Antibacterial Sphingolipid and Steroids from the Black Coral *Antipathes dichotoma*

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From the black coral *Antipathies dichotoma*, a sphingolipid (2*S,3*S**,4*E*,8*E*)-2*N*-[tetradecanoyl]-4(*E*),8(*E*)-icosadiene-1, 3-diol (1) and a steroid (22*E*)-methylcholesta-5,22-diene-1α,3β,7α-triol (2) were isolated. Other known compounds, 3β,7α-dihydroxy-cholest-5-ene (3) (22*E*,24*S*),5α,8α-epidioxy-24-methylcholesta-6,22-dien-3β-ol (4) and (22*E*,24*S*),5α,8α-epidioxy-24-methylcholesta-6,9(11),22-trien-3β-ol (5). The structures were established on the basis of NMR spectroscopic analysis and comparison with literature. The antibacterial activity of five compounds was evaluated.**

Key words black coral; *Antipathes dichotoma*; sphingolipid; trihydroxy steroid; antibacterial

Antipathes dichotoma (Pallas) belongs to zoanthoid black coral. It has some pharmaceutical uses, such as relieving fever and softening hard mass. Few literatures about the chemical constituents of black corals that reported nine steroids from *A. subpinnata*,[1,2] and four alkaloids from *A. dichotoma*.[3] Sphingolipids form a biologically important class of compounds,[4] some of which have been reported to exhibit antihepatotoxic, antitumor and immunostimulatory activities,[5,6] inhibition of atherosclerosis[7] and as secondary messengers.[8]

Results and Discussion

Compound **1** has a molecular formula of $C_{34}H_{65}NO_3$ which was determined from high resolution (HR)-FAB-MS data (*m*/*z* 558.4876 $[M+Na]^+$). Calcd 558.4862, electron ionization-mass spectra (EI-MS) (*m*/*z* 535) and ^{13}C-NMR. The IR spectra showed the hydroxyl and amide NH group bands at 3340 and 3320 cm^{-1}, the band at 1640 cm^{-1} was due to CONH group.

