the side chain. In the ^{13}C-NMR spectrum of **5**, the imine signal of **4** is replaced by a carbon signal at δ = 67.8. Interpretation of the COSY data revealed a spin sequence from C-20 to C-29 indicative of a saturated pyrrolidine ring. The ^{1}H-NMR spectrum of **5** also features a signal at δ = 2.35 (s, 3 H), indicating an additional *N*-methyl group compared to compound **4**. HMBC correlations (Table 3) suggest that this function is located on the nitrogen atom of the pyrrolidine ring, which is supported by the ^{13}C-NMR data.

Cytotoxicity Tests

When tested against human bronchopulmonary non-small-cell lung carcinoma cells (NSCLC-N6), all compounds exhibited in vitro cytotoxic activity. Compounds **3**, **4** and **5** showed activity at the tested concentrations, with IC_{50} values of 3.6 μg/mL, 5.7 μg/mL, and 4.9 μg/mL, respectively, while plakinamine D (**2**) was cytotoxic with IC_{50} < 3.3 μg/mL. Furthermore, the compounds showed anti-HIV activity, which was monitored by the efficiency of the substrate to inhibit *syncytia* formation after HIV infection of an MT_4 cell line, as described previously.[9,10] A slight delay of infection was observed with compound **4** at 0.05 μg/mL, with plakinamine C (**1**) at 0.1 μg/mL, and with compound **5** at 0.1 μg/mL. The remaining compounds were toxic at the concentrations tested.

Experimental Section

General: NMR measurements were performed with a Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer, using the UXNMR software package. Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were recorded by employing the conventional pulse sequence.[11] The HMQC and ROESY experiments were performed according to Bax et al.[12] The ROESY experiment was acquired in phase-sensitive mode (TPPI). ^{1}H-detected heteronuclear multiple bond correlation (HMBC) spectroscopy was performed according to Bax and coworkers.[12,13] – Mass spectra were recorded with a VG Prospec instrument equipped with an FIB source (Cs^+ ion bombardment) using a glycerol or glycerol/thioglycerol (3:1) matrix.

Animal Material: Samples of the sponge *Corticium* sp. were collected at a depth of 12–18 m at Efaté, Porth Havannah, Vanuatu, South Pacific, in July 1996. The samples were frozen immediately after collection and lyophilized to yield 180 g of dry mass. The sponge was identified by Dr. John Hooper of the Queensland Museum, Brisbane, Australia, as *Corticium* sp. (Homosclerophorida,

Table 3. ^{1}H- (500 MHz) and ^{13}C- (125 MHz) -NMR assignments and HMBC correlations of the side chains in compounds **4** and **5** (CD_3OD)

	4			**5**		
C-	δ_H[a]	δ_C	HMBC[b]	δ_H	δ_C	HMBC
20	1.80	35.3		1.57	35.4	
21	0.90 (6.2)	19.2	C-17,C-20,C-22	1.04 d (6.2)	19.7	C-17,C-20,C-22
22	2.47, 2.04	39.7	C-23	1.60, 1.35	42.5	
23		183.0		2.22	67.8	NCH_3
24	2.88 br. t	56.4		1.83	53.3	
25	2.14	29.3		1.79	31.4	
26	1.04 d (7.0)	22.1	C-24,C-25,C-27	0.97 d (6.8)	17.6	C-24,C-25,C-27
27	0.76 d (7.0)	16.5	C-24,C-25,C-26	0.91 d (6.4)	22.7	C-24,C-25,C-26
28	1.90, 1.79	30.7		1.82, 1.68	25.1	
29	3.71 br. t	59.7	C-23	2.96 t (7.5), 2.26	57.4	
NCH_3				2.35 s	40.8	C-23,C-29

[a] Coupling constants (in Hz) are given in parentheses; ^{1}H assignments aided by COSY experiments. – [b] HMBC optimized for $^{2,3}J_{CH}$ = 10 Hz.

Plakinidae). A voucher specimen (R1718) has been deposited at the ORSTOM Center in Nouméa, New Caledonia.

