Journal of the Korean Magnetic Resonance Society **2007**, *11*, 122 - 128

Three Sterol Sulfates Isolated from a Marine Sponge *Acanthodoryx Fibrosa*

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Received June 30, 2007

Abstract : Three sterol sulfates were isolated from AMPK activity-guided fraction of a marine sponge *Acanthodoryx fibrosa*. Their structures were determined by an extensive NMR analysis, MS data, and two compounds were confirmed as unusual phosphorylated sterol sulfates by comparing with NMR data of the known compounds. Compound **3** was given to be a new dephosphated sterol sulfate derivative. Compound **1** moderately showed AMPK activation effect on L6 myoblast cell through Western blot analysis.
Keywords : 1D and 2D NMR, sponge *Acanthodoryx fibrosa*, AMPK activation, sterol sulfate

INTRODUCTION

Marine organisms have been recognized to be plentiful producers of novel compounds which show a variety of biological activities including cytotoxic, antimicrobial, antifeedant, antidiabetic activity, and antiinflammatory properties.[1-4] In our screening for AMP-activated protein kinase (AMPK) activator from the extracts of marine organisms with Western Blot analysis, we found moderate activity in the methanol fraction of a marine sponge *Acanthodoryx fibrosa* collected in Philippine. AMPK is a key sensor and regulator in the cellular energy metabolic system. AMPK stimulates downstream pathways which increase energy production (glucose transport, fatty acid oxidation) and switched off pathways which consume energy (lipogenesis, gluconeogenesis). This

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allowed AMPK to be an attractive target for the treatment of metabolic syndrome like obesity and type 2 diabetes.[5, 6]

Bioassay-guided isolation afforded three unusual sterol sulfates whose structures were elucidated by NMR spectroscopy and MS data. Two of these compounds were found to be identical with phosphorylated sterol sulfates reported in the literature[7,8] and the other was given as a new dephosphated derivative. This paper describes isolation and structure elucidation of three compounds.

EXPERIMENTAL

Extraction and Isolation

A marine sponge *Acanthodoryx fibrosa* (sample no. 06ph-7) was collected by hand using SCUBA at Philippine in 2006. The specimen (0.5Kg) transferred under dried condition was twice extracted with MeOH at room temperature. The crude extract was first partitioned between H_2O and methylene chloride (MC), and then the aqueous layer re-partitioned between butanol and H_2O solvent. The butanolic phase was in turn subjected to reversed phase vacuum flash chromatography eluting with stepwise gradients of MeOH in H_2O(50%, 60%, 70%, 80%, 90%, 100%). Among them, the 90% MeOH fraction moderately showed the AMPK activation effect on L1 Myoblast cell with Western Blot analysis. First, this was separated by reversed phase HPLC(YMC ODS-A column, 250mm × 10mm, Varian RI detector) using a solvent system (H_2O/MeOH=17/83) to yield eight fractions (rp1- rp8). Second fraction (rp2) was further purified by reversed-phase HPLC with an eluant (H_2O/MeOH=10/90) to give compound **1** (7.8mg) and **2** (2.7mg). In the same way, the fifth fraction (rp5) was also purified by a solvent system (/MeCN/H_2O/MeOH=35/15/50) to obtain compound **3** (2.0 mg).

NMR experiment

The 1D and 2D NMR spectra were obtained on a Varian NMR system working at 500MHz for proton and 125MHz for carbon. The ^1H and ^{13}C NMR chemical shifts refer to CD3OD at 3.30 and 49.0 ppm, respectively. For all experiments, the temperature was stabilized at 297K. The parameters used for 2D NMR spectra were as follows; The gradient

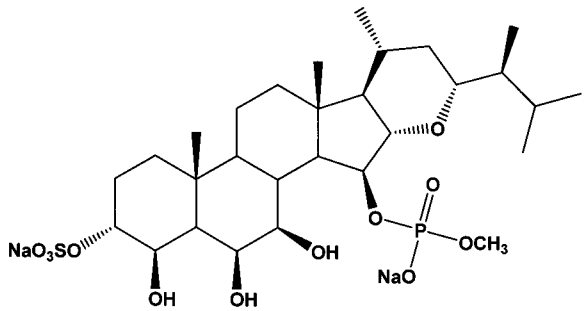
COSY spectra were collected with a spectral width 2567 Hz in a 512(t1) × 1024 (t2) matrix applying the pulse gradient of 1ms duration with a strength 10G/m and processed with a sinebell function. The gradient HSQC spectra were measured in a 128(t1) × 1024(t2) matrix with J_{CH}=140 Hz and processed in a 256(t1) × 1024(t2) matrix by a linear prediction method for a higher resolution. The gradient HMBC experiment was optimized for the long-range coupling constant of 7Hz. The ROESY experiment was carried out with mixing time of 300ms.

