Two New Polyhydroxylated Steroids from the Hainan Soft Coral *Sinularia* sp.

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Two new polyhydroxylated steroids, (2*β*,3*β*,4*α*,5*α*,8*β*,11*β*)-4-methylergost-24(28)-ene-2,3,8,11-tetrol-(**1**) and (3*β*,5*α*,6*β*)-ergosta-22,24(28)-diene-3,5,6,19-tetrol (**3**), together with the six known related steroids **2** and **4**–**8**, were isolated from the Hainan soft coral *Sinularia* sp. Their structures were elucidated on the basis of spectroscopic analysis and by comparison with previously reported data. The structure of the known compound **9** (hyrtiosterol) was revised as **2** by extensive analysis of the ROESY data and by the NOE difference experiment.

Introduction. – Soft corals are Coelenterates (class Anthozoa, subclass Octocorallia, order Alcyonacea, family Alcyoniidae), of which some are symbiotic associations of coral animals (polyps) with their algal partners (Zooxanthellae) [1]. They are rich sources of terpenoids, notably cembranoid diterpenes, and steroids, of which many were reported to have various biological activities ranging from antitumor, antiallergy, to anti-inflammatory [2]. The genus *Sinularia*, which is very speciose in the South China Sea, is reputed for its versatile chemical constituents and their biological activity. Terpenoids [3–6] and steroids [7–9] compose the main secondary metabolites isolated from the genus. In particular, many biological activities were reported for these metabolites, including cytotoxicity [10], enhancement of glucose transport in rat adipocytes [11], and histamine-release inhibition [12].

In our previous study [13], we have reported the isolation and structural elucidation of two new 19-acetylated steroids from an undetermined species of *Sinularia* (*01HN-75*). However, strangely enough, we did not find the presence of diterpenoids from the mentioned collection. Recently, in the course of our continuous efforts aiming at searching for new bioactive molecules from South China Sea invertebrates [14–17], we made another collection of the soft coral *Sinularia* sp. (*02HN-75*), which showed the same appearance as the sample *01HN-75* and was identified by Prof. *R.-L. Zhou* of the South China Sea Institute of Oceanology-CAS to be the same species as the first collection from the same place but different location. Chemical investigation of the Et_2O-soluble fraction of this animal resulted in the isolation of two new polyhydroxylated steroids, named (2*β*,3*β*,4*α*,5*α*,8*β*,11*β*)-4-methylergost-24(28)-ene-2,3,8,11-tetrol-(**1**), (3*β*,5*α*,6*β*)-ergosta-22,24(28)-diene-3,5,6,19-tetrol (**3**), along with the six known related steroids **2** and **4**–**8** (*Fig. 1*). Once again, we did not discover diterpene constituents from this collection. In the present paper, we report the isolation and structure elucidation of the new compounds.

