Journal of the Korean Magnetic Resonance Society **2016**, *20*, 41-45
DOI 10.6564/JKMRS.2016.20.2.041

Bile acids from a Marine Sponge-Associated Fungus Penicillium sp.

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Received Apr 6, 2016; Revised May 2, 2016; Accepted May 19, 2016

Abstract Chemical investigation of a marine-derived fungus, *Penicillium* sp. 108YD020, resulted in the discovery of six bile acid derivatives, glycocholic acid (**1**), glycocholic acid methyl ester (**2**), cholic acid (**3**), glycochenodeoxycholic acid (**4**), glycodeoxycholic acid methyl ester (**5**), and cholic acid methyl ester (**6**). The structures of six bile acid derivatives **1**–**6** were determined by the detailed analysis of 1D, 2D NMR and LC-MS data, along with chemical methods and literature data analysis.

Keywords Marine natural products, cholic acid, glycocholic acid, bile acids, marine fungus, *Penicillium* sp.

Introduction

Bile acids are principal ingredients of bile synthesized in the liver of vertebrates such as mammals and fish, and secreted into the duodenum to play an important role in lipid metabolism[1]. They include cholic acid, chenocholic acid, deoxycholic acid and their conjugates with glycine and taurine contribute to bile formation[2]. A variety of bile acids have a steroid skeleton and carboxylic side chain. In addition, bile acids have been reported that they are different in the number and substituted position of the hydroxyl group and side chain length[1]. Here, we report the isolation and structure determination of six bile acid derivatives from a marine fungus.

Experimental Methods

General Experimental Procedures- The ^{1}H, ^{13}C, and HMBC NMR spectra were acquired on a Varian Unity 500 MHz spectrometer. High performance liquid chromatography (HPLC) was conducted with a PrimeLine pump (Analytical Scientific Instrument, Inc., El Sobrante, CA, USA) with RI 2000 refractive index detector (JORDI Labs, Mansfield, UK). Semi-preparative HPLC was performed using an ODS column (YMC-pack-ODS-A, 250 × 10 mm i.d., 5 μm).

Isolation and Identification of the Strain 108YD020- The strain 108YD020 was isolated from a marine sponge sample collected at Wangdolcho, East Sea, South Korea, in 2010, by serial dilution technique and grown on Bennett's agar plates (1% glucose, 0.2% tryptone, 0.1% yeast extract, 0.1% beef extract, 0.5% glycerol, 1.7% agar powder, salinity 32g/L, pH 7.0 before sterilization). The plates were incubated for seven days at 28℃, and the resulting colony of the strain 108YD020 was isolated and stocked in 40% (*v/v*) glycerol-seawater solution. According to BLAST and phylogenetic analysis based on 18s

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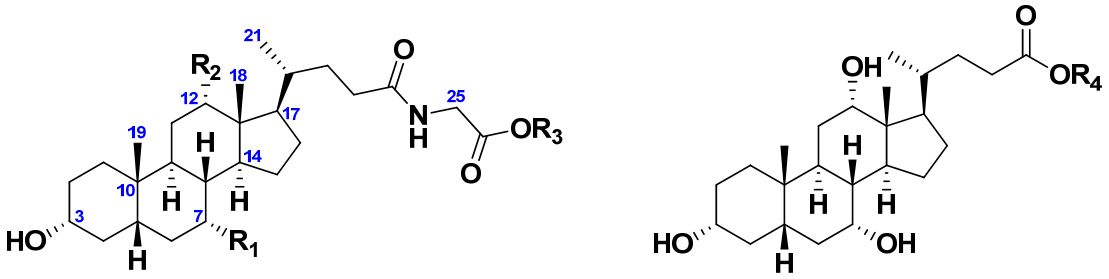

1 (R_1=OH, R_2=OH, R_3=H, Glycocholic acid)
2 (R_1=OH, R_2=OH, R_3=CH_3, Glycocholic acid methyl ester)
4 (R_1=OH, R_2=H, R_3=H, Glycochenodeoxycholic acid)
5 (R_1=H, R_2=OH, R_3=CH_3, Glycodeoxycholic acid methyl ester)

3 (R_4=H, Cholic acid)
6 (R_4=CH_3, Cholic acid methyl ester)

Figure 1. The structures of 1–6 isolated from the culture extract of *Penicillium* sp. 108YD020.

rRNA gene sequences, the strain was identified as *Penicillium* sp. The strain is currently preserved in the Microbial Culture Collection, Korea Institute of Ocean Science and Technology (KIOST), under the curatorship of Hee Jae Shin.

