Polyhydroxylated Steroids and Other Constituents of the Soft Coral *Nephthea chabroli*

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Polyhydroxylated steroids and other metabolites were isolated from the soft coral *Nephthea chabroli* collected in South China Sea. The structures of two new compounds were determined to be 24-methylcholesta-9(11), 24(28)-diene-3β,12α,19-triol and 4α-methyl-3β,14β-dihydroxy-5α-ergost-24(28)-en-23-one on the basis of spectroscopic analysis. Cytotoxic activities of the polyhydroxylated sterols were evaluated on prostate carcinoma LNCaP cell line.

Key words *Nephthea chabroli*; soft coral; polyhydroxylated steroid; cytotoxicity

Soft corals contain a variety of bioactive compounds such as diterpenes, sesquiterpenes, and steroids.[1)] In the course of our search for biologically active substances from marine organisms,[2—4)] a collection of the soft coral *Nephthea chabroli* was obtained from the South China Sea, and from this collection, cembrane diterpenes and norditerpenes (chabrolols A—C) were obtained.[2)] Another chemical constituent, lemnabourside, was found to show anti-proliferative and 5α-reductase inhibitory effect on human prostate cancer cells.[3)] In the literature, secondary metabolites including benzoquinone derivatives, sesquiterpenes, and sterols have been reported from *Nephthea chabroli*.[5—9)] In the present paper, we report the isolation of eight sterols (**1**—**8**), a diterpene glycoside (**9**), a ceramide (**10**), a quinoline (**11**), and fatty acid derivatives (**12**—**13**). Of these constituents, **6** and **8** are new structures.

Compounds **1**—**6** displayed characteristic spectral features of 19-hydroxylated steroids,[10,11)] showing in their NMR spectra a pair of methylene proton doublets at about δ_H 3.7 (1H, J=12 Hz) and 3.9 (1H, J=12 Hz), as well as a methylene carbon signal at δ_C 63.2 (t). The structures of **1**—**5** were determined to be 24-methylcholesta-5,24(28)-diene-3β,19-diol (**1**),[12,13)] 24-methylcholesta-5,24(28)-diene-3β,7β,19-triol (**2**),[12,13)] 24-methyl-7β-acetoxycholesta-5,24(28)-diene-3β,19-diol (**3**),[12,13)] 24-methylcholest-24(28)-ene-3β,5α,6β,19-tetraol (**4**),[12,13)] and 24-methyl-7-oxocholesta-5,24(28)-diene-3β,19-diol (**5**).[12)] The structural identities of these compounds were established by comparing the physical and spectroscopic data with literature values.

Compound **6**, obtained as a colorless solid, exhibited positive result in the Liebermann–Burchard test. A molecular formula of $C_{28}H_{46}O_3$ was deduced from electron impact (EI)-MS result (m/z 430 $[M]^+$) and elemental analysis. The IR absorption bands indicated the presence of hydroxyls (3350 cm^{-1}) and a terminal methylene (1640, 900 cm^{-1}). The presence of a primary hydroxyl and two secondary hydroxyl groups was revealed by ^{1}H- and ^{13}C-NMR data [δ_H 3.88 (1H, d, J=12 Hz) and 3.68 (1H, d, J=12 Hz), δ_C 63.2 (t); δ_H 3.63 (1H, m), δ_C 71.2 (d); δ_H 3.90 (1H, d, J=4 Hz), δ_C 65.1 (d)]. Four methyls, a terminal methylene, and a trisubstituted double bond were also present as demonstrated by the NMR data. The double quantum filtered correlation spectroscopy (DQF-COSY) results suggested that the proton bearing a secondary alcohol (δ 3.90, H-12) was coupled to an olefinic proton (δ 5.94, H-11). The assignment of a partial structure of 9(11)-ene-12-ol was further supported by the heteronuclear multiple bond connectivity (HMBC) results. Thus, HMBC correlations were observed between δ_H 0.74 (18-CH_3) and δ_C 65.1 (C-12), between δ_H 3.62 (H-19a)/3.88 (H-19b) and δ_C 141.5 (C-9), as well as between δ_H 5.94 (H-11) and δ_C 42.5 (C-10). With the assistance of computer-aided molecular modeling (MOPAC molecular modeling program), the energy-minimized stereostructure of **6** showed the dihedral angle (φ) between H_{11}–C_{11}–C_{12}–H_{12} to be 37.7°, corresponding to a coupling constant of 2—5 Hz as estimated from the Karplus correlation graph. Given a coupling constant of 4 Hz for H-12, β-orientation of 12-H was assumed. Indeed, the nuclear Overhauser effect spectroscopy (NOESY) spectrum displayed correlation signals between H_{12}/H_{18} as well as between H_{11}/H_{12}. These NOE results were in support of the stereochemical assignment. Hence, all available data indicated a structure of 24-methylcholesta-9(11),24(28)-diene-3β,12α,19-triol for **6**.