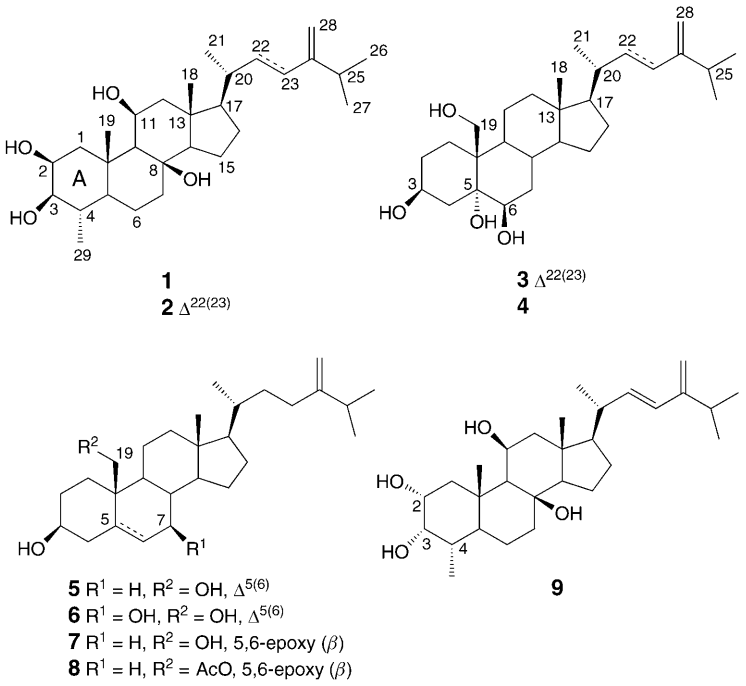

Fig. 1. *The structure of compounds* **1**–**9**

Results and Discussion. – The usual workup [13] of the Et_2O-soluble fraction of the acetone extract of the specimen (*02HN-75*) yielded compounds **1**–**8**. The known compounds were identified as (2*β*,3*β*,4*α*,5*α*,8*β*,11*β*)-4-methylergosta-22,24(28)-diene-2,3,8,11-tetrol (**2**) [18], (3*β*,5*α*,6*β*)-24-methylcholest-24(28)-ene-3,5,6,19-tetrol (=(3*β*,5α,6*β*)-ergost-24(28)-ene-3,5,6,19-tetrol; **4**) [19], (3*β*)-24-methylcholesta-5,24(28)-diene-3,19-diol (=(3*β*)-ergosta-5,24(28)-diene-3,19-diol; **5**) [20], (3*β*,7*β*)-ergosta-5,24(28)-diene-3,7,13-triol (**6**) [21], 5,6-epoxylitosterol (=(3*β*,5*β*,6*β*)-5,6-epoxyergost-24(28)-ene-3,19-diol; **7**) [20], and armatinol A (=(3*β*,5*β*,6*β*)-5,6-epoxyergost-24(28)-ene-3,19-diol 19-acetate; **8**) [22], by analysis of their NMR spectra and comparison with the data reported in the literature.

Compound **1** was obtained as a viscous liquid with a molecular formula of $C_{29}H_{50}O_4$, as established by its HR-ESI-MS and NMR spectra, indicating five degrees of unsaturation. The ^{1}H- and ^{13}C-NMR (*Table*), ^{1}H,^{1}H-COSY and HMBC (*Fig. 2*), HMQC, and NOESY data (*Fig. 3*) allowed to deduce its structure.

The ^{1}H-NMR spectrum of **1** revealed resonances for 46 protons including two tertiary Me signals at δ 1.09 (Me(18)) and 1.52 (Me(19)) and four secondary Me resonances at δ 0.93 (Me(21)), 1.01 (Me(26)), 1.00 (Me(27)), and 0.98 (Me(29)). Additionally, signals for two terminal CH_2 protons at δ 4.64 (H–C(28)) and 4.70 (H–C(28)), together with three OCH protons at δ 4.05 (H–C(2)), 3.07 (H–C(3)), and 4.45 (H–C(11)) were observed. The ^{13}C-NMR spectrum of **1** displayed 29 signals including 6 Me, 9 CH_2, 10 CH, and 4 quaternary C-atoms. The four signals between δ 69.8 and 77.4 were indicative of oxygenated C-atoms. Three of the oxygenated C-atoms were assigned to OH-bearing CH moieties and one

Table. *^{1}H-NMR Data of Compounds* **1** *and* **3** *and ^{13}C-NMR Data of Compounds* **1**–**4**