Seed and Mass Cultures of the Strain 108YD020- The seed and mass cultures of the strain 108YD020 were carried out in malt extract medium (2% malt extract, 2% glucose, 0.1% tryptone, 3.2% sea salt, pH 7.0 before sterilization). The medium (300 mL) was provided into a 500 mL conical flask and sterilized. A single colony of the strain from the agar plate was inoculated into the flask and incubated at 28℃ for four days on a rotary shaker at 120 rpm. An aliquot (0.1% *v/v*) from the seed culture was inoculated into 2 L flasks (total 23 flasks) containing 1 L of the medium. The mass culture was incubated under the same conditions as the seed culture for seven days and then harvested.

Extraction and isolation of compounds- The culture broth (total 23 L) was harvested by high speed centrifugation (60,000 rpm) and then extracted with EtOAc (two times). The EtOAc extract was evaporated to obtain crude extract (2.70 g). The crude extract was subjected to an ODS open column chromatography followed by stepwise gradient elution with MeOH/H_2O (*v/v*) (1:4, 2:3, 3:2, 4:1, and 100:0) as eluent. The subfraction eluted with MeOH/H_2O (4:1) was purified by a reversed-phase HPLC (YMC ODS-A column, 250 × 10 mm i.d., 5 μm; 70% MeOH in H_2O; flow rate 1.5 mL/min; RI detector) to yield pure compounds **1** (27.9 mg) and **2** (6.6 mg). The subfraction eluted with MeOH/H_2O (100:0) was also purified by a reversed-phase HPLC (YMC ODS-A column, 250×10 mm i.d., 5 μm; 90% MeOH in H_2O; flow rate 1.5 mL/min; RI detector) to yield pure compounds **3** (4.9 mg), **4** (2.1 mg), **5** (2.1 mg), and **6** (2.2 mg).

Results

The fungal strain 108YD020 was isolated from a marine sponge sample collected at Wangdolcho, the Republic of Korea's Eastern reef, and identified as *Penicillium* sp. by 18s rRNA sequencing. The strain 108YD020 was cultured in malt extract medium at 28°C for seven days. The culture broth was extracted with ethyl acetate and the crude extract was purified by flash open chromatography and a reversed-phase high-performance liquid chromatography (HPLC). The bile acid derivatives were identified as glycocholic acid (**1**), glycocholic acid methyl ester (**2**), cholic acid (**3**), glycochenodeoxycholic acid (**4**), glycodeoxycholic acid methyl ester (**5**), and cholic acid methyl ester (**6**) by comparative analysis of their NMR, MS and optical rotation data with those reported in the literature.

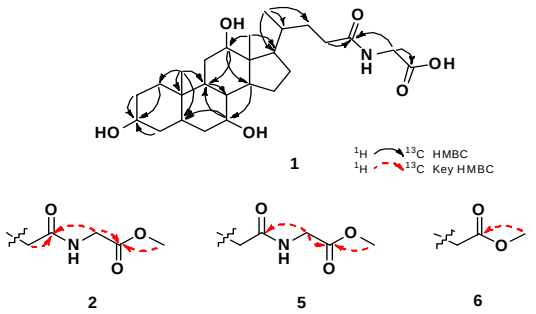

Figure 2. Key HMBC correlations of **1**-**6**.

Compound **1** was isolated as a brown amorphous solid. The molecular formula was determined to be $C_{26}H_{43}NO_6$ based on the LR-ESI-MS data *m/z* 464.28 $[M-H]^-$. The ^1H and ^{13}C NMR spectra of **1** showed key signals of three methyl groups (δ_H 0.72 (s, H-18)/δ_C 13.2 (C-18), δ_H 0.92 (s, H-19)/δ_C 23.3 (C-19), δ_H 1.04 (d, J = 6.5 Hz, H-21/δ_C 17.9 (C-21)), three hydroxyl groups (δ_H 3.37 (m, H-3)/δ_C 73.0 (C-3), δ_H 3.80 (brs, H-7)/δ_C 69.2 (C-7), δ_H 3.96 (brt, H-12)/δ_C 74.2 (C-12)) and gylcine moiety (δ_H 3.89 (m, H-25)/δC 41.9 (C-25), δ_C 173.2 (C-26)) in side chain. The hydroxyl group was located at C-3, 7, 12 by judging the chemical shifts of the oxygenated methine. The position of three methyl groups and glycine moiety were determined by the key HMBC correlations (Figure 2): H-18 (δ_H 0.72)/C-12 (δ_C 74.2), C-17 (δ_C 48.2); H-19 (δ_H 0.92)/C-1 (δ_C 36.6), C-9 (δ_C 28.0), C-10 (δ_C 36.0); H-21 (δ_H 1.04)/C-17 (δ_C 48.2), C-20 (δ_C 36.9), C-22 (δ_C 33.2); H-23 (δ_H 2.18, 2.32)/C-24 (δ_C 177.3); H-25 (δ_H 3.89)/C-24 (δ_C 177.3), C-26 (δ_C 173.2). These data suggested a structural similarity of **1** to the glycocholic acid[3].