Compound **7** was determined to be 24-methylcholesta-5,24(28)-dien-3β-ol by interpretation of its spectroscopic data and comparison with literature values.[12)]

Compound **8** was obtained as a white solid. The molecular formula $C_{29}H_{48}O_3$ was deduced from EI-MS (m/z 444 $[M]^+$) and elemental analysis. The compound reacted positively to the Liebermann–Burchard reagent, and displayed characteristic NMR features of a steroidal compound. The IR spectrum exhibited absorption bands at ν_{max} 3340, 2985, 1675, 1630, 880 cm^{-1}, suggesting the presence of hydroxyls, α,β-unsaturated ketone, and a terminal methylene group. The existence of an α,β-unsaturated ketone was also implied by an UV absorption at 240 nm. An HMBC experiment was then employed to establish the side-chain structure. HMBC correlations were observed between the carbonyl carbon signal C-23 (δ_C 203.0) and δ_H 2.38 (H-20), 2.68 (H-22), 5.66 (H-28b), and 5.91 (H-28a). The H-28 signals showed long range coupling with δ_C 156.2 (C-24), which was further coupled with δ_H 2.94 (H-25), 1.01 (H-26), and 1.02 (H-27). These data led to the establishment of connectivity between C_{23}–C_{26}. In addition, the DQF-COSY displayed correlations between δ_H 2.38 (H-20) and 2.68 (H-22), as well as between H-20 and δ_H 0.86 (H-21), suggesting a spin system contain-

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Table 1. ^{13}C-NMR Data of **6** and **8**[a)]

Carbon	**6**	**8**
1	33.6 t	28.2 t
2	31.0 t	41.2 t
3	71.2 d	77.3 d
4	42.2 t	49.7 d
5	50.6 d	57.4 d
6	39.6 t	19.2 t
7	21.6 t	20.2 t
8	39.0 d	38.9 d
9	141.5 s	51.8 d
10	42.5 s	36.6 s
11	128.7 d	18.6 t
12	65.1 d	40.1 t
13	42.3 s	43.5 s
14	42.9 d	73.9 s
15	24.1 t	37.8 t
16	28.2 t	30.8 t
17	55.8 d	56.6 d
18	12.0 q	13.8 q
19	63.2 t	13.8 q
20	35.7 d	33.3 d
21	18.8 q	19.6 q
22	34.8 t	45.3 t
23	31.8 t	203.0 s
24	156.9 s	156.2 s
25	33.9 d	28.1 d
26	22.0 q	22.3 q
27	21.9 q	22.2 q
28	106.0 t	121.1 t
29	—	15.4 q

a) Assignments are made by interpretation of two-dimensional NMR techniques. Values are expressed in ppm downfield from TMS standard. Spectra were run in $CDCl_3/CD_3OD$ (for **6**) and $CDCl_3$ (for **8**) respectively.

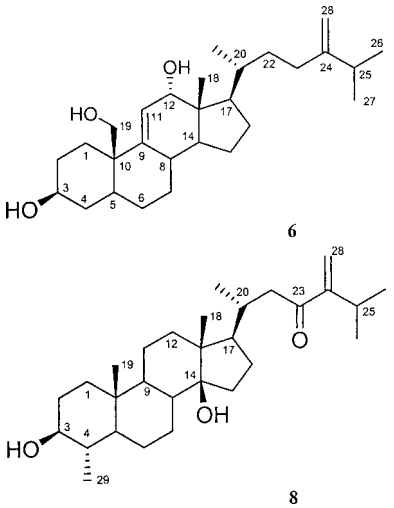

Fig. 1. Structures of Compounds **6** and **8**

ing H_{22}–H_{20}–H_{21}. On the other hand, the assignment of 29-CH_3 group to the biogenetically favoured position C-4 was supported by HMBC correlations between δ_H 0.96 (H_3-29) and δ_C 77.3 (C-3)/59.7 (C-4). Finally, a tertiary hydroxyl group was assigned to C-14 based on the HMBC results. Thus, δ_C 73.9 (C-14) was found to display long range correlations with δ_H 0.98 (H-18) and 1.80 (H-15). The stereochemistry of **8** was determined by interpretation of the DIF NOE data, which showed enhancements between H_3/H_{29}, H_4/H_{19}, H_5/H_{29}, and 14-OH/H_{18} signals, indicating the spatial arrangements of 3α-H, 4β-H, 5α-H, and 14β-OH, respectively. From all available data, **8** was deduced to be 4α-methyl-3β,14β-dihydroxy-5α-ergost-24(28)-en-23-one. To the best of our knowledge, this is a new structure.