	1[a])		**2**[a])	**3**[b])		**4**[b])
	δ(H)	δ(C)	δ(C)	δ(H)	δ(C)	δ(C)
CH_2(1)	1.12–1.16 (*m*), 2.24–2.28 (*m*)	43.8 (*t*)	43.8 (*t*)	2.11–2.15 (*m*), 1.34–1.40 (*m*)	30.1 (*t*)	31.4 (*t*)
H–C(2)	4.05 (br. *s*)	69.9 (*d*)	69.9 (*d*)	2.15–2.20 (*m*), 2.42–2.47 (*m*)	32.7 (*t*)	32.6 (*t*)
H–C(3)	3.07 (*dd*, *J*=7.1, 2.7)	77.4 (*d*)	77.4 (*d*)	4.93–4.97 (*m*)	67.5 (*d*)	67.5 (*d*)
H–C(4) or CH_2(4)	1.72–1.77 (*m*)	32.6 (*d*)	32.6 (*d*)	2.37–2.43 (*m*), 3.05–3.09 (*m*)	43.0 (*t*)	42.8 (*t*)
H–C(5) or C(5)	0.68–0.75 (*m*)	52.7 (*d*)	52.7 (*d*)	–	76.4 (*s*)	76.3 (*s*)
CH_2(6) or H–C(6)	1.47–1.52 (*m*), 1.53–1.59 (*m*)	19.7 (*t*)	19.7 (*t*)	4.16 (br. *s*)	76.0 (*d*)	75.8 (*d*)
CH_2(7)	1.18–1.22 (*m*), 1.70–1.75 (*m*)	39.9 (*t*)	39.9 (*t*)	2.38–2.43 (*m*)	35.2 (*t*)	35.0 (*t*)
C(8) or H–C(8)	–	75.6 (*s*)	75.6 (*s*)	2.80–2.85 (*m*)	32.5 (*d*)	32.4 (*d*)
H–C(9)	0.84 (br. *d*, *J*=2.8)	57.9 (*d*)	58.0 (*d*)	1.93–1.97 (*m*)	46.7 (*d*)	46.5 (*d*)
C(10)	–	36.8 (*s*)	36.9 (*s*)	–	43.9 (*s*)	43.8 (*s*)
H–C(11) or CH_2(11)	4.45 (br. *s*)	69.8 (*d*)	69.8 (*d*)	1.23–1.26 (*m*)	22.7 (*t*)	22.6 (*t*)
CH_2(12)	1.34–1.38 (*m*), 2.30–2.33 (*m*)	49.2 (*t*)	49.1 (*t*)	1.36–1.39 (*m*)	41.3 (*t*)	41.3 (*t*)
C(13)	–	42.1 (*s*)	42.0 (*s*)	–	43.7 (*s*)	43.6 (*s*)
H–C(14)	1.17–1.22 (*m*)	60.4 (*d*)	60.5 (*d*)	2.27–2.30 (*m*)	57.8 (*d*)	57.6 (*d*)
CH_2(15)	1.40–1.44 (*m*), 1.51–1.54 (*m*)	19.2 (*t*)	19.2 (*t*)	1.70–1.73 (*m*)	29.1 (*t*)	28.6 (*t*)
CH_2(16)	1.24–1.32 (*m*), 1.82–1.88 (*m*)	27.4 (*t*)	27.8 (*t*)	1.35–1.39 (*m*), 2.10–2.15 (*m*)	29.7 (*t*)	29.7 (*t*)
H–C(17)	0.98–1.04 (*m*)	58.1 (*d*)	58.1 (*d*)	2.29–2.32 (*m*)	56.5 (*d*)	56.5 (*d*)
Me(18)	1.09 (*s*)	15.3 (*q*)	15.6 (*q*)	0.92 (*s*)	13.2 (*q*)	12.8 (*q*)
Me(19) or CH_2(19)	1.52 (*s*)	18.3 (*q*)	18.3 (*q*)	4.33 (*d*, *J*=12.0), 4.93 (*d*, *J*=12.0)	64.8 (*t*)	64.7 (*t*)
H–C(20)	1.38–1.43 (*m*)	35.1 (*d*)	39.7 (*d*)	2.16–2.22 (*m*)	40.9 (*d*)	36.1 (*d*)
Me(21)	0.93 (*d*, *J*=6.5)	18.3 (*q*)	20.1 (*q*)	1.11 (*d*, *J*=6.7)	20.9 (*q*)	18.9 (*q*)
CH_2(22) or H–C(22)	1.06–1.10 (*m*), 1.49–1.54 (*m*)	34.2 (*t*)	135.4 (*d*)	5.69 (*dd*, *J*=15.8, 8.9)	136.6 (*d*)	35.1 (*t*)
CH_2(23) or H–C(23)	1.84–1.88 (*m*), 2.02–2.09 (*m*)	30.9 (*t*)	129.5 (*d*)	6.08 (*d*, *J*=15.8)	129.4 (*d*)	24.6 (*t*)
C(24)	–	156.7 (*s*)	152.9 (*s*)	–	153.4 (*s*)	156.8 (*s*)
H–C(25)	2.17–2.23 (*m*)	33.8 (*d*)	29.4 (*d*)	2.50–2.55 (*m*)	29.8 (*d*)	34.1 (*d*)
Me(26)	1.01 (*d*, *J*=7.0)	21.8 (*q*)	22.4 (*q*)	1.12 (*d*, *J*=6.6)	22.3 (*q*)	22.0 (*q*)
Me(27)	1.00 (*d*, *J*=6.7)	22.0 (*q*)	22.0 (*q*)	1.12 (*d*, *J*=6.6)	22.3 (*q*)	22.1 (*q*)
CH_2(28)	4.64 (br. *s*), 4.70 (br. *s*)	106.0 (*t*)	109.7 (*t*)	4.96 (br. *s*), 5.04 (br. *s*)	110.0 (*t*)	106.6 (*t*)
Me(29)	0.98 (*d*, *J*=6.5)	14.9 (*q*)	14.8 (*q*)			
OH–C(11)	2.72 (br. *s*)					