The structure of compounds **2**, **4** and **5** exhibited features similar to those of **1** (Figure 1) and signals of methyl groups, hydroxyl groups and carbonyl carbons were found in NMR data. In compounds **2** and **5**, the methoxy groups (δ_H 3.71/δ_C 52.7 in **2**; δ_H 3.72/δ_C 52.7 in **5**) were observed in HSQC spectra and the methoxy protons showed HMBC correlations to C-25 (δ_C 172.0 and δ_C 172.1 in **2** and **5**, respectively) (Figure 2). The differences between these compounds were that glycocholic acid (**1**) has a carboxyl acid at C-26 but compounds **2** and **5** have a methyl ester at C-26. So, compound **2** was given the name glycocholic acid methyl ester.

In addition, in proton NMR spectra **(**Figure 3D and 3E**),** H-12 of **4** and H-7 of **5** were up-field shifted than those of **1**, suggesting that compounds **4** and **5** were dehydroxylated from glycocholic acid (**1**) at C-12 and C-7, respectively. These data suggested compounds **2**, **4** and **5** as other members of the bile acid family and compounds **2**, **4** and **5** were given the

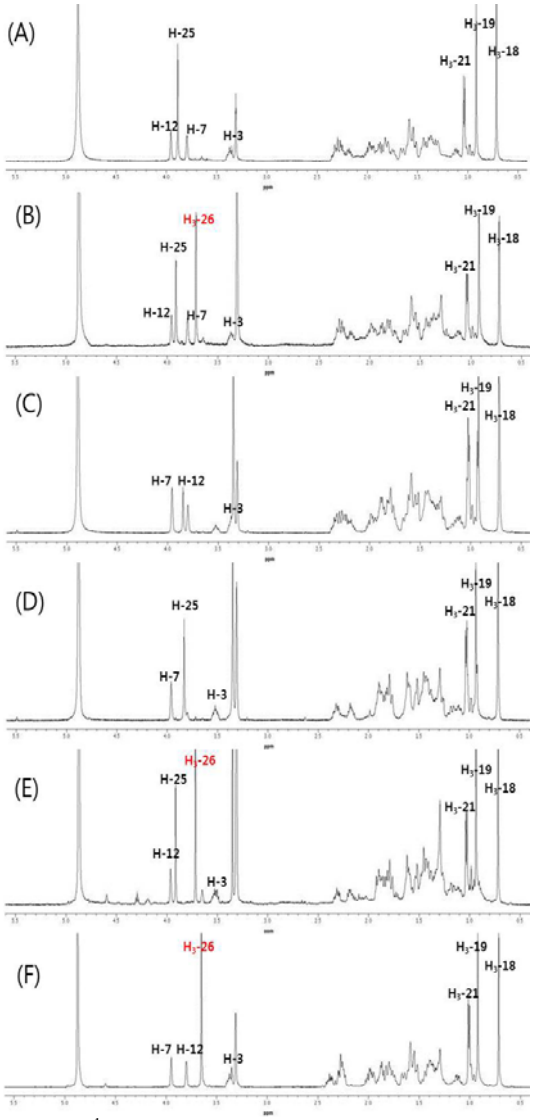

Figure 3. ^{1}H-NMR spectra of compounds **1** (A), **2** (B), **3** (C), **4** (D), **5** (E), and **6** (F).

name glycocholic acid methyl ester, glycochenodeoxycholic acid, glycodeoxycholic acid methyl ester, respectively.

