Oxygenated 4-Methylidene Sterols from the South China Sea Sponge *Theonella swinhoei*

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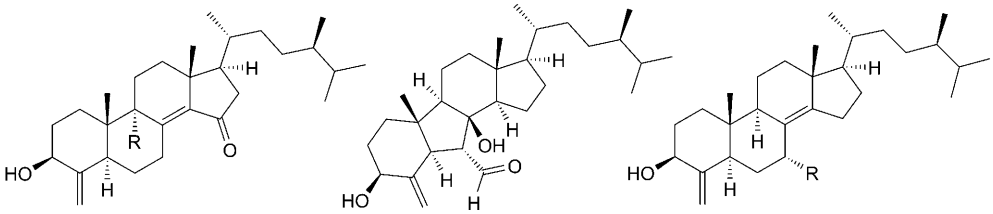
Two new O-bearing 4-methylidene sterols, 9α-hydroxy-15-oxoconicasterol (**1**) and 8β-hydroxy-B-norconicasta-6α-aldehyde (**2**), were isolated from the EtOH extract of the marine sponge *Theonella swinhoei* collected from the South China Sea. Their structures were elucidated by spectroscopic data and comparison with known compounds. In addition, spectroscopic data reported for the known 4-methylidene sterol 7α-hydroxyconicasterol (**3**) were revised.

Introduction. – Marine sponges of the genus *Theonella* have attracted a great deal of attention for their bioactive secondary metabolites, including polyoxygenated aliphatic compounds, unusual cyclic peptides, macrolides, polyethers, polyketides, alkaloids, and sterols [1–3]. Interestingly, the sterols isolated from the genus *Theonella*, mainly from *T. swinhoei*, were found possessing unique 4-methylidene moieties [3–8], and some of them also have 8,14-seco-frameworks [7][8]. As part of ongoing investigation on bioactive chemical constituents of marine sponges collected from the South China Sea, studies on the marine sponge *T. swinhoei* led to the isolation and determination of two new O-bearing 4-methylidene sterols, 9α-hydroxy-15-oxoconicasterol (**1**) and 8β-hydroxy-B-norconicasta-6α-aldehyde (**2**), together with a known 4-methylidene sterol 7α-hydroxyconicasterol (**3**), from the EtOH extract of this sponge. Here, we report the details of isolation and structure elucidation of these compounds.



1 R = OH
4 R = H

2

3 R = OH
5 R = H

Results and Discussion. – The EtOH extract of the marine sponge *T. swinhoei* was subjected to solvent partition, column chromatography (CC), or vacuum liquid chromatography (VLC) (on SiO_2, *ODS*, and *Sephadex LH-20*), and RP-HPLC to afford two new O-bearing 4-methylidene sterols, 9α-hydroxy-15-oxoconicasterol (**1**) and 8β-hydroxy-B-norconicasta-6α-aldehyde (**2**), and a known 4-methylidene sterol, 7α-hydroxyconicasterol (**3**). Their structures were elucidated by HR-ESI-MS, and 1D- and 2D-NMR techniques, including ^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY (or ROESY).

Compound **1** was isolated as colorless needles from $CHCl_3$, and its molecular formula was established as $C_{29}H_{46}O_3$ from HR-TOF-ESI-MS (*m/z* 465.3340 ([*M* + Na]$^+$)) and ^{13}C-NMR data. Seven degrees of unsaturation implied by the formula were ascribed to four rings, two C=C bonds (δ(C) 152.8, 147.7, 141.7, and 103.9), and one C=O group (δ(C) 208.4). The ^{1}H-NMR spectrum exhibited signals for six Me groups (δ(H) 0.71 (*s*), 0.78 (*d*, *J* = 6.7), 0.80 (*d*, *J* = 6.8), 0.85 (*d*, *J* = 6.8), 0.97 (*s*), and 1.02 (*d*, *J* = 6.2)), two olefinic H-atoms (δ(H) 5.13 (br. *s*) and 4.69 (br. *s*)), and one O-bearing CH group (δ(H) 4.03 (*dd*, *J* = 12.0, 5.2)). The ^{13}C-NMR and DEPT spectra showed 29 signals including those of six Me, ten CH_2, six CH groups, and seven quaternary C-atoms (*Table*). The ^{1}H- and ^{13}C-NMR spectra of compound **1** were characteristic of an O-bearing sterol [9], which was confirmed by extensive 2D-NMR spectroscopic analysis. The strong HMBCs from the six Me groups to associated C-atoms indicated three typical fragments of a steroid corresponding to two angular Me groups and nearby C-atoms, and the partial side chain (*Fig. 1*). The HMBCs from CH_2(29) to C(3), C(4), and C(5), and the allylic ^{1}H,^{1}H-COSY correlations of CH_2(29) with H−C(3) and H−C(5) confirmed the existence of exocyclic CH_2 group at C(4). The ^{1}H,^{1}H-COSY correlations of H_β−C(2) (δ(H) 1.39 (*qd*, *J* = 12.0, 4.0)) with H_α−C(1) and H−C(3) validated the assignment of ring *A*. The ^{1}H,^{1}H-COSY correlations H−C(5)/CH_2(6), CH_2(6)/CH_2(7), and CH_2(11)/CH_2(12), and the HMBCs from CH_2(7) to C(5), C(6), C(8), C(9), and C(14), and from H_β−C(12) to C(9), allowed the establishment of rings *B* and *C*. The ring *D* was established by the ^{1}H,^{1}H-COSY correlation CH_2(16)/H−C(17), and the HMBCs from CH_2(16) to C(13), C(14), C(15), C(16), and C(17). The ^{1}H,^{1}H-COSY correlation CH_2(22)/CH_2(23) provided the final connection of the alkyl side chain.