[a]) Measured in $CDCl_3$ with a *Varian-INOVA-600* spectrometer; chemical shifts δ [ppm] are referred to $CHCl_3$ (δ(H) 7.26) and $CDCl_3$ (δ(C) 77.0); *J* in Hz. [b]) Measured in C_5D_5N with a *Bruker-DRX*-500-MHz spectrometer; chemical shifts δ [ppm] are referred to C_5H_5N (δ(H), 7.20, 7.57, 8.73) and C_5D_5N (δ(C), 123.6, 135.8, 150.0); *J* in Hz.

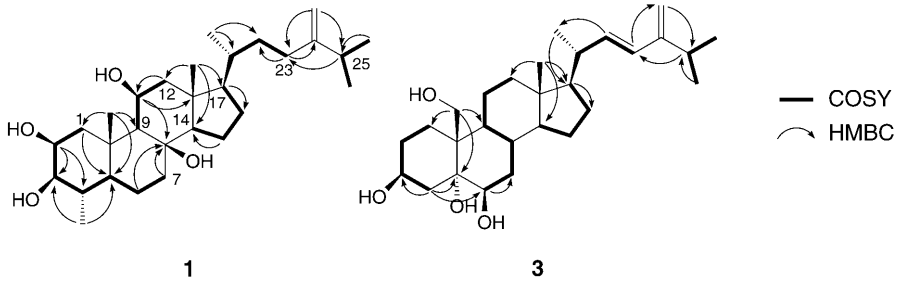

Fig. 2. *The ^{1}H,^{1}H-COSY and selected key HMBC correlations of* **1** *and* **3**

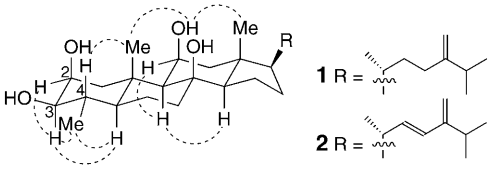

Fig. 3. *Selected key NOESY correlations of* **1** *and* **2**

to a quaternary C-atom on the basis of DEPT and HMQC data. Five signals for Me groups (δ 15.3, 18.3, 18.3, 21.8, and 22.0) were typical for a sterol having a cholestane skeleton. The sixth secondary Me group at δ 14.9 was indicative for a 4α-positioned Me group, which is well known in zooxanthellae sterols [23].