The ^{1}H-NMR spectrum (Table 1) revealed the presence of two primary methyls at δ 0.88 (6H, t, J=7.2 Hz), two hetero bearing-methines at δ 3.91 and 4.32 and oxygenated methylene protons at δ 3.70 and 3.95, four olefinic protons at δ 5.54, 5.78, 5.43 and 5.36, an NH proton at δ 6.20 and a huge methylene envelope at δ 1.3 (Table 1). The ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectral data of **1** were supportive of the above analysis, showing a carbonyl group at δ_C 174.1, two double bonds at δ_C 134.3, 131.4, 129.1 and 128.9, three oxygenated or other hetero atomized carbons at δ_C 74.6, 62.4 and 54.4, aliphatic methylenes at δ_C 22.7—36.8 and two methyls at δ_C 14.2. The downfield doublet at δ 6.28 (NH) was deuterium-exchangeable, and there was no any correlation between this signal and any carbon in the heteronuclear multiple quantum coherence (HMQC) spectrum. On the other hand, a correlation from δ 6.28 (NH) to δ 3.91 (m), and the correlations from δ 6.28 (NH) to δ_C 174.1(C-1′), 36.8 (C-2′), 62.4 (C-1), 54.4 (C-2) and 74.6 (C-3) were observed in the ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) spectra, respectively. All the above data suggested **1** is a ceramide (sphingolipid).[9,10] In order to determine the lengths of sphingosine and fatty acid chains, the positions of double bonds and the absolute configuration of **1**, the acid methanolysis method of Gaver and Sweeley[11] which yield a fatty acid methyl ester (FAME) methyl tetradecanoate *m*/*z* 242 detected by GCMS, the presence of tetradecanoyl moiety confirmed by the characteristic ion at *m*/*z* 211 $[CH_3(CH_2)_{12}CO]^+$. So the molecular formulas of FAME and sphingosine are $C_{15}H_{30}O$ and $C_{20}H_{39}NO_2$, respectively. The double bonds and hydroxyl groups should be in sphingosine moiety and their positions could be determined by inspection of ^{1}H–^{1}H COSY spectrum, two methylene (C-1) protons at δ 3.70 and 3.95 correlated with the methine proton (C-2) at δ 3.91 which is correlated with the methine (C-3) proton at δ 4.32, the methine (C-3) proton at δ 4.32 correlated with the olefinic (C-4) proton at δ 5.54 (dt, J=15.0, 6.0 Hz) which is in turn correlated with another olefinic (C-5) proton at δ 5.78 (dt, J=15.0, 6.6 Hz), the olefinic proton at δ 5.78 correlated with two methylene (C-6) at δ 2.15 (q, J=6.6 Hz) that correlated with another two methylene (C-7) protons at δ 2.08 (q, J=6.6 Hz), which correlated with the olefinic (C-8) proton at δ 5.36 (dt, J=15.0, 6.0 Hz), that proton correlated with the olefinic (C-9) proton at δ 5.43 (dt, J=15.0, 6.0 Hz) that is correlated with the two methylene (C-10) protons at δ 1.97 (q, J=7.8 Hz). The above discussion implied that the two OH groups are at C-1 and C-3, and two double bonds one at C-4/C-5 and another between C-8/C-9 (double bonds are *trans* oriented owing to the values of chemical shifts of allylic methylene δ_C>30 and the J values).[12] Consideration of biogenesis and steric hinderance of sphingolipids, generally were acknowledged to determine the absolute stereochemistry of the phytosphingosine moiety. On the basis of the ^{13}C-NMR spectral data, the relative stereochemistries at C-2 (δ 54.4) and C-3 (δ 74.6) were deduced to be 2*S* and 3*S*.[7] Thus, the structure of **1** was established as (2*S**,3*S**,4*E*,8*E*)-2*N*-[tetradecanoyl]-4(*E*),8(*E*)-icosadiene-1,3-diol.

Compound **2** has a molecular formula of $C_{28}H_{46}O_3$ which was determined from HR-FAB-MS data (*m*/*z* 453.3357 $[M+Na]^+$). Calcd 453.3341, EI-MS (*m*/*z* 535) and EI-MS (*m*/*z* 430) together with ^{13}C-NMR, implying six degrees of unsaturation. The presence of hydroxyl and olefinic functionalities was deduced from IR absorptions at 3355 and 1643 cm^{-1}. Three hydroxyls in the molecule were estimated from ion peaks appearing at *m*/*z* 412 $(M-H_2O)^+$, and 394

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Table 1. NMR Spectral Data of Compounds **1** and **2** in $CDCl_3$ (δ, ppm, *J*, Hz).

No.	**1**		No.	**2**	
	δ ^{1}H (m, *J*, Hz)	δ ^{13}C		δ ^{1}H (m, *J*, Hz)	δ ^{13}C
1a	3.70 (dd, 10.8, 3.6)	62.4	1	3.63 (br s)	73.6
1b	3.95 (dd, 11.4, 3.6)		2α	2.06 (m)	23.9
2	3.91 (m)	54.4	2β	1.77 (m)	—
3	4.32 (br s)	74.6	3	4.08 (tt, 10.8, 6, 4.8)	67.7
4	5.54 (dt, 15, 6)	128.9	4α	2.17 (m)	40.6
5	5.78 (dt, 15, 6.6)	131.4	4β	2.14 (m)	—
6	2.15 (q, 6.6)	32.4	5	—	144.0
7	2.08 (q, 6.6)	32.2	6	5.36 (br d, 2.4)	117.5
8	5.36 (dt, 15, 6.6)	134.3	7	3.65 (s)	75.9
9	5.43 (dt, 15, 6.6)	129.1	8	1.45 (m)	39.4
10	1.97 (q, 7.8)	32.6	9	1.66 (m)	37.1
11—18	1.30 (m)	29	10	—	43.1
19	1.30 (m)	25.8	11α	1.50 (m)	22.9
20	0.88 (t, 7.2)	14.2	11β	1.44 (m)	—
1′		174.1	12α	1.18 (m)	33.19
2′	2.23 (t, 7.2)	36.8	12β	2.01 (m)	—
3′	1.64 (m)	32.3	13	—	43.42
4′—12′	1.30 (m)	29	14	1.20 (m)	55.8
13′	1.30 (m)	22.7	15α	1.85 (m)	28
14′	0.88 (t, 7.2)	14.2	15β	1.43 (m)	—
NH	6.28 (d, 7.2)	—	16α	1.90 (m)	30.0
			16β	1.28 (m)	—
			17	1.15 (m)	54.7
			18	0.60 (s)	12.33
			19	1.09 (s)	19.7
			20	1.80 (m)	39.2
			21	1.02 (d, 6.6)	22.33
			22	5.16 (dd, 15, 7.2)	135.6
			23	5.18 (dd, 15, 7.2)	132.2
			24	2.02 (m)	43.73
			25	1.47 (m)	31
			26	0.82 (d, 6.6)	22.1
			27	0.84 (d, 6.6)	20.8
			28	0.92 (d, 6.8)	18.9