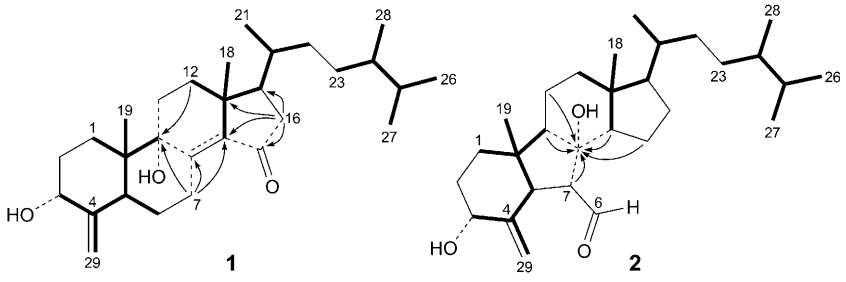


Fig. 1. *Selected HMBC* (— and →) *and COSY* (—) *correlations of* **1** *and* **2**. Dotted lines indicate bonds without COSY correlations

Table. *Data of ^{1}H-NMR at 600 MHz and ^{13}C-NMR at 150 MHz for* **1**, **2**, *and* **3** *in $CDCl_3$.* δ in ppm, J in Hz.

Position	**1**		**2**		**3**	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
1	1.99–2.05 (*m*, H_α), 1.47–1.52 (*m*, H_β)	29.6 (*t*)	1.48–1.52 (*m*, H_α), 1.74–1.80 (*m*, H_β)	37.3 (*t*)	1.36–1.41 (*m*, H_α), 1.74–1.78 (*m*, H_β)	36.5 (*t*)
2	2.02–2.07 (*m*, H_α), 1.39 (*qd*, J = 12.0, 4.0, H_β)	32.4 (*t*)	2.02–2.06 (*m*, H_α), 1.49–1.54 (*m*, H_β)	32.6 (*t*)	1.99–2.04 (*m*, H_α), 1.35–1.42 (*m*, H_β)	33.0 (*t*)
3	4.03 (*dd*, J = 12.0, 5.2)	72.8 (*d*)	4.14 (*dd*, J = 10.3, 5.0)	72.9 (*d*)	4.05 (*dd*, J = 10.4, 5.2)	73.3 (*d*)
4	–	152.8 (*s*)	–	148.0 (*s*)	–	152.6 (*s*)
5	2.62 (br. *d*, J = 12.0)	41.4 (*d*)	2.66 (br. *d*, J = 12.6)	51.9 (*d*)	2.34 (br. *d*, J = 13.6)	42.3 (*d*)
6	1.65–1.69 (*m*, H_α), 1.49–1.55 (*m*, H_β)	24.5 (*t*)	9.78 (*d*, J = 3.1)	205.0 (*t*)	1.77 (*dt*, J = 13.8, 2.4), 1.60 (*td*, J = 13.8, 3.0, H_β)	31.3 (*t*)
7	2.04–2.08 (*m*, H_α), 4.00 (*ddd*, J = 14.4, 4.2, 2.5, H_β)	22.2 (*t*)	2.61 (*dd*, J = 12.6, 3.1)	60.1 (*d*)	4.65 (br. *t*, J = 2.8)	66.5 (*d*)
8	–	147.7 (*s*)	–	87.2 (*s*)	–	127.6 (*s*)
9	–	74.5 (*s*)	1.74–1.78 (*m*)	62.9 (*d*)	2.27 (*td*, J = 10.2, 2.4)	43.8 (*d*)
10	–	44.3 (*s*)	–	44.6 (*s*)	–	39.7 (*s*)
11	1.64–1.68 (*m*, H_α), 1.97 (*td*, J = 14.8, 3.6, H_β)	28.2 (*t*)	1.56–1.59 (*m*, H_α), 1.27–1.33 (*m*, H_β)	19.7 (*t*)	1.86–1.92 (*m*, H_α), 1.43–1.50 (*m*, H_β)	19.8 (*t*)
12	1.57–1.63 (*m*, H_α), 2.01–2.05 (*m*, H_β)	33.4 (*t*)	1.43–1.47 (*m*, H_α), 1.74–1.78 (*m*, H_β)	36.4 (*t*)	1.14–1.20 (*m*, H_α), 1.97(*dt*, J = 12.4, 3.3, H_β)	36.9 (*t*)
