Vietnam Journal of Chemistry, International Edition, **55**(2): 207-210, 2017
DOI: 10.15625/2525-2321.2017-00445

Compounds from culture broth of marine bacterium *Micromonospora* sp. (G047)

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Received 16 September 2016; Accepted for publication 11 April 2017

Abstract

Five compounds L-phenyl alanine (**1**), 1-methyl-6-acetyl-α-D-glucopyranoside (**2**), thymine (**3**), uracil (**4**), and (2*R*,3*S*)-butane-2,3-diol (**5**) were isolated and characterized from the culture broth of the marine bacterium *Micromospora* sp. (G047 strain), which was isolated from the sediment collecting from the coast of Co To – Quang Ninh. The structures of all isolates were determined by spectroscopic analysis including MS and 2D NMR, as well as by comparison with reported data in the literature. Compound **2** has been previously synthesized, however, this is the first its report from a natural source.

Keywords. *Micromospora* sp., marine bacterium, 1-methyl-6-acetyl-α-D-glucopyranoside, thymine, uracil, L-phenyl alanine.

1. INTRODUCTION

Actinomycetes are aerobic, spore forming gram-positive bacteria, belonging to the order Actinomycetales characterized with substrate and aerial mycelium growth. They are rich source of secondary metabolites with diverse biological activity [1]. Being a large group of microbial resources of wide practical use and high commercial value, actinomycetes contribute to around 70 % of the source of antibiotics and also produce numerous non-antibiotic bioactive metabolites, such as enzymes, enzyme inhibitors, immunological regulators, anti-oxidation reagents, and so on [2]. Actinomycetes are widely distributed in natural habitats, especially soil and ocean. Among the marine microorganisms actinomycetes comprise an important group. They have tremendous potential to synthesize bioactive secondary metabolites [3].

In this paper, we report the isolation and structural characterization of five compounds (**1-5)** from the fermentation broth of an actinomycete strain G047, which was isolated from the sponge *Ircinia echinata* collected at Co To – Quang Ninh. The EtOAc extract of a G047 fermentation exhibited antimicrobial activity against both gram-positive (*Enteroccocus faecalis* and *Bacillus cereus*) and the fungus strain (*Candida albicans*). The taxonomic identification of the strain G047 was achieved by analysis of 16S rRNA gene sequences. On the basis of morphological and phylogenetic evidence, the actinomycete strain G047 was assigned to the genus *Micromospora*. Strain G047 was identified as *Micromonospora aurantiaca* S97 China with 99 %.

2. EXPRIMENTAL

2.1. General procedures

Optical rotations were determined on a JASCO P-2000 polarimeter (Hachioji, Japan). The ESI-MS was measured on Agilent 6120 series single quadrupole LC/MS systems (USA). NMR spectra were recorded on a Bruker AM500 FT-NMR spectrometer using TMS as an internal standard. Column chromatography (CC) was performed using a silica gel (Kiesel gel 60, 70-230 mesh and 230-400 mesh, Merck, Germany). Thin layer chromatography (TLC) used pre-coated silica gel 60 F_{254} (Merck, Germany).

2.2. Marine actinomycetes material

The samples of sponge *Ircinia echinata* were collected in Co To - Thanh Lan in April 2014, and were identified by Prof. Do Cong Thung. Voucher specimens were deposited at the Institute of Marine Biochemistry and Institute of Marine Environment and Resources of Vietnam Academy of Science and Technology, Hanoi, Viet Nam.

The sponge sample (1 g) was added to 10 mL of sterile sea water in a conical flask. The flask was

agitated for about one hour. The marine sediment was filtered and the filtrate was serially diluted to give 10^{-1} to 10^{-7} dilutions using sterilized sea water. An aliquot of 100 µL of each dilution was spread on the media. Different media like Starch Casein Agar (SCA), Glycerol Asparagine Agar (GA Agar), Humic acid-B vitamin agar (HV Agar) and Glucose yeast malt extract agar (GYM) were used for isolation of actinomycetes. The media containing 50 % of sterile sea water were supplemented with rifampicin (5 µg/mL) and nystatin (25 µg/mL) (Himedia Mumbai) to inhibit bacterial and fungal contamination, respectively. The petriplates were incubated up to 3 weeks at 28 °C. The isolated discrete colonies were observed and used for identification.

