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SECONDARY METABOLITES FROM MARINE BACTERIUM *STREPTOMYCES* SP. G039

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Abstract

Nine secondary metabolites, cylo-(Leu-Pro) (**1**), cyclo-(Pro-Tyr) (**2**), indole-3-carboxylic acid (**3**), 3-hydroxyacetylindole (**4**), phenethyl 2-phenyl acetate (**5**), *n*-butyl–isobutyl phthalate (**6**), 4-(2-hydroxyethyl) phenol (**7**), 2-phenylacetic acid (**8**), and uracil (**9**) were isolated and identified from marine *Streptomyces sp.* G039. Their structures were determined by spectroscopic analysis including MS and 2D NMR, as well as by comparison with reported data in the literature.

Keywords. *Streptomyces* sp. G039 strain, marine microorganism, cylo-(Leu-Pro), cyclo-(Pro-Tyr), indole-3-carboxylic acid, 3-hydroxyacetylindole.

1. INTRODUCTION

The studies of the secondary substances produced by marine micro-organisms have obtained many significant achievements in the world [1]. Among the secondary metabolites from marine microorganisms, there are many compounds having interesting biological activities that should be useful to development for their pharmaceutical uses. Meanwhile, the search of bioactive secondary metabolites from marine microorganisms is not widely explored in Vietnam [2, 3]. In the course of our screening program, the EtOAc extract of a *Streptomyces sp.* from marine sediment of Ha Long Bay exhibited an inhibition activity against *Staphylococcus aureus*, *Echerichia coli* and *Fusarium oxysporum*. In this paper, we reported the isolation and structural elucidation of nine secondary metabolites from the cultures broth of *Streptomyces* sp.

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. Actinomycete material

The marine sediment was collected in Ha Long Bay – Quang Ninh in April 2014. The sediment sample (1 g) was added to the 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 obtain 10^{-1} to 10^{-7} dilutions using the 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 upto 3 weeks at 28 °C. The isolated discrete colonies were observed and used for identification.

2.3. Fermentation, extraction and isolation

Strain G039 (*Streptomyces* sp.) was cultured in high-nutrient medium (30 g of instant ocean, 10 g of starch, 4 g of yeast, 2 g of peptone, 1 g of calcium carbonate, 40 mg of iron sulfate, and 100 mg of potassium bromate) for 7 days at 25°C while shaking at 200 rpm. The culture solution (30 L) was extracted with EtOAc (15 L × 3 times). The EtOAc extract solution was concentrated to dryness. The residue (4.03 g) was separated by CC on silica gel, eluted with CH_2Cl_2/MeOH gradient to afford 6 fractions. Fraction 3 (350 mg) was separated by CC on silica gel, eluted with *n*-hexane/acetone (5 to 50% acetone in *n*-hexane) to give compound **6** (59 mg). Fraction 4 (250 mg) was subjected to CC on Sephadex LH-20 using MeOH followed by preparative TLC to afford compound **5** (10 mg). Fraction 5 (230 mg) was purified by a Sephadex LH-20 CC eluting with MeOH to afford 5 subfractions. Subfraction 1 (65 mg) was recrystallized in CH_2Cl_2 to provide compound **1** (15 mg). Fraction 6 (1.2 g) was subjected to a CC on silica gel, using CH_2Cl_2/MeOH gradient to give 6 subfractions. Subfraction 2 (150 mg) was separated on silica gel CC, eluted with 5 % acetone in methanol, providing compound **7** (8 mg). Subfraction 3 (160 mg) was purified by Sephadex LH-20 CC (MeOH), followed by the preparative TLC to give compound **3** (7 mg). Subfraction 5 (45 mg) was separated by silica gel CC, eluted with CH_2Cl_2/acetone gradient affording compound **2** (8 mg). Subfraction 6 (84 mg) was recrystallized in CH_2Cl_2 to yield compound **4** (13 mg).

Cylo-(Leu-Pro) (1): White amorphous solid, m.p 147-148 ^oC, $[\alpha]_D^{25}$ -85 (*c* 0.20, MeOH); ESI-MS: *m/z* 249 $[M+K]^+$. ^1H NMR (500 MHz, CD_3OD): 0.98 (3H, d, *J* = 6.5 Hz, CH_3-13), 0.99 (3H, d, *J* = 6.5 Hz, CH_3-12), 1.54 (1H, m, H-10α), 1.91 (1H, m, H-11), 1.93 (1H, m, H-4α), 1.98 (1H, m, H-4β), 2.07 (1H, m, H-10β), 2.08 (1H, m, H-5α), 2.34 (1H, m, H-5β), 3.55 (2H, m, CH_2-3), 4.14 (1H, m, H-9), 4.28 (1H, t, *J* = 7.5 Hz, H-6). ^{13}C NMR (125 MHz, CD_3OD): 21.2 (C-13), 22.7 (C-4), 23.3 (C-12), 24.7 (C-11), 28.1 (C-5), 38.7 (C-10), 45.5 (C-3), 53.4 (C-9), 59.0 (C-6), 166.2 (C-1), 170.2 (C-7).

Cyclo-(Pro-Tyr) (2): White amorphous solid, m.p 156-158 ^oC, ESI-MS: *m/z* 261 $[M+H]^+$. ^1H NMR (500 MHz, $CDCl_3$): 1.87 (2H, m, CH_2-10), 1.99 (1H, m, H-5b), 2.31 (1H, m, H-5a), 2.79 (1H, dd, *J* = 9.5, 14.5 Hz, H-10b), 3.44 (1H, dd, *J* = 9.5, 14.5 Hz, H-10a), 3.53 (1H, m, H-3b), 3.64 (1H, m, H-3a), 4.06 (1H, dd, *J* = 1.5; 7.5 Hz, H-6), 4.22 (1H, dd, *J* = 2.5, 9.5 Hz), 6.07 (1H, s, NH), 6.77 (2H, d, *J* = 8.5 Hz, H-3'), 7.03 (2H, d, *J* = 8.5 Hz, H-2').

Indole-3-carboxylic acid (3): White powder; mp.231-232^oC; ESI-MS: *m/z*162 $[M+H]^+$; ^1H NMR (500 MHz, CD_3OD): 7.17 (1H, dd, *J* = 1.5, 7.0 Hz, H-5), 7.22 (1H, dd, *J* = 1.5, 7.0 Hz, H-6), 7.45 (1H, dd, *J* = 1.0, 7.0 Hz, H-7), 7.96 (1H, s, H-2), 8.08 (1H, dd, *J* = 1.5, 7.0 Hz, H-4). ^{13}C NMR (125 MHz, CD_3OD): 108.6 (C-3a), 112.9 (C-7), 122.0 (C-4), 122.4 (C-5), 123.6 (C-6), 127.5 (C-3a), 133.4 (C-2),138.2 (C-7a), 169.2 (COOH).

3-Hydroxyacetylindole (**4**)**:** Amorphous solid; ^1H NMR (500 MHz, $CDCl_3$): 8.74 (1H, br. s, NH), 8.28 (1H, m, H-4), 7.92 (1H, d, *J* = 4.0 Hz), H-2), 7.45 (1H, m, H-7), 7.35 (1H, m, H-5), 7.33 (1H, m, H-6), 4.78 (2H, s, CH_2-9). ^{13}C NMR (125 MHz, $CDCl_3$): 65.4 (C-9), 111.6 (C-7), 114.3 (C-3), 122.0 (C-4), 123.2 (C-5), 124.2 (C-6), 125.3 (C-3a), 130.5 (C-2), 136.2 (C-7a), 193.2 (C-8).

Phenethyl 2-phenyl acetate (5): Yellow oil; ESI-MS: *m/z* 241 $[M+H]^+$, ^1H NMR (500 MHz, $CDCl_3$): 2.94 (2H, t, *J* = 7.0 Hz, CH_2-7'), 3.62 (2H, s, CH_2-7), 4.33 (2H, t, *J* = 7.0 Hz, CH_2-8'), 7.16-7.35 (10H, m, 2Ph-H). ^{13}C NMR (125 MHz, $CDCl_3$): 35.1 (C-7'), 41.4 (C-7), 65.3 (C-8'), 126.5-129.3 (Ph-CH), 133.9 (C-1), 137.7 (C-1'), 171.4 (C-8).

***n*-Butyl–isobutyl phthalate** (**6**): ^1H NMR (500 MHz, $CDCl_3$): 0.96 (3H, t, *J* = 7.0 Hz, CH_3-4'), 0.98 (6H, d, *J* = 6.5 Hz, CH_3-3'' and CH_3-4''), 1.44 (2H, sext, *J* = 7.0 Hz, CH_2-3'), 1.72 (2H, quint, *J* = 7.0 Hz, CH_2-2'), 2.04 (1H, m, *J* = 6.5 Hz, H-2''), 4.09 (2H, d, *J* = 7.0 Hz, CH_2-1''), 4.30 (2H, t, *J* = 7.0 Hz, CH_2-1'), 7.52 (2H, m, H-4 and H-5), 7.72 (2H, m, H-3 and H-6). ^{13}C NMR (125 MHz, $CDCl_3$): 13.7 (C-4'), 19.2 (C-3'' and C-4''), 27.7 (C-2''), 30.6 (C-2'), 65.6 (C-1'), 71.8 (C-1''), 128.8 (C-4 and C-5), 130.9 (C-3 and C-6), 132.3 (C-1), 132.4 (C-2), 167.6 (C-7), 167.7 (C-8).

4-(2-Hydroxyethyl) phenol (7): White powder, m.p. 91-92 ^oC, ESI-MS: *m/z* 139 $[M+H]^+$; ^1H NMR (500 MHz, $CDCl_3$): 2.73 (2H, t, *J* = 7 Hz, H-7), 3.71 (2H, t, *J* = 7.0 Hz, H-8), 6,72 (2H, d, *J* = 8.5 Hz; H-2+ H-6), 7.05 (2H, d, *J* = 8.5 Hz, H-3 and H-5).

2-Phenylacetic acid (8): white needle crystal, m.p. 76-77 ^oC; ESI-MS: *m/z* 138 $[M+H]^+$; ^1H NMR (500 MHz, $CDCl_3$): 3.59 (2H, s, CH_2), 7.25-7.33 (5H, m, Ph-H). ^{13}C NMR (125 MHz, $CDCl_3$): 41.4 (CH_2), 127.2 (C-4), 128.6 (C-2 and C-6), 129.4 (C-3 and C-5), 123.6 (C-6), 133.8 (C-1), 175.2 (COOH).

Uracil (**9**)**:** White powder, mp. 320-321 ^oC; ESI-MS: *m/z* 111 $[M+H]^+$; ^1H NMR (500 MHz, DMSO-d_6): 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): 100.3 (C-5), 142.3 (C-6), 151.62 (C-2), 164.5 (C-4).

3. RESULTS AND DISCUSSION

Compound **1** was isolated as white amorphous

solid and optically active, $[\alpha]_D^{25}$ -85 (*c* 0.20, MeOH). The ESI-MS indicated the pseudomolecular ion peak at *m/z* 249 $[M+K]^+$. The ^{1}H NMR spectrum of **1** displayed signals of 2 methyl groups as doublet of doublet at δ_H 0.98 (d, *J* = 6.5 Hz, CH_3-13), 0.99 (d, *J* = 6.5 Hz, CH_3-12) and signals of ten aliphatic protons ranging from 1.54 to 4.28 ppm. Analysis of the ^{13}C NMR and DEPT spectra of **1** revealed the presence of 11 carbons, including two methyl groups at δ_C 21.2 (C-13) and 23.3 (C-12), three methines at δ_C 24.7 (C-11), 53.4 (C-9) and 59.0 (C-6), four methylenes at δ_C 22.7 (C-4), 28.1 (C-5), 38.7 (C-10), 45.5 (C-3), and two carbonyl at δ_C 166.2 (C-1) and 170.2 (C-7). The chemical shifts of CH_2-3, CH-6 and CH-9 suggested their linkage to nitrogen atoms. This data suggested the presence of two amino acid units, proline and leucine in the structure of **1**. Detailed analysis of NMR spectra and comparison with reported values in the literature [4, 5], the structure of **1** was determined to be cylo-(Leu-Pro). This cylodipeptide was previously described from *Michelia champaca* and *Ulva pertusa* [4, 5].

Compound **2** was isolated as a white amorphous solid. The ESI-MS indicated the pseudo-molecular ion peak at *m/z* 261 $[M+H]^+$. The 1D NMR spectrum of **2** displayed signals of the proline unit as **1**. However, in comparison with **1**, the presence of an A_2B_2 aromatic system [δ_H 6.77 (2H, d, *J* = 8.5 Hz, H-3') and 7.03 (2H, d, *J* = 8.5 Hz, H-2')] instead of signals of the 2-propyl group was noted for **2**. This observation strongly suggested that the leucine unit of **1** was replaced by the 4-hydroxyphenylanaline moiety in **2**. Comparison with the literature [5], compound **2** was identified as cyclo-(Pro-Tyr). This cyclodipeptide was previously isolated from *Streptomyces* sp. and *Colletotrichum gloeosporioides* [6, 7]. It was found that this compound had antibacterial activity against both Gram-positive and Gram-negative bacteria and antifungal property [7].

Compound **3** was obtained as white powder. The ESI-MS indicated the pseudo-molecular ion peak at *m/z* 162 $[M+H]^+$. Its ^{1}H-NMR spectrum of **3** showed signals of 5 aromatic protons at δ_H 7.17 (dd, *J* = 1.5, 7.0 Hz, H-5), 7.22 (dd, *J* = 1.5, 7.0 Hz, H-6), 7.45 (dd, *J* = 1.0, 7.0 Hz, H-7), 7.96 (s, H-2) and 8.08 (dd, *J* = 1.5, 7.0 Hz, H-4). In the ^{13}C-NMR and DEPT spectra of **3**, signals of nine carbons were noted, including five aromatic methine groups, a carboxylic group (δ_C 169.2, C-8) and three sp^2 quaternary carbons at δ_C 108.6 (C-3), 127.5 (C-3a) and 138.2 (C-7a). Thus, the presence of an 1,2-disubstituted benzene ring was defined. Carefully analysis of the NMR spectra and comparison with reported data [8], compound **3** was identified as indole-3-carboxylic acid which was reported to have antibacterial activity [8].