During the course of isolation, non-steroidal compounds were also purified from the extract and examined spectroscopically. They were determined to be lemnabourside (a diterpene glycoside, **9**),[14] *N*-palmitoyl-octadecasphinga-4(*E*),8(*E*)-dienine (a ceramide derivative, **10**),[15] 7-hydroxy-8-methoxy-4(1*H*)-quinolone (**11**),[16] (13*Z*)-octadecenoic acid (**12**),[17] and (13*Z*)-ethyl octadecenoate (**13**).[17]

The steroidal compounds (**1**—**8**) were subjected to cytotoxicity assay using human prostate cancer LNCaP cell line. Compounds **2** and **4** exhibited EC_{50} values of 4.9 and 7.4 μg/ml, respectively. Compounds **3**, **6**, **5**, **8**, **7** and **1** (EC_{50}=15.5, 23.4, 29.5, 38.4, 41.5 and 56.7 μg/ml, respectively) were considered inactive.

Experimental

All solvents used were of analytical grade. Silica gel (230—400 mesh), TLC grade silica gel and RP-18 Si gel were used for chromatography. NMR spectra were recorded on JEOL JNM-EX-400-FT-NMR spectrometer, the IR absorption spectra on Perkin Elmer 16 PC FT-IR spectrometer, and mass spectra on Finnigan TSQ 7000 mass spectrometer. Optical rotations were measured on Perkin Elmer 241 polarimeter.

Animal Material The soft coral *Nephthea chabroli* was collected in Xisha island located in the South China Sea. A voucher specimen was deposited in the research center of organic natural products chemistry, Zhongshan University, Guangzhou, China.

Extraction and Isolation The samples of *N. chabroli* (5 kg, dry weight) was cut into small species and extracted with EtOH (5×5 l) at room temperature. The extraction was evaporated under reduced pressure to obtain a residue that was partition between H_2O and $CHCl_3$. The $CHCl_3$–soluble fraction (70 g) was taken to dryness in vacuum. Vacuum liquid chromatography of the residue on silica gel using hexane–acetone gradients afforded eight fractions (A—H). Fraction A was rechromatographed to yield **7** (57 mg), **12** (85 mg) and **13** (70 mg). From fraction B, **8** (10 mg) was obtained. Fraction D was subjected to chromatography on LH-20 (MeOH) and silica gel to afford **1** (47 mg) and **10** (11 mg). Fraction E afforded **3** (38 mg) and **9** (154 mg) after chromatography on silica gel and RP-18 gel. When fraction F was chromatographed on silica gel and then on RP-18 gel, it yielded **2** (49 mg), **5** (22 mg), and **6** (32 mg). Fraction G afforded **4** (174 mg). When the water soluble fraction was further extracted by *n*-BuOH and chromatographed on RP-18 gel, a crop of **11** (20 mg) was obtained.

24-Methycholesta-9(11),24(28)-diene-3β,12β,19-triol (**6**): Colorless solid, mp 163—164 °C, $[\alpha]_D^{25}$ −141.9° (c=0.031, CH_3OH). IR (KBr) cm^{-1}: 3350, 1640, 900. ^{1}H-NMR ($CDCl_3/CD_3OD$, 1 : 1; 400 MHz) δ: 0.74 (3H, s, H-18), 0.96 (3H, d, J=6.4 Hz, H-21), 1.02 (3H, d, J=5.2 Hz, H-27), 1.03 (3H, d, J=6.1 Hz, H-26), 3.86 (1H, d, J=11.7 Hz, H-19a), 3.88 (1H, d, J=11.7 Hz, H-19b), 3.90 (1H, d, J=4 Hz, H-12), 4.65 (1H, br s, H-28a), 4.71 (1H, br s, H-28b), 5.94 (1H, d, J=4 Hz, H-11). ^{13}C-NMR (100 MHz) data: see Table 1. EI-MS m/z (rel. int.): 430 $[M]^+$ (20), 412 (100), 363 (60), 297 (5), 287 (38), 239 (25), 224 (15), 197 (35), 161 (74), 145 (74), 91 (54), 69 (68), 55 (72). *Anal.* Calcd for $C_{28}H_{46}O_3$: C, 78.08; H, 10.77. Found: C, 77.92; H, 10.86.