13	–	43.2 (*s*)	–	44.8 (*s*)	–	43.0 (*s*)
14	–	141.7 (*s*)	1.59 (*dd*, J = 9.8, 4.2)	58.1 (*d*)	–	148.3 (*s*)
15	–	208.4 (*s*)	1.90–1.95 (*m*, H_α), 1.62–1.68 (*m*, H_β)	22.0 (*t*)	2.33–2.41 (*m*, H_α), 2.33–2.41 (*m*, H_β)	25.1 (*t*)
16	2.42 (*dd*, J = 18.9, 7.5, H_α), 2.06 (*dd*, J = 18.9, 11.0, H_β)	42.5 (*t*)	1.89–1.94 (*m*, H_α), 1.34–1.40 (*m*, H_β)	29.6 (*t*)	1.86–1.92 (*m*, H_α), 1.38–1.46 (*m*, H_β)	26.9 (*t*)
17	1.51–1.58 (*m*)	50.4 (*d*)	1.43–1.48 (*m*)	56.9 (*d*)	1.17–1.21 (*m*)	56.3 (*d*)
18	0.97 (*s*)	17.2 (*q*)	0.87 (*s*)	22.0 (*q*)	0.85 (*s*)	17.9 (*q*)
19	0.71 (*s*)	16.3 (*q*)	0.63 (*s*)	14.9 (*q*)	0.57 (*s*)	12.3 (*q*)
20	1.55–1.61 (*m*)	34.6 (*d*)	1.33–1.38 (*m*)	35.0 (*d*)	1.44–1.50 (*m*)	33.5 (*d*)
21	1.02 (*d*, J = 6.2)	19.2 (*q*)	0.89 (*d*, J = 6.6)	18.6 (*q*)	0.94 (*d*, J = 6.8)	19.0 (*q*)
22	1.27–1.33 (*m*, H_a), 1.13–1.18 (*m*, H_b)	33.8 (*t*)	1.31–1.37 (*m*, H_a), 1.03–1.11 (*m*, H_b)	34.1 (*t*)	1.34–1.38 (*m*, H_a), 1.13–1.18 (*m*, H_b)	34.5 (*t*)
23	1.23–1.28 (*m*, H_a), 1.06–1.11 (*m*, H_b)	29.8 (*t*)	1.31–1.37 (*m*, H_a), 1.06–1.12 (*m*, H_b)	30.5 (*t*)	1.21–1.25 (*m*, H_a), 1.08–1.14 (*m*, H_b)	30.0 (*t*)
24	1.18–1.24 (*m*)	38.8 (*d*)	1.16–1.22 (*m*)	38.9 (*d*)	1.19–1.23 (*m*)	38.9 (*d*)
25	1.49–1.56 (*m*)	32.4 (*d*)	1.48–1.55 (*m*)	32.4 (*d*)	1.51–1.56 (*m*)	32.4 (*d*)
26	0.85 (*d*, J = 6.8)	20.1 (*q*)	0.85 (*d*, J = 6.8)	20.2 (*q*)	0.86 (*d*, J = 6.8)	20.2 (*q*)
27	0.80 (*d*, J = 6.8)	18.2 (*q*)	0.80 (*d*, J = 6.8)	18.3 (*q*)	0.81 (*d*, J = 6.8)	18.2 (*q*)
28	0.78 (*d*, J = 6.7)	15.4 (*q*)	0.77 (*d*, J = 6.8)	15.4 (*q*)	0.79 (*d*, J = 6.6)	15.4 (*q*)
29	5.13 (br. *s*, H_a), 4.69 (br. *s*, H_b)	103.9 (*t*)	5.08 (br. *s*, H_a), 4.52 (br. *s*, H_b)	103.9 (*t*)	5.10 (br. *s*, H_a), 4.62 (br. *s*, H_b)	102.8 (*t*)