$(M-2H_2O)^+$ and 376 $(M-3H_2O)^+$ in the EI-MS spectrum, and from the NMR signals (Table 1) resonating at δ_C 75.9, 73.6 and 67.7 (each CH) and δ 3.65 (1H, s), δ 3.63 (1H, br s), δ 4.08 (1H, tt, J=10.8, 6, 4.8 Hz), respectively. Two double bonds (δ_C 144.0, q_c, 135.6, CH, 132.2, CH, 117.5, CH; δ 5.36, 5.18, 5.16 each 1H) were also indicated in compound **2**. Moreover, the ^{1}H-NMR spectrum exhibited two singlets at 1.08 and 0.60 (each 3H, s) and four doublets at δ 0.82, 0.84, 0.92 and 1.02 (each 3H, d, J=6.6 Hz). These findings together with the 28 carbon signals in the ^{13}C-NMR suggested a trihydroxy methylcholesta skeleton. Analysis of ^{1}H–^{1}H COSY and HMBC correlations established structure of compound **2** as illustrated in Fig. 1, where positions of 3OH groups and two double bonds were determined to be at C-1, C-3, C-7, C-5/C-6 and C-22/C-23, respectively. The stereochemistry of the three hydroxy groups was established on the basis of nuclear Overhauser effect spectroscopy (NOESY) correlation. The hydroxyl group at C-1 and C-3 were determined to be α and β orientations, respectively, depending on strong nuclear Overhauser effect (NOE) interaction of H_3-19 (δ 1.09, s) with H-1 (δ 3.63, br s), H_3-19 with H-4β (δ 2.14, m), and H-4α (δ 2.17, m) with H-3 (δ 4.08, tt, J=10.8, 6.0, 4.8 Hz) (Fig. 2). The β orientation of the OH group at C-7 was deduced on the basis of significant correlation of H-7 (δ 3.65, s) with H-8 (δ 1.45) and H-6 (δ 5.36, br d, J=2.4 Hz) but no with H-9 (δ 1.66, m) or H-14 (δ 1.20, m). Also from H-NMR data, the orientations of OH groups at C-1, C-3 and C-7 were further confirmed to be α, β and α, respectively, on the basis of peak shape and coupling constants where H-1 (δ 3.65, br s), H-3 (δ 4.08, tt, J=10.8, 6, 4.8 Hz) and H-7 (δ 3.63, m). The *E* geometry of the double bond at C-22/C-23 supported from NOE interactions of H-22 (δ 5.16, dd, J=15, 7.2 Hz) with H_3-28 (δ 0.92, d, J=6.8 Hz) and H-23 (δ 5.18, dd, J=15, 7.2 Hz) with H-20 (δ 1.80, m) and H-23 with H-25 (δ 1.47, m). Furthermore the double bond at C-22/C-23 is *trans* oriented owing to the values of chemical shifts of allylic methine δ_C>30 and the J values (15 Hz).[12] On the basis of the above findings, the structure of compound **2** was established as (22*E*)-methylcholesta-5,22-diene-1α,3β,7α-triol.