Extraction and Isolation: The lyophilized sponge (180 g) was extracted by blending with MeOH (4 × 1 L). The combined extracts were concentrated and subjected to Kupchan′s partitioning scheme to give four extracts: *n*-hexane (4.8 g), CCl_4 (1.4 g), $CHCl_3$ (2.7 g), and *n*BuOH (5.9 g), which were each tested against the NSCLC-N6 cell line. The *n*-hexane fraction proved inactive, while the CCl_4, $CHCl_3$, and *n*BuOH fractions showed cytotoxic activity with IC_{50} values of 17.9 μg/mL, < 1.1 μg/mL, and 3.3 μg/mL, respectively. − The more cytotoxic $CHCl_3$ and *n*BuOH fractions were subsequently purified. The $CHCl_3$ extract was fractionated by DCCC using $CHCl_3$/MeOH/H_2O (7:13:8) in the ascending mode (the lower phase was the stationary phase). Fractions (6 mL each) were collected and examined by TLC on SiO_2 with $CHCl_3$/MeOH/H_2O (80:18:2) as eluent. Fractions 6−28 were pooled and purified by HPLC on a C_{18} μ-Bondapak column (30 cm × 3.9 mm i.d.) eluting with MeOH/H_2O/TEA (92:8:0.5) to yield pure plakinamine C (**1**) (10.0 mg), plakinamine D (**2**) (2.5 mg), and compound **5** (8.4 mg). Fractions 32−47 from the DCCC were chromatographed on a Sephadex LH-20 column (3 × 80 cm), eluting with MeOH, to give three main fractions: 1−30, 31−69, and 70−120. Fractions 31−69 were then purified by HPLC (C_{18} μ-Bondapak column, 30 cm × 3.9 mm i.d.) under the same conditions as above to give pure compound **4** (7.5 mg). − The *n*BuOH extract was submitted to DCCC with *n*BuOH/Me_2CO/H_2O (3:1:5) in the descending mode (the upper phase was used as stationary phase). The obtained fractions were then separated by reversed-phase HPLC (C_{18} μ-Bondapak column, 30 cm × 3.9 mm i.d.) with MeOH/H_2O/TEA (92:8:0.5) as the eluent, to give pure compound **3** and two modified steroidal alkaloids.[4]

Plakinamine C (1): Yield: 10.0 mg; $[\alpha]_D$ = +29.4 (*c* = 0.016, $CHCl_3$/MeOH, 1:1); ^{1}H- and ^{13}C-NMR spectral data of the steroid nucleus are given in Table 1, and those of the side chain in Table 2.

Plakinamine C Acetate (1a): A solution of **1** (1 mg) in acetic anhydride/pyridine (1:1) was left to stand at room temperature for about 12 h. The solvents were then evaporated under reduced pressure affording 1 mg of the monoacetate **1a**.

Plakinamine D (2): Yield: 2.5 mg; $[\alpha]_D$ = +25.2 (*c* = 0.013, $CHCl_3$/MeOH, 1:1); ^{1}H- and ^{13}C-NMR spectral data of the steroid nucleus are given in Table 1, and those of the side chain in Table 2.

Compound 3: Yield: 25.2 mg; $[\alpha]_D$ = +35.4 (*c* = 0.014, $CHCl_3$/MeOH, 1:1); ^{1}H- and ^{13}C-NMR spectral data of the steroid nucleus are given in Table 1, and those of the side chain in Table 2.

Compound 4: Yield: 7.5 mg; $[\alpha]_D$ = +7.4 (*c* = 0.015, $CHCl_3$/MeOH, 1:1); ^{1}H- and ^{13}C-NMR spectral data of the steroid nucleus are given in Table 1, and those of the side chain in Table 3.

Compound 5: Yield: 8.4 mg; $[\alpha]_D$ = +23.0 (*c* = 0.020, $CHCl_3$/MeOH, 1:1); ^{1}H- and ^{13}C-NMR spectral data of the steroid nucleus are given in Table 1, and those of the side chain in Table 3.

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