The coupling constants between H–C(3) (4.03 (*dd, J* = 12.0, 5.2)) and CH_2(2), and the NOESY correlations of H–C(3) with H_α–C(1) and H–C(5), indicated that H–C(3) was axial. The NOESY correlations H–C(5)/H_α–C(7), H_β–C(6)/Me(19), and Me(19)/H_β–C(11) implied that HO–C(9) was α-oriented (*Fig. 2*), which was further supported by the *syn*-axial γ-effects of HO–C(9) [10]. Compared with the C(9)-unsubstituted compound **4**, upfield shifts of 7.3, 7.9, and 5.1 ppm for C(1), C(5), and C(7), respectively, of compound **1** were observed [7]. The configuration of C(24) was determined by comparison of ^{13}C-NMR data with the epimeric steroidal side chain [11]. On the basis of the foregoing analysis, compound **1** was determined as 9α-hydroxy-15-oxoconicasterol.

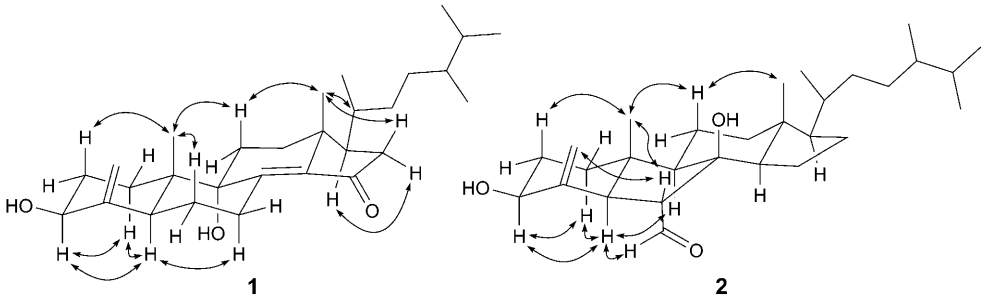


Fig. 2. *Key NOESY or ROESY correlations of* **1** *and* **2**

Compound **2** was obtained as colorless needles from $CHCl_3$, and its molecular formula, $C_{29}H_{48}O_3$, was deduced from HR-TOF-ESI-MS (*m/z* 467.3503 ($[M+Na]^+$)) and ^{13}C-NMR data. This formula implied six degrees of unsaturation, which were assigned to four rings, one C=C bond (δ(C) 148.0, 104.0), and one aldehyde C=O group (δ(C) 205.0). The ^{1}H-NMR spectrum showed signals for six Me groups (δ(H) 0.63 (*s*), 0.77 (*d*, *J* = 6.8), 0.80 (*d*, *J* = 6.8), 0.85 (*d*, *J* = 6.8), 0.87 (*s*), and 0.89 (*d*, *J* = 6.6)), two olefinic H-atoms (δ(H) 5.08 (br. *s*), 4.52 (br. *s*)), one O-bearing CH group (δ(H) 4.14 (*dd*, *J* = 10.3, 5.0)), and one aldehyde H-atom (δ(H) 9.78 (*d*, *J* = 3.1)). The ^{13}C-NMR and DEPT spectra exhibited 29 signals including those of six Me, nine CH_2, and ten CH groups, as well as four quaternary C-atoms (*Table*). Three typical fragments of a steroid could also be established by the strong diagnostic HMBCs from the six Me groups to the nearby C-atoms (*Fig. 1*). The existence of an exocyclic CH_2 group at C(4) was verified by the HMBCs from CH_2(29) to C(3), C(4), and C(5), and the allylic ^{1}H,^{1}H-COSY correlations between CH_2(29), and H–C(3) and H–C(5). The ^{1}H,^{1}H-COSY correlations between CH_2(2), and CH_2(1) and H–C(3) allowed us to establish ring *A*. The ^{1}H,^{1}H-COSY correlations between H–C(7), and H–C(5) and H–C(6) indicated that the aldehyde group was connected to C(7). The HMBCs from H–C(7), H–C(9), H_β–C(11), H–C(14), and CH_2(15) to C(8), together with the ^{1}H,^{1}H-COSY correlations of H_β–C(11) with H–C(9) and H_α–C(12), confirmed the assignment of rings *B* and *C*. The ^{1}H,^{1}H-COSY correlations H–C(14)/H_α–C(15), H_β–C(15)/H_α–C(16), and H_α–C(16)/H–C(17) allowed the establishment of ring *D*. The ^{13}C-NMR, HMBC, and ^{1}H,^{1}H-COSY data indicated that compound **2** possessed the same side chain as compound **1**.