RESULTS AND DISCUSSION

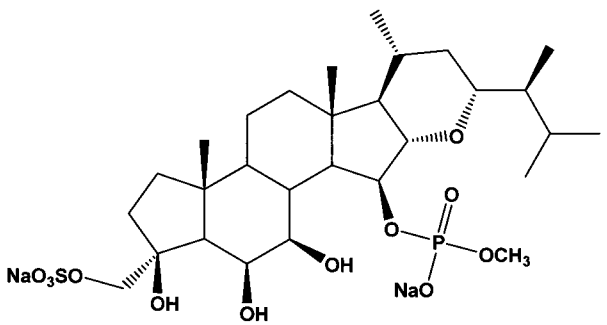
Three natural compounds **1-3** were purified from the 90% methanolic fraction showing the AMPK activation effect on L6 myoblast cell. Compound **1** showed a prominent molecular ion peak at m/z 653 $[M+H-2Na]^+$ in the ESI mass spectrum. The IR spectrum contained strong bands at 1210 and 1055cm^{-1}, indicative of the presence of a sulfate group.

^1H NMR spectrum contained severely overlapped signals with two methyl singlet and four methyl doublet resonances in the upfield region and also seven protons attached to carbons bearing oxygen atom between δ 3.39 and 4.76. This data, together with ^{13}C NMR spectrum, suggested that compound **1** is characteristic of a C28 sterol with an additional sulfate moiety. On the basis of strong HMBC correlations of two methyl singlet protons with adjacent carbons, careful analysis of 1D, COSY, and HSQC NMR data allowed us to assign the sterol ring A-D. The methine proton located at C-5 was coupled with two protons at δ 4.18 (H-4) and 4.10 (H-6), geminally to hydroxyl group, in the COSY spectrum. And then each proton was again correlated with protons at δ 4.38 (H-3) and 3.39 (H-7), respectively, consisting of two pairs in the vicinal oxygen-substituted carbons. Another methine proton at δ 4.76 gave a correlation with the proton (δ 3.78) on C-16. This information enabled six protons between δ 3.39 to 4.76 to be located on the ring A-D frame, and the last proton (δ 3.48) on oxygen-substituted carbons was assigned to C-23 position from the HMBC correlation of three methyl protons Me-26, -27, -28 with C-23, C-24, and C-25. Furthermore, downfield chemical shifts of C-16 and -23 suggested the presence of an ether ring. This was confirmed by the ROESY cross peak between two protons (δ 3.48 and

3.78) corresponding to their carbons. On the other hand, one methoxy moiety at δ 3.62 gave a doublet split signal (J = 10.8 Hz), but did not show any correlations with protons or carbons.



1



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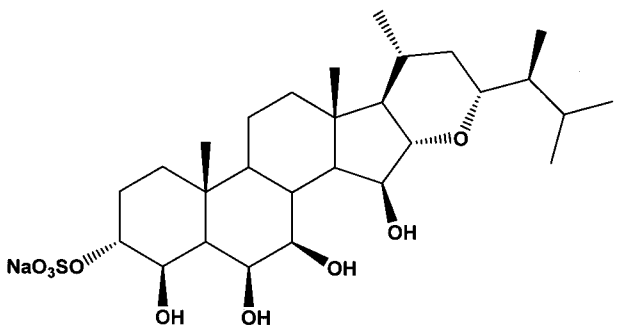

3

The literature survey on the partial structure of polyhydroxyl sterol proposed that compound **1** is the haplosamate **A**, a phosphorylated sterol sulfate.[7] The chemical shift of protons and carbons of **1** were very consistent with those of haplosamate **A**. Besides, a phosphorus signal at δ 2.12 was also observed in the ^{31}P NMR spectrum which led the methoxy protons at δ 3.62 to the methyl doublet pattern. Finally, the relative stereochemistry of **1** determined from ROESY data was also equal to that of the known haplosamate **A**.