2.3. Fermentation, extraction and isolation

An agar grown culture of G047 was inoculated into 1 L of a medium comprising starch, yeast extract, peptone, artificial sea salt and distilled water with a ratio of 10.0 g/4.0 g/2.0 g/30.0 g/1.0 L, respectively. After 7 days of incubation at 28 °C with agitation, the first stage was used to inoculate the production fermentation into 10 L of a culture medium (starch, yeast extract, peptone, $CaCO_3$, $FeSO_4$, KBr, artificialseasalt and distilled water with a ratio of 10.0 g/4.0 g/2.0 g/1.0 g/40 mg/100 mg/30.0 g/1.0 L, respectively). The fermentation was incubated at 28 °C with agitation and harvested on the seventh day.

The fermentation broth (10 L) was extracted with ethyl acetate (5×8 L). The combined ethyl acetate extracts were concentrated under reduced pressure. The ethyl acetate extract (2.4 g) was subjected to column chromatography (CC) on silica gel, eluted with CH_2Cl_2/MeOH gradient to give 6 fractions (F1-F6). Fraction 2 (561 mg) was fractionated by CC on silica gel, eluted with *n*-hexane/dichloromethane gradient to give 5 subfractions (F2.1-F2.5). Subfraction F2.2 (351 mg) was chromatographed by silica gel column, eluted with *n*-hexane/acetone gradient affording compound **5** (18 mg). Fraction 3 (520 mg) was subjected to CC on silica gel, eluted with *n*-hexane/acetone gradient, giving three subfractions (F3.1-F3.3). Subfraction F3.3 (125 mg) was purified by CC on Sephadex LH-20 (MeOH/CH_2Cl_2: 9/1) to furnish compounds **2** (12 mg). Fraction 4 (434 mg) was fractionated again on a silica gel CC (CH_2Cl_2/acetone gradient) yielding 4 subfractions (F4.1-F4.4). Subfraction F4.3 (78 mg) was subjected to a CC on silica gel (CH_2Cl_2/acetone gradient) to give compounds **3** (16 mg). Fraction 5 (347 mg) was chromatographed by a silica gel column (CH_2Cl_2/MeOH gradient) and recrystallized in CH_2Cl_2 to yield compound **4** (14 mg). Fraction 6 (419 mg) was subjected to a CC on silica gel, using CH_2Cl_2/MeOH gradient to give 3 subfractions (F6.1-F6.3). Subfraction F6.2 (81 mg) was purified by CC on Sephadex LH-20 (MeOH/CH_2Cl_2: 9.5/1.5) followed by preparative TLC (CH_2Cl_2/ MeOH: 9/1) to furnish compounds **1** (7 mg).

L-phenyl alanine (1): White powder, mp. 145-146 ^oC; ESI-MS: *m/z* 166 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ_H (ppm) 3.02 (1H, dd, *J* = 9.0, 14.5 Hz, H_a-3), 3.36 (1H, dd, *J* = 4.5, 14.5 Hz, H_b-3), 3.79 (1H, dd, *J* = 4.5, 9.0 Hz, H-2), 7.29-7.38 (5H-Ph); ^{13}C-NMR (125 MHz, CD_3OD): δ_C (ppm) 38.3 (C-3), 57.6 (C-2), 128.4 (C-4'), 129.9 (C-2', C-6'), 130.4 (C-3', C-5'), 137.3 (C-1'), 173.7 (COOH).

1-methyl-6-acetyl-α-D-glucopyranoside (2): White powder; mp. 145-146 ^oC; $[\alpha]^{20}_D$ +157 (*c* 0.1, H_2O), ESI-MS: *m/z* 237 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD): δ_H (ppm) 2.07 (3H, s, $COCH_3$), 3.42 (3H, s, OCH_3), 3.35 (1H, m, H-4), 3.62 (1H, m, H-2), 3.72 (1H, m, H-3), 3.74 (1H, m, H-5), 4.22 (1H, m, H_b-6), 4.37 (1H, dd, *J* = 2.0, 11.5 Hz, H_a-6), 4.67 (1H, d, *J* = 4.0 Hz, H-1); ^{13}C-NMR (125 MHz, CD_3OD): δ_C (ppm) 20.7 (CH_3CO), 55.6 (OCH_3), 64.9 (C-6), 70.9 (C-4), 71.8 (C-5), 73.5 (C-2), 75.0 (C-3), 101.2 (C-1), 172.8 (C=O).