The ^{1}H,^{1}H-COSY and HMQC data revealed that **1** has four distinct ^{1}H,^{1}H spin systems including the structural fragments C(1) to C(7), C(9) to C(12), C(14) to C(23), and C(25) to C(27) (see *Fig. 2*). Interpretation of the COSY and HMQC data allowed to locate three OH groups at C(2) (δ 69.9), C(3) (δ 77.4), and C(11) (δ 69.8). These assignments were supported by the HMBC cross-peaks H–C(2)/C(3), C(4), H–C(3)/C(4), C(29), H–C(9)/C(11), and CH_2(12)/C(11) (*Fig. 2*). The existence of the fourth OH moiety at C(8) (δ 75.6) was implied by the HMBC cross-peaks CH_2(6)/C(8), CH_2(7)/C(8), H–C(9)/C(8), and H–C(11)/C(8).

The relative configuration of **1** was determined by a ROESY experiment (*Fig. 3*). Specifically, NOE correlations observed between H–C(4) and Me(19) indicated that H–C(4) was β-oriented. Similarly, NOE interactions between H–C(2) and H–C(3), H–C(3) and Me(29), Me(29) and H–C(5), and H–C(3) and H–C(5) indicated that H–C(2), H–C(3), Me(29), and H–C(5) were all α-oriented. These assignments were confirmed by an NOE difference experiment performed with **1**. Thus, irradiation of the H–C(3) signal resulted in enhancements of the H–C(2), Me(29), and H–C(5) signals implying that all these protons occurred on the same face (α) of the ring A. Furthermore, NOE correlations observed between OH–C(11) and Me(19), OH–C(11) and Me(18), H–C(11) and H–C(9), H–C(9) and H–C(14) indicated that H–C(9), H–C(11), and H–C(14) were all α-oriented. The β-configuration of the OH moiety at C(8) was assigned by comparison of the chemical shift value of C(8) with literature data [24].

Compound **2**, a colorless oil, showed ^{1}H-NMR data very similar to those of **1**, indicating the same framework and similar functional groups in the molecule. In addition, the NMR spectra exhibited signals attributable to an additional disubstituted C=C bond at the side chain. The splitting pattern and the coupling constants of the olefinic protons (δ 5.52 (*dd*, J = 15.7, 8.9 Hz) and 5.92 (*d*, J = 15.7 Hz)) not only suggested the position of the olefin at C(22)=C(23) but also indicated its (*E*) nature. The ESI-MS of **2** showed a quasi-molecular ion peak at *m/z* 483 ($[M+Na]^+$), two mass units less than **1**, supporting the dehydrogenation at C(22) and C(23). Apparently, the proposed

structure of **2** is a C(2) and C(3) epimer of hyrtiosterol (**9**) [18], which was isolated previously from the Red Sea sponge *Hyrtios* sp. However, careful comparison of ^{1}H- and ^{13}C-NMR data of **2** with those of **9** did not show the expected differences due to the different configurations at C(2) and C(3). In fact, their NMR data are almost identical.

It raises a necessity to verify the correctness of structure **9**. In [18], the α configurations at C(2), C(3), and C(4) of **9** were reported to be determined by ROESY cross-peaks observed between H–C(2) and H–C(3), and H–C(3) and H–C(4) and by analysis of the splitting pattern and coupling constants of H–C(2), H–C(3), and H–C(4). However, the more significant NOEs between H–C(1) and H–C(4) and between H–C(4) and Me(19) were not reported for **9**. In our case, we observed a series of significant NOEs as depicted in *Fig. 3* by performing both ROESY and NOE difference experiments with **2**. In addition, the coupling constants ($J=7.1$ Hz) between H–C(4) and H–C(3) and a broad formal *s* of H–C(2) seem more suitable for H_{ax}–C(4), H_{ax}–C(3) and H_{eq}–C(2) assignments based on the *Dreiding*-model analysis (*Fig. 3*). It may be worthy to point out that H–C(4) (δ 1.75) was heavily overlapped, and its coupling constants ($J=11.7$, 7.1 Hz, reported in [18]) was actually unrecognizable. In light of these observations, we suggest that the configurations at C(2) and C(3) of **9** probably should be revised and be the same as those of **1**.