Antibacterial Activity Biological activity of the compounds **1**—**5**, were screened for their antibacterial activity by disk-diffusion technique[13] against Gram-positive (*Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa*), at 1 mg/ml concentration. The inhibited zone diameters were measured and recorded in Table 2. Furacin was used as a standard compound. The minimal inhibitory concentration (MIC) of the strongly active compounds were also measured and listed in Table 2.

The data obtained from Table 2 shows that sphingolipid

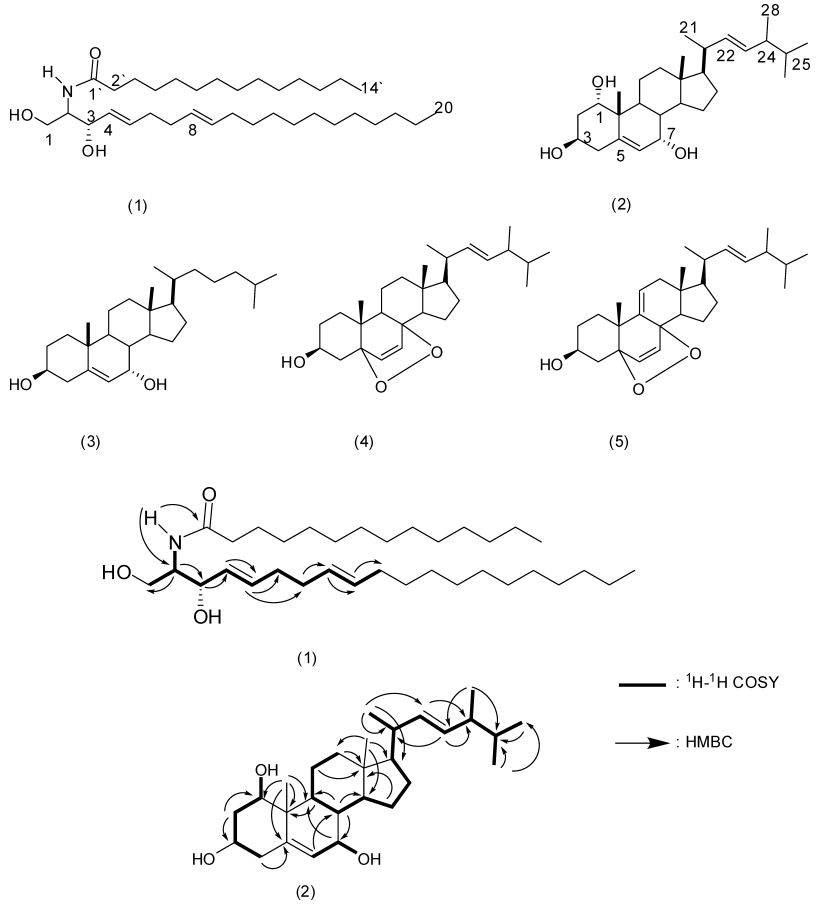

Fig. 1. Selected ^{1}H–^{1}H COSY and HMBC Correlations of **1** and **2**

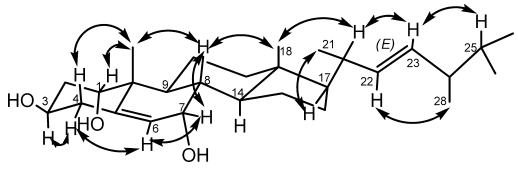

Fig. 2. Key NOESY Correlations of **2**

Table 2. Diameter of Inhibition Zone in mm at Concentration Level of 1 mg/ml and Minimum Inhibitory Concentration (MIC) in mg/ml

Compound	*B. subtilis* MIC (mg/ml)	*P. aeruginosa* MIC (mg/ml)
1	17.9	18.2
	0.4	0.2
2	12.7	9.8
	0.6	—
3	6.3	9.0
	—	—
4	17.9	10.0
	0.3	—
5	17.6	11.1
	0.5	—

(1) exhibits potent activity against *B. subtilis* and *P. aeruginosa*. While the epidioxy steroids (4, 5) and the trihydroxy steroid (2) show potent activity against *B. subtilis*.