Compound **2** had the molecular weight identical to that of compound **1**. The interpretation of 1D and 2D NMR spectra indicated that **2** was very similar to **1** except for the ring A part. Compound **2** possessed an oxygenated methylene group [δ_H 3.85 (1H, d, 9.8); 3.99 (1H, d, 9.8); δ_C 73.0, s] and an oxygenated quaternary carbon at δ 81.3, and exhibited strong IR absorption bands at 1218 and 1066 cm^{-1} corresponding to sulfate group. HMBC experiment showed that two oxygenated methylene protons and H-2 and -5 had a long range coupling with the oxygenated quaternary carbon, forming a five-membered ring. Other structural features of **2** were identical with those of compound **1**. This compound also turned out to be reported in the literature.[8]

The molecular weight of compound **3** was obtained from the molecular ion peak at *m/z* 559 [M+H−2Na−PO_3CH_3] of the ESI mass spectrum. The IR spectrum exhibited characteristic sulfate bands at 1213 and 1048 cm^{-1}. The ^{1}H NMR spectrum of **3** are similar to that of **1**, but different in the downfield range. First of all, the methoxy signal at δ 3.62 present in **1** were missing in the case of **3** and, more importantly, the methine proton at δ 4.15 (H-15) was shifted to somewhat upfield compared to that of **1**. In the ^{13}C NMR data, there was notable difference in the chemical shift for C-15 from δ 81.4 in **1** to 76.4 in **3**. Based on the chemical shift considerations and comparison with literature values for hydroxylated and phosphorylated sterols, **3** was proposed to have the hydroxyl group in the position of C-15.[9,10] All other assignments of **3** were completed through a combination of 1D and 2D NMR data. To conclude, the structure of 3 was determined to be a new dephosphated derivative of haplosamate **A**.

Steroidal sulfates are widely distributed in marine organisms such as sponges and starfish, but steroids with both sulfates and phosphate groups are very rare.[11] Though two phosphorylated sterol sulfates are known compounds, compound **1** moderately showed AMPK activation effect on L6 myoblast cell to the concentration of 10μM.

Table 1. NMR spectral data for compounds **1** and **3** in CD_3OD

position	δH (**1**)	δH (**3**)	δC (**1**)	δC (**3**)
1	1.33 (1H, m); 1.41 (1H, m)	1.34 (1H, m); 1.41 (1H, m)	34.9, t	35.0, t
2	1.88 (1H, m); 2.07 (1H, m)	1.88 (1H, m); 2.05 (1H, m)	23.7, t	23.7, t
3	4.38 (1H, br d, 2.5)	4.38 (1H, m)	77.7, d	77.6, d
4	4.18 (1H, m)	4.17 (1H, m)	75.7, d	76.1, d
5	1.54 (1H, m)	1.50 (1H, m)	45.3, d	45.0, d
6	4.10 (1H, m)	4.01 (1H, m)	77.7, d	77.5, d
7	3.39 (1H, m)	3.44 (1H, dd, 7.8, 3.4)	79.8, d	78.0, d
jex8	2.26 (1H, m)	2.17 (1H, m)	35.3, d	34.4, d
9	0.901 (1H, m)	0.91 (1H, m)	54.1, d	54.5, d
10			36.4, s	36.2, s
11	1.43 (1H, m); 1.52 (1H, m)	1.51 (2H, m)	19.9, t	20.2, t
12	1.21 (1H, m); 1.87 (1H, m)	1.19 (1H, m); 1.82 (1H, m)	40.9, t	41.2, t
13			43.0, d	42.8, s
14	1.42 (1H, m)	1.30 (1H, m)	58.7, d	60.0, d
15	4.75 (1H, m)	4.15 (1H, dd, 6.4, 3.4)	81.4, d	76.4, d
16	3.78 (1H, br d, 8.8)	3.55 (1H, dd, 10.3, 3.4)	93.0, d	92.1, d
17	0.76 (1H, dd, 10.3, 10.3)	0.74 (1H, dd, 10.3, 10.3)	62.5, d	62.2, d
18	0.98 (3H, s)	0.97 (3H, s)	15.6, q	15.4, q
19	1.33 (3H, s)	1.33 (3H, s)	17.8, q	18.1, q
20	1.81 (1H, m)	1.81 (1H, m)	34.1, d	34.0, d
21	1.00 (3H, 6.9)	0.97 (3H, 6.4)	20.7, q	20.7, q
22	0.91 (1H, m); 1.63 (1H, m)	0.87 (1H, m); 1.65 (1H, m)	39.3, t	39.9, t
23	3.48 (1H, dd, 8.3, 8.3)	3.39 (1H, dd, 7.8, 2.9)	83.2, d	82.8, d
24	1.42 (1H, m)	1.42 (1H, m)	45.1, d	45.1, d
25	1.97 (1H, m)	2.01 (1H, m)	28.8, d	28.5, d
26	0.81 (3H, d, 6.9)	0.81 (3H, d, 6.9)	18.0, q,	17.6, q
27	0.90 (3H, d, 6.9)	0.90 (3H, d, 7.3)	21.9, q	21.8, q
28	0.80 (3H, d, 6.9)	0.78 (3H, d, 6.9)	10.8, q	10.8, q
OCH_3	3.62 (3H, d, 10.4)		53.3, q	

Acknowledgement

This work was supported by the BK21 project of the Ministry of Education, Korea.

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