The structure of **6** was established from NMR data analysis using 1D and 2D NMR techniques including ^{1}H, ^{13}C, and HMBC spectra. The ^{13}C NMR spectrum showed 25 signals assigned as three methyl carbons [δ_C 13.1 (C-18); δ_C 23.3 (C-19); δ_C 17.7 (C-21)], three oxygenated carbons [δ_C 73.0 (C-3); δ_C 69.2 (C-7); δ_C 74.1 (C-12)], and one carbonyl quaternary carbon [δ_C 176.7 (C-24)]. The HMBC correlation between H-25 (δ_H 3.65) and C-24 (δ_C 176.7) (Figure 2) established the presence methyl ester. Comparison of the ^{1}H and ^{13}C NMR data of **6** with those of **3**, revealed that the structures of these compounds are similar, except for the signal differences at the side chain. Therefore, compound **6** was named as cholic acid methyl ester.

As all the compounds have similar optical rotation values [**1**: $[\alpha]_D^{25}$ +21.33° (*c* 0.5, MeOH); **2**: $[\alpha]_D^{25}$ +10.00° (*c* 0.5, MeOH); **3**: $[\alpha]_D^{25}$ +27.33° (*c* 0.5, MeOH); **4**: $[\alpha]_D^{25}$ +26.67° (*c* 0.5, MeOH); **5**: $[\alpha]_D^{25}$ +28.00° (*c* 0.5, MeOH); **6**: $[\alpha]_D^{25}$ +24.00 (*c* 0.5, MeOH)], it was suggested that compounds **1**-**6** and other bile acid derivatives isolated from the marine organisms[4-6] share the same absolute configuration.

Glycocholic acid (**1**): brown amorphous solid; $C_{26}H_{43}NO_6$; $[\alpha]_D^{25}$ +21.33° (*c* 0.5, MeOH); ESI-MS *m/z* 464.28 $[M-H]^-$.

Glycocholic acid methyl ester (**2**): brown amorphous solid; $C_{27}H_{45}NO_6$; $[\alpha]_D^{25}$ +10.00° (*c* 0.5, MeOH); APCI-MS *m/z* 446.29 $[M-H]^-$.

Cholic acid (**3**): pink amorphous solid; $C_{24}H_{40}NO_5$; $[\alpha]_D^{25}$ +27.33° (*c* 0.5, MeOH); APCI-MS *m/z* 407.27 $[M-H]^-$.

Glycochenodeoxycholic acid (**4**): pink amorphous solid; $C_{26}H_{43}NO_5$; $[\alpha]_D^{25}$ +26.67° (*c* 0.5, MeOH); APCI-MS *m/z* 448.44 $[M-H]^-$.

Glycodeoxycholic acid methyl ester (**5**): brown amorphous solid; $C_{27}H_{45}NO_5$; $[\alpha]_D^{25}$ +28.00° (*c* 0.5, MeOH); APCI-MS *m/z* 462.29 $[M-H]^-$.

Cholic acid methyl ester (**6**): brown amorphous solid; $C_{25}H_{42}NO_5$; $[\alpha]_D^{25}$ +24.00 (*c* 0.5, MeOH); APCI-MS *m/z* 421.27 $[M-H]^-$.

Discussion

As a result, six bile acid derivatives, glycocholic acid (**1**), glycocholic acid methyl ester (**2**), cholic acid (**3**) and glycochenodeoxycholic acid (**4**), glycodeoxycholic acid methyl ester (**5**), cholic acid methyl ester (**6**) were isolated from the ethyl acetate extract of the culture broth by chromatographic technique. Different molecular forms of bile acids can be synthesized in the liver by different species. Primary bile acids are those synthesized by the liver. Secondary bile acids are synthesized by bacteria in the colon. Bile acids have various functions, including eliminating cholesterol from the body, driving the flow of bile to eliminate certain catabolites, emulsifying fat-soluble vitamins to enable their absorption, and aiding in motility and the reduction of the bacteria flora found in the small intestine and biliary tract.[7] *Penicillium* sp. 108YD020 synthesizes cholesterol from mevalonic acid, but no information regarding the biosynthesis of bile acids from cholesterol by this organism has been reported so far. So, this result suggests the new finding that bile acids are also produced by a marine fungus *Penicillium* sp. The production of bile acids in the genus of *Penicillium* isolated from a marine sponge will be an interesting subject for further study. However, more studies are needed to know the bioactivities of **2**, **4**, **5** and **6**.

Acknowledgements

This research was supported in part by the Korea Institute of Ocean Science and Technology (Grant PE99273) and the Ministry of Oceans and Fisheries (Grant PM58670 and PM59120), Korea.

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