Compound **3** was obtained as an optically active oil. The molecular formula, $C_{28}H_{46}O_4$, was established by the pseudo-molecular ion at *m/z* 469.3402 ($[M+Na]^+$) in the HR-ESI-MS, indicating six degrees of unsaturation. The ^{1}H- and ^{13}C-NMR (*Table*) and ^{1}H,^{1}H-COSY and HMBC (*Fig. 2*) data, as well as their comparison with known data of similar compounds, established the structure of **3**.

Inspection of the ^{13}C-NMR data for **3** revealed the presence of a disubstituted C=C (δ 136.6 (*d*) and 129.4 (*d*)), a terminal C=C bond (δ 153.4 (*s*), 110.0 (*t*)), 9 CH_2, 8 CH, and 4 Me groups, and 3 tertiary C-atoms. The total of 28 C-atoms suggested that **3**, like **1** and **2**, is also a sterol. Since in the ^{1}H-NMR spectrum, the signal at *ca.* δ 1.0 typical for Me(19) was absent but signals assignable to four Me groups at δ 0.92 (*s*, Me(18)), 1.11 (*d*, $J=6.7$ Hz, Me(21)), and 1.12 (*d*, $J=6.6$ Hz, Me(26) and Me(27)) were present, **3** was readily recognized as a 19-hydroxylated steroid. Furthermore, downfield signals appeared at δ 4.95–4.97 (*m*, 1 H) and 4.16 (*m*, 1 H) for OH-bearing CH protons, at δ 4.96 (br. *s*, 1 H) and 5.04 (br. *s*, 1 H) for the terminal CH_2 protons, and at δ 4.33 (*AB* (*d*), $J=12.0$ Hz, 1 H) and 4.93 (*AB* (*d*), $J=12.0$ Hz, 1 H) typical for a CH_2 group bearing an OH group. The foregoing spectral data were strongly reminiscent of those of the co-occurring (3β,5α,6β)-ergost-24(28)-ene-3,5,6,19-tetrol (**4**) [19]. A comparison of the overall ^{1}H- and ^{13}C-NMR data of **3** and **4** revealed that the only difference between them concerns the side chain, where the former contained a disubstituted C(22)=C(23) bond, in agreement with the molecular mass difference of two mass units observed between **3** and **4**. Moreover, the nearly identical chemical shifts of the ^{1}H- and ^{13}C-NMR signals assigned to the moiety from C(20) to C(28) of **3** and co-occurring **2** indicated that the side chain is the same in both compounds. Thus, **3** is the 22,23-didehydro derivative of **4**.

Although many steroids were reported from the genus *Sinularia*, the steroids with a 4α-positioned methyl group have, to the best of our knowledge, not been found from this genus. Furthermore, the abundant production and accumulation of steroids in the title specimen is intriguing, as it seems unlikely that these compounds act solely as repellents against predators. Instead, they may play an as yet unknown physiological role in these benthic animals.

The crude Et_2O extract of the title soft coral exhibited cytotoxity toward a limited panel of cancer cell lines. Unfortunately, compounds **1**–**8** proved inactive toward the

growth of the A-549, KB, and P388 cells at a concentration of 20 μg/ml. Other bioassays such as antibacterial, anti-inflammatory, etc., are currently ongoing.