Thymine (3): White powder; mp. 320-323 ^oC; ESI-MS: *m/z* 127 $[M+H]^+$; ^{1}H-NMR (500 MHz, CD_3OD &$CDCl_3$): δ_H (ppm) 1.87 (3H, s, CH_3), 7.15 (1H, s, H-6); ^{13}C-NMR (125 MHz, CD_3OD & $CDCl_3$): δ_C (ppm) 12.2 (CH_3), 110.3 (C-5), 138.6 (C-6), 156.5 (C-2), 168,9 (C-4).

Uracil (4): White powder; mp. 155-156 ^oC; ESI-MS: *m/z* 113 $[M+H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ_H (ppm) 5.45 (1H, d, *J* = 7.5 Hz, H-5), 7.38 (1H, d, *J* = 7.5 Hz, H-6); ^{13}C-NMR (125 MHz, DMSO-d_6)): δ_C (ppm) 100.3 (C-5), 142.3 (C-6), 151.6 (C-2), 164.5 (C-4).

(2*R*,3*S*)-butane-2,3-diol (5): Colorless oil, $[\alpha]_D^{25}$ -13,6 (*c* 0,25, MeOH); ESI-MS: *m/z* 91 $[M+H]^+$; ^{1}H-NMR (500 MHz, DMSO-d_6): δ_H (ppm) 1.01 (3H, d, *J* = 6.0 Hz, CH_3), 3.32 (1H, q, *J* = 6.0 Hz, CH), 2.77 (2H, brs. -OH); ^{13}C-NMR (125 MHz, DMSO-d_6):): δ_C (ppm) 18.5 (CH_3), 71.8 (CH).

3. RESULTS AND DISCUSSION

Compound **1** was isolated as a white powder. In its positive ESI mass spectrum, the pseudo-molecular ion was observed at *m/z* 166 $[M+H]^+$. The ^{1}H-NMR spectrum of **1** indicated the presence of five aromatic protons at δ_H 7.29-7.38, and three protons in the aliphatic region (δ_H 3.02-3.79). The ^{13}C-NMR and DEPT spectra of **1** showed the

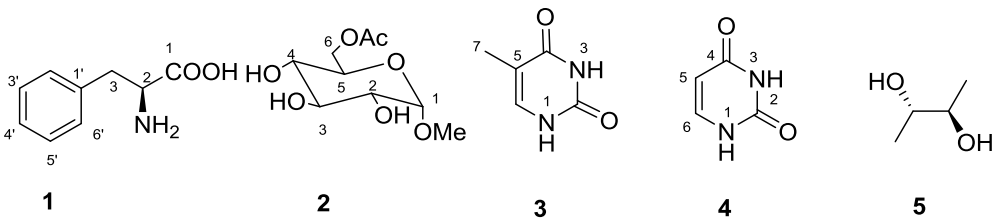
presence of 9 carbon atoms, including one carbonyl groups at δ_C 173.7, one sp^3 methine at δ_C 57.6 (C-2), five aromatic methines at δ_C 128.4 (C-4'), 129.9 (C-2', C-6') and 130.4 (C-3', C-5'), one methylene at δ_C 38.3 (C-3), and one quaternary carbon at δ_C 137.3 (C-1'). The chemical shifts of CH-2 (δ_H 3.85, δ_C 57.5) indicated their bonding to nitrogen atom. Comparison of the ^{1}H-NMR spectrum and TLC of **1** with L-phenyl alanine which was available in our laboratory revealed their similarity. Thus, **1** was determined as L-phenyl alanine [4].