The β-orientation of HO–C(3) was determined by the coupling constants between axial H–C(3) (4.14 (*dd, J* = 10.3, 5.0)) and CH_2(2) , and the ROESY correlations H–C(3) with H_α–C(1) and H–C(5). The ROESY correlations H–C(5)/H–C(6), H–C(7)/H_b–C(29), and H–C(7)/Me(19), suggested that the aldehyde group was α-oriented (*Fig. 2*). The β-orientation of HO–C(8) was deduced from the upfield chemical shift of C(11) (δ(C) 19.7) and the lowfield chemical shift of Me(18) (δ(C) 22.0), which were due to the *syn*-axial γ-effect and δ-effect, respectively [10][12]. Therefore, compound **2** was elucidated as 8β-hydroxy-B-norconicasta-6α-aldehyde.

Compound **3** was obtained as a white powder, and its molecular formula was found to be $C_{29}H_{48}O_2$ from ESI-MS (*m/z* 451.36 ($[M+Na]^+$), 467.35 ($[M+K]^+$)) and ^{13}C-NMR data. The ^{1}H-NMR spectrum exhibited signals of six Me groups (δ(H) 0.57 (*s*), 0.79 (*d*, *J* = 6.8), 0.81 (*d*, *J* = 6.8), 0.85 (*s*), 0.86 (*d*, *J* = 6.8), and 0.94 (*d*, *J* = 6.8)), two olefinic H-atoms (δ(H) 5.10 (br. *s*), 4.62 (br. *s*)), and two O-bearing CH groups (δ(H) 4.05 (*dd*, *J* = 10.4, 5.2), 4.65 (*t*, *J* = 2.8)). The ^{13}C-NMR and DEPT spectra showed 29 signals including those of six Me, ten CH_2, and nine CH groups, as well as of five quaternary C-atoms (*Table*). The C-atoms resonating at δ(C) 152.6, 148.3, 127.6, and 102.8 indicated the presence of two C=C bonds. The ^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY spectra displayed that compound **3** was a 4-methylidene sterol with a 7α-hydroxy-8(14)-ene fragment, which was previously isolated from Hachijo marine sponge *T. swinhoei* [7]. The HO–C(3) was determined to be β-oriented from the vicinal coupling constants of H–C(3) (4.05 (*dd, J* = 10.4, 5.2)), and the NOESY correlations of H–C(3) with H_α–C(1) and H–C(5). Although the ^{1}H-NMR data of compound **3** were very similar to the literature data, their ^{13}C-NMR data exhibited a significant difference for C(7). The chemical shift of C(7) of compound **3** was 66.5, while in the literature it was reported as 79.9 ppm, implying that these two HO–C(7) had different configurations, which were consistent with the observed allylic 7α- and 7β-OH substituent effects, respectively [13][14]. By comparison with the ^{13}C-NMR data of conicasterol (**5**), upfield shifts of 7.3 and 5.5 ppm for C(5) and C(9), respectively, of compound **3** were observed [7], which were ascribed to the *syn*-axial γ-effects [10]. Therefore, HO–C(7) of compound **3** was determined as α-oriented, and HO–C(7) in the literature should be revised as β-oriented.

To the best of our knowledge, there were 17 4-methylidene sterols isolated from *T. swinhoei* and one from *T. conica*, unaccompanied by conventional sterols [3–8]. These 4-methylidene sterols were commonly oxygenated at C(3), C(7), or C(15), and almost all of them had 8(14)-ene group except two, which possessed 8(14)-seco-skeleton [7][8]. Compounds **1** and **2** had novel HO–C(9) or HO–C(8) group, and B-nor-framework, which were relatively rare even in conventional sterols. Besides from the genus *Theonella*, only one 4-methylidene sterol, but without other substituted features of that from sponge *Theonella*, was isolated from the marine sponge *Clathria fasciculata* [15], which is questionable since its structure was identified only by LC/MS data. Based on our results and available literature, oxygenated 4-methylidene sterols and secosterols may be considered as ideal taxonomic markers for the genus *Theonella*, especially for the sponge *T. swinhoei*.