Experimental

General Experimental Procedure Optical rotations were measured on ATAGO POLAX-L 2 polarimeter. EI/MS analyses were carried out on a Shimadzu-QP 2010. ^{1}H-NMR spectra were recorded at 600 MHz, and ^{13}C-NMR at 75 MHz. Chemical shifts are given in ppm relative to tetramethyl silane (TMS) as internal standard. Thin layer chromatography was performed on silica gel (Kieselgel 60, F254) of 0.25 mm layer thickness. Gel filtration was carried out using Sephadex LH-20. Spots were detected by using ethanol/sulfuric acid as spray reagent. The black coral (Coelenterata, Hexacorallia) *A. dichotoma*, fam. Antipatharia identified by Prof. Manfred Grasshoff, and was collected using SCUBA, from Hakel area, Saudi Arabia, in the Red Sea in Jan. 2009. A voucher specimen was deposited in the Faculty of Marine Science (B.C. 2009.77).

Extraction and Isolation The air dried black coral (1.5 kg) was soaked in a mixture of chloroform/methanol (1 : 1) and allowed to stand at room temperature for few days. Filtration and evaporation to dryness under reduced pressure. Half of the residue (30 g) was fractionated by column chromatography over silica gel with gradient hexane–EtOAc and then with gradient dichloromethane–acetone. Fractions of 50 ml were collected, and those containing a single compound were combined and further purified to give the following compounds.

(2*S,3*S**,4*E*,8*E*)-2*N*-[Hexadecanoyl]-4(*E*),8(*E*)-octadecadiene-1,3-diol (1)** Colorless amorphous powder; mp 123—125 °C (30 mg, 0.002%); *Rf*=0.52 (Si gel, *n*-hexane–EtOAc, 1 : 1); $[\alpha]_D$ +83 (c=0.1, $CHCl_3$). IR (neat) ν cm^{-1}: OH and amide NH (3340—3200 cm^{-1}), amide carbonyl (1640 cm^{-1}) and olefinic double bond (1620 cm^{-1}); HR-FAB-MS data (*m/z* 558.4876 [M+Na]$^+$). Calcd 558.4862; EI-MS *m/z* 535 [M, $C_{34}H_{65}NO_3$]$^+$. ^{1}H- and ^{13}C-NMR: Table 1.

Methanolysis of 1 Compound **1** (20.0 mg) was refluxed with 0.9 M HCl in 82% aq. MeOH (10 ml) for 18 h. The mixture was extracted with *n*-hexane and evaporation of the hexane yielded a fatty acid methyl ester (FAME). GC-MS analysis of FAM gave a single fatty acid, methyl tetradecanoate *m/z* 242 [M$^+$, $C_{15}H_{30}O_2$], 211 [M−OMe]$^+$, 199 [M−C_3H_7]$^+$.

(22*E*)-Methylcholesta-5,22-diene-1α,3β,7α-triol (2) White powder;

mp 138—140 °C; (36 mg, 0.0024%); *Rf*=0.55 (Si gel, *n*-hexane–acetone, 6 : 4); $[\alpha]_D$ −30 (*c*=0.1, $CHCl_3$); IR (neat) ν_{max}: 3355 (OH), 2959, 2930, 2870, 1653 (olefinic double bond) cm^{-1}; HR-FAB-MS data (*m/z* 453.3357 $[M+Na]^+$). Calcd 453.3341; EI-MS *m/z* 430 (100, $[M, C_{28}H_{46}O_3]^+$), 418 (5.7, $[M-H_2O]^+$), 399 (3.5, $[M-H_2O-Me]^+$), 394 (1.2, $[M-2H_2O]^+$), 376 (19.5, $[M-3H_2O]^+$) 314 (11.7), 303 (41.1), 285 (7.3), 249 (11.2),173 (11.3), 145 (13.0), 133 (15.0), 123 (51.2), 105 (40.8):^{1}H- and ^{13}C-NMR: Table 1.