Compound **2** was obtained as white solids. Its ESI-MS showed the protonated molecular ion at *m/z* 237 $[M+H]^+$. The ^{1}H-NMR spectrum of 2 indicated the presence of one methoxy group at δ_H 3.42 (3H, s, OCH_3), one acetyl group at δ_H 2.07 (3H, s, $COCH_3$), one anomer proton at δ_H 4.67 (1H, d, J = 4.0 Hz, H-1), and and seven protons at δ_H 3.35-4.37. In the ^{13}C NMR and DEPT spectra, the presence of carbon signals of a methoxy group at δ_C 55.6 (OCH_3), a acetyl group at [δ_C 20.7 (CH_3), 172.8 (C=O)], a glucosyl moiety from an anomeric carbon at δ_C 101.2 (C-1), a methylene at δ_C 64.9 (C-6), and five methines at δ_C 70.9 (C-4), 71.8 (C-5), 73.5 (C-2), 75.0 (C-3), and 101.2 (C-1) were noted. The coupling constant of the anomeric proton of glucosyl moiety was 4.0 Hz, indicating *α*-configuration. Complete analysis of NMR spectra and comparison of the optical activity confirmed its structure as 1-methyl-6-acetyl-α-D-glucopyranoside [5, 6]. This compound has been previously synthesized [5]. However, this is the first report from a natural source.

Compound **3** was isolated as white solid. Its ESI-MS data showed a proton adduct ion $[M + H]^+$ at *m/z* 127. The ^{1}H-NMR spectrum of **3** showed one olefinic methine at δ_H 7.15 (1H, s, H-6), and a methyl δ_H 1.87 (3H, s, CH_3). The ^{13}C-NMR and DEPT spectra of **3** exhibited the signal of 5 carbons, including one methyl group at δ_C 12.2 (CH_3), two carbonyl at δ_C 156.5 (C-2), 168.9 (C-4), one methine group at δ_C 138.6 (C-6), and one sp^2 quaternary carbons at δ_C 110.3 (C-5). Complete analysis of NMR-spectra and comparison of the NMR data indicated that this compound was thymine [7].

Compound **4** was obtained as white solids, mp 320-323 ^{o}C. Its ESI-MS showed the protonated molecular ion at *m/z* 113 $[M+H]^+$. The ^{1}H-NMR spectrum of **4** showed two olefinic methine at δ_H 5.45 (1H, d, J = 7.5 Hz, H-5) and 7.38 (1H, d, J = 7.5 Hz, H-6). The ^{13}C-NMR and DEPT spectra of **4** exhibited the signal of 4 carbons, including two carbonyl at δ_C 151.6 (C-2), 164.4 (C-4), two methine group at δ_C 100.3 (C-5), 142.2 (C-6). Complete analysis of NMR-spectra and comparison of the NMR data indicated this compound was uracil [8].

Compound **5** was isolated as colorless oil and optically active compound with $[\alpha]_D^{25}$ -13.6 (*c* 0.25, MeOH). Its ESI-MS presented the protonated molecular ion at *m/z* 91 $[M+H]^+$. The ^{13}C-NMR and DEPT spectra of **5** presented the signals of methyl and methine groups at δ_C 18.5 and 71.8, respectively. The ^{1}H-NMR spectrum in $CDCl_3$ of **5** showed the presence of a methyl doublet at δ_H 1.10 (3H, d, J = 6.0 Hz), proton of a methine at δ_H 3.52 (1H, q, J = 6.0 Hz) and an exchangeable proton at δ_H 2.77. This observation indicated that compound **5** has a symmetric structure. The chemical shifts of methine groups suggested their linkage to oxygen. The structure of **5** was then confirmed by analyses of 2D-NMR spectra which allowed establishing it as 2,3-butan-diol. Comparison of optical activity of **5** with that of *meso*-butan-diol revealed their identical configuration [9].



1 **2** **3** **4** **5**

Figure 1: Compounds isolated (**1-5**) from the culture broth of *Micromonospora* sp. (G047)

4. CONCLUSION

From the fermentation broth of an actinomycete strain G047, which was isolated from the sponge *Ircinia echinata* collected at Co To – Quang Ninh, five compounds L-phenyl alanine (**1**), 1-methyl-6-acetyl-α-D-glucopyranoside (**2**), thymine (**3**), uracil (**4**), and (2*R*,3*S*)-butane-2,3-diol (**5**) were isolated and characterized. The structures of all isolates were determined by spectroscopic analysis including MS and 2D NMR. Compound **2** is the reported for the first time from a natural source.

Acknowledgment. *The authors thank the Vietnam Academy of Science and Technology (VAST) for financial support (Project: VAST.TĐ.ĐAB.04/13-15).*

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