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Experimental Part

General. HPLC: *Waters 1525/2998* liquid chromatograph. CC: *Sephadex LH-20* (*Pharmacia*) and *YMC ODS-A* (50 μm). Vacuum liquid chromatography (VLC): SiO_2 (200–300 mesh; *Yantai*, P. R. China); the fractions were monitored by TLC (*HSGF 254*; *Yantai*, P. R. China), and spots were visualized by heating SiO_2 plates sprayed with 10% H_2SO_4 in H_2O. M.p.: *SGW X-4* melting-point apparatus; uncorrected. Optical rotations: *Perkin-Elmer 341* polarimeter. NMR Spectra: *Bruker AVANCE-600* spectrometer. ESI- and HR-TOF-ESI-MS spectra: *Q-Tof micro YA019* mass spectrometer.

Animal Material. Specimen of *T. swinhoei* was collected around Yongxing Island and seven connected islets in the South China Sea in June 2007, and was identified by Prof. *Jin-He Li* (Institute of Oceanology, Chinese Academy of Sciences, P. R. China). A voucher sample (No. DS-TS01) was deposited with the Laboratory of Marine Drugs, Department of Pharmacy, Changzheng Hospital, Second Military Medical University, P. R. China.

Extraction and Isolation. The fresh sponges (3.5 kg, wet weight) were extracted with EtOH at r.t. The EtOH extracts were concentrated under reduced pressure to give 537 g of brown gum, which was partitioned between AcOEt and H_2O to afford 216 g of AcOEt-phase extract. The AcOEt-phase extract was partitioned between MeOH/H_2O 9 :1 and petroleum ether (PE) to afford 170 g of PE-phase extract. The MeOH/H_2O phase was diluted to 3 :2 with H_2O and extracted with CH_2Cl_2 to give 15 g of CH_2Cl_2-phase extract. This extract was subjected to VLC (SiO_2; CH_2Cl_2/MeOH 50 :1, 20 :1, 15 :1, 10 :1, 5 :1, and 2 :1) to afford ten fractions; *Frs. 1–10*. The *Fr. 4* (750 mg) was subjected to chromatography repeatedly on *Sephadex LH-20* and SiO_2 to give compound **3** (18.5 mg). *Fr. 5* (250 mg) was subjected to chromatography repeatedly on *Sephadex LH-20* and *YMC ODS-A* (50 μm), and further purified by HPLC (*YMC-Pack ODS-A C18*, 5 μm, 10 × 250 mm, 1.5 ml/min, UV detection 210 nm) with MeOH/H_2O 90 :10 to yield pure compounds **1** (5.6 mg) and **2** (2.5 mg).

9α-Hydroxy-15-oxoconicasterol (=*(3β,24*R*)-3,9-Dihydroxy-4-methylideneergost-8(14)-en-15-one*; **1**). Colorless needles ($CHCl_3$). M.p. 209–211°. $[\alpha]_D^{19} = +221$ ($c = 0.100$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: *Table*. HR-TOF-ESI-MS: 465.3340 ($C_{29}H_{46}NaO_3^+$; calc. 465.3345).

8β-Hydroxy-B-norconicasta-6α-aldehyde (=*(3*R*,3a*R*,5b*S*,8*S*,10*R*,10a*R*)-3-[(2*R*,5*R*)-5,6-Dimethylheptan-2-yl]hexadecahydro-8,10a-dihydroxy-3a,5b-dimethyl-9-methylidenecyclopenta[*a*]fluorene-10-carbaldehyde*; **2**). Colorless needles ($CHCl_3$). M.p. 128–130°. $[\alpha]_D^{19} = +109$ ($c = 0.125$, $CHCl_3$). ^{1}H- and ^{13}C-NMR: *Table*. HR-TOF-ESI-MS: 467.3503 ($C_{29}H_{48}NaO_3^+$; calc. 467.3501).

7α-Hydroxyconicasterol (=*(3β,7α,24*R*)-4-Methylideneergost-8(14)-ene-3,7-diol*; **3**). White powder. ^{1}H- and ^{13}C-NMR: *Table*. ESI-MS: 451.36 ($[M+Na]^+$), 467.35 ($[M+K]^+$), 879.71 ($[2M+Na]^+$).

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