3β,7α-Dihydroxy-cholest-5-ene (3) The fraction eluted with hexane–EtOAc (6 : 4), was further purified by preparative TLC of silica gel using same eluant system to give a pure material 130—133 °C (15 mg, 0.0001%); $[\alpha]_D$ −98 (*c*=0.1, $CHCl_3$). Compound **3** was identified by comparison of its ^{1}H- and ^{13}C-NMR with ref. 14.

(22*E*,24*S*),5α,8α-Epidioxy-24-methylcholesta-6,22-dien-3β-ol (4) and (22*E*,24*S*),5α,8α-Epidioxy-24-methylcholesta-6,9(11),22-trien-3β-ol (5) The fraction eluted with hexane–EtOAc (7 : 3), was further purified by preparative TLC of silica gel using same eluant system to give a material thought to be pure, which when subjected to ^{1}H-NMR analysis gave a mixture of **4** and **5** in ratio of 14 : 1. Compounds **4** and **5** were identified from their NMR spectral data and by comparison with refs. 15 and 16, respectively.

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References

1) Aiello A., Fattorusso E., Menna M., *Steroids*, **56**, 513—517 (1991).
2) Aiello A., Fattorusso E., Menna M., *J. Nat. Prod.*, **55**, 321—325 (1992).
3) Qi S. H., Su G. C., Wang Y. F., Liu Q. Y., Gao C. H., *Chem. Pharm. Bull.*, **57**, 87—88 (2009).
4) Kottler T., Sandhoff K., *Angew. Chem., Int. Ed.*, **38**, 1532—1568 (1999).
5) Atta-Ur-Rahman, Zareen S., Choudhary M. I., Akhtar M. N., Ngounou F. N., *Phytochemistry*, **69**, 2400—2405 (2008).
6) Cetani F., Zilic J., Zacchigna M., Procida G., *Fitoterapia*, **81**, 97—103 (2010).
7) Tao W. W., Yang N. N., Liu L., Duan J. A., Wu D. K., Qian D. W., Tang Y. P., *Fitoterapia*, **81**, 196—199 (2010).
8) Murakami T., Furusawa K., Tamai T., Yoshikai K., Nishikawa M., *Bioorg. Med. Chem. Lett.*, **15**, 1115—1119 (2005).
9) Yaoita Y., Ishizuka T., Kakuda R., Machida K., Kikuchi M., *Chem. Pharm. Bull.*, **48**, 1356—1358 (2000).
10) Loukaci A., Bultel-ponce V., Longeon A., Guyot M., *J. Nat. Prod.*, **63**, 799—782 (2000).
11) Gaver R. C., Sweeley C. C., *J. Am. Oil Chem. Soc.*, **42**, 294—298 (1965).
12) Su B.-N., Takaishi Y., *J. Nat. Prod.*, **62**, 1325—1327 (1999).
13) Gruickshank R., Duguid J. R., Marmion B. P., Swain R. H. A., "Medicinal Microbiology," Vol. 11, Churchill Livingstone, New York, 1975, p. 190.
14) Notaro G., Piccialli V., Sica D., *J. Nat. Prod.*, **55**, 1588—1594 (1992).
15) Wang J. F., Huang Y.J., Fang M. J., Xie W. L., Su W. J., Zhao Y. F., *Acta Crystallogr. E*, **60**, 764—765 (2004).
16) Gunatilaka A. A. L., Gopichand Y., Schmitz F. J., Djerassi C., *J. Org. Chem.*, **46**, 3860—3866 (1981).