4α-Methyl-3β,14β-dihydroxy-5α-ergost-24(28)-en-23-one (**8**): White solid, mp 150—151 °C, $[\alpha]_D^{25}$ +66.2° (c=0.09, CH_3OH). IR (KBr) cm^{-1}: 3340, 2985, 1675, 1630, 880. UV ($CHCl_3$) λ_{max} nm: 240. ^{1}H-NMR ($CDCl_3$) δ: 0.86 (3H, d, J=4.5 Hz, H-21) 0.96 (3H, s, H-29), 0.98 (3H, s, H-18), 1.00 (3H, s, H-19), 1.01 (3H, d, J=6.6 Hz, H-26), 1.02 (3H, d, J=6.6 Hz, H-27), 2.68 (2H, dd, J=3.0, 15.6 Hz, H-22), 3.86 (1H, d, J=11.7 Hz, H-19a), 5.66 (1H, br s, H-28a), 5.91 (1H, br s, H-28b). ^{13}C-NMR data: see Table 1. EI-MS m/z (rel. int.): 444 $[M]^+$ (15), 429 (18), 401 (12), 333 (8), 317 (48), 285 (40), 247 (12), 187 (16), 181 (68), 147 (40), 121 (72), 109 (78), 97 (100), 69 (96), 55 (76). *Anal.* Calcd for $C_{29}H_{48}O_3$: C, 78.32; H, 10.89. Found: C, 78.14; H, 10.96.

Cytotoxicity Assay Human prostate cancer LNCaP cell line was obtained from the American Type Culture Collection (CRL1740, ATCC) and maintained in RPMI supplemented with 10% fetal bovine serum (FBS), 100 mg/l streptomycin and 100 IU/ml penicillin at 37 °C in a humidified at-

mosphere of 5% CO_2. Steroid compounds were dissolved in DMSO at a concentration of 80 mg/ml which was then diluted to appropriated concentrations with culture medium when use. The final concentration of DMSO did not exceed 0.5% in any experiment. LNCaP cells (2×10^4 cells/0.1 ml/well) were incubated with serial dilutions of steroid compounds in 96-well culture plates (Costar, U.S.A.) for 48 h, and their cytotoxicity was measured by fluorometric determination of DNA quantity with the fluorochrome Hoechst 33342 (Molecular Probe Inc. H3570, Oregon, U.S.A.) using a Millipore fluorometer (Cytophor 2350, Millipore, U.S.A.). Data points represented the mean values and standard deviations of triplicate assays.

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References

1) Faulker D. J., *Nat. Prod. Rep.*, **16**, 155—198 (1999).
2) Zhang W. H., Williams I. D., Che C. T., *Tetrahedron Lett.*, **42**, 4681—4685 (2001).
3) Liu W. K., Wong N. L. Y., Huang H. M., Ho J. K. C., Zhang W. H., Che C.T., *Life Sci.*, **69**, 843—853 (2002).
4) Zhang W. H., Che C. T., *J. Nat. Prod.*, **64**, 1489—1492 (2001).
5) Bowden B. F., Coll J. C., *Aust. J. Chem.*, **34**, 2677—2681 (1981).
6) Ray A. K., Datta P. K., Das T., Bhattacharyya P., Barua A. K., *J. Nat. Prod.*, **54**, 854—855 (1991).
7) Anjaneyulu A. S. R., Prakash C. V. S., *Indian J. Chem.*, **34B**, 32—39 (1995).
8) Handayani D., Edrada R. A., Proksch P., Wray V., Witte L., ven Ofwegen L., Kunzmann A., *J. Nat. Prod.*, **60**, 716—718 (1997).
9) Rao M. R., Venkatesham U., Venkateswarlu Y., *J. Nat. Prod.*, **62**, 1584—1585 (1999).
10) Wang G., Li F., Zeng L., Ma L., Tu G., *Chem. J. Higher Ed. Inst.* (China), **13**, 623—627 (1992).
11) Iguchi K., Saitoh S.,Yamada Y., *Chem. Pharm. Bull.*, **37**, 2553—2554 (1989).
12) Duh C. Y., Wang S. K., Chu M. J., Dai C. F., Sheu J. H., *J. Fish. Soc. Taiwan*, **24**, 127—135 (1997).
13) Duh C. Y., Wang S. K., Chu M. J., Sheu J. H., *J. Nat. Prod.*, **61**, 1022—1024 (1998).
14) Zhang M., Long K., Wu H., Ma K., *J. Nat. Prod.*, **57**, 155—160 (1994).
15) Shin J., Seo Y., *J. Nat. Prod.*, **58**, 948—953 (1995).
16) Long K., Ju Z., Lin Y., Xu S., Xie Q., Xie R., *J. Zhongshan Univ. Nat. Sci. Sect.* (China), **4**, 85—92 (1984).
17) Singh B., Agrawal P. K., Thakur R. S., *J. Nat. Prod.*, **52**, 180—183 (1989).