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Experimental Part

General. All solvents were of anal. grade (*Shanghai Chemical Plant*, Qingdao, People's Republic of China). Column chromatography (CC): *Sephadex LH-20 gel* (*Amersham Biosciences*) or commercial silica gel (200–300 and 400–600 mesh; *Qing Dao Hai Yang Chemical Group Co.*). TLC: precoated silica gel plates (*G60 F_{254}; Yan Tai Zi Fu Chemical Group Co.*). Reversed-phase HPLC: *Agilent-1100* liquid chromatography, *VWD-G1314A* detector at 210 nm; semi-prep. *ODS-HG-5* (5 m, 10 mm (i.d.×25 cm) column. Optical rotations: *Perkin-Elmer-341MC* polarimeter. IR Spectra: *Nicolet-Magna-FT-IR 750* spectrometer; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker-DRX-500* spectrometer with the residual $CDCl_3$ (δ(H) 7.26, δ(C) 77.0) and *Varian-INOVA-600* spectrometer with the residual C_5D_5N (δ(H) 7.20, 7.57, 8.73; δ(C) 123.6, 135.8, 150.0) as an internal standard. ESI-MS and HR-ESI-MS: *Q-TOF-Micro-LC-MS-MS* spectrometer.

Biological Material. The specimen of the *Sinularia* sp., identified by Prof. *R.-L. Zhou* of the South China Sea Institute of Oceanology, Chinese Academy Sciences, was collected off the coast of Ximao Island, Sanya, Hainan Province, China, in December 2002, at a depth of −20 m, and was frozen immediately after collection. A voucher specimen is available for inspection at the Institute of Materia Medica, SIBS-CAS (No. *02HN-75*).

Extraction and Isolation. The frozen material (dry weight 241 g) was extracted with acetone at r.t. The acetone extract was evaporated and the resulting residue partitioned between H_2O and Et_2O. The Et_2O extract (3.8 g) was subjected to CC (silica gel, gradient elution from $CHCl_3$ to MeOH): *Fractions 1–6*. *Fr. 2* was subjected to CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1): *Fr. 2.1* and *2.2. Fr. 2.1* was subjected to CC (silica gel (400–600 mesh), $CHCl_3$/MeOH 98:2): **8** (12.3 mg). *Fr. 2.2* was further purified by reversed-phase HPLC (semi-prep. *ODS-HG-5* (5 μ, 250×10 mm) MeOH/H_2O 92:8, 2 ml/min, det. at 210 nm): **5** (3.6 mg) and **7** (2.2 mg). *Fr. 3* was subjected to CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1) and then purified by reversed-phase HPLC (semi-prep. *ODS-HG-5* (5 μ, 250×10 mm), MeOH/H_2O 93:7, 2 ml/min, det. at 210 nm): **1** (5.6 mg) and **2** (4.2 mg). *Fr. 4* was purified by CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1), followed by CC (silica gel (400–600 mesh), $CHCl_3$/MeOH 95:5): **6** (7.2 mg). *Fr. 6* was subjected to CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1) and then purified by reversed-phase HPLC (semi-prep. *ODS-HG-5* (5 μ, 250×10 mm), MeOH/H_2O 95:5, 2 ml/min, det. at 210 nm): **3** (1.6 mg) and **4** (5.2 mg).

(*2β,3β,4α,5α,8β,11β)-4-Methylergost-24(28)-ene-2,3,8,11-tetrol* (**1**): Viscous liquid. $[\alpha]_D^{20} = +62.1$ ($c = 0.39$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: *Table*. IR: 3390, 2929, 1738, 1456, 1049, 887. ESI-MS: 485.4 ($[M+Na]^+$). HR-ESI-MS: 485.3619 ($[M+Na]^+$, $C_{29}H_{50}NaO_4^+$; calc. 485.3607).

(3β,5α,6β)-Ergosta-22,24(28)-diene-3,5,6,19-tetrol (**3**): Colorless oil. $[\alpha]_D^{20} = +5$ ($c = 0.11$, MeOH). ^{1}H- and ^{13}C-NMR: *Table.* ESI-MS: 469 $[M+Na]^+$. HR-ESI-MS: 469.3402 ($[M+Na]^+$, calc. for $C_{28}H_{46}NaO_4$, 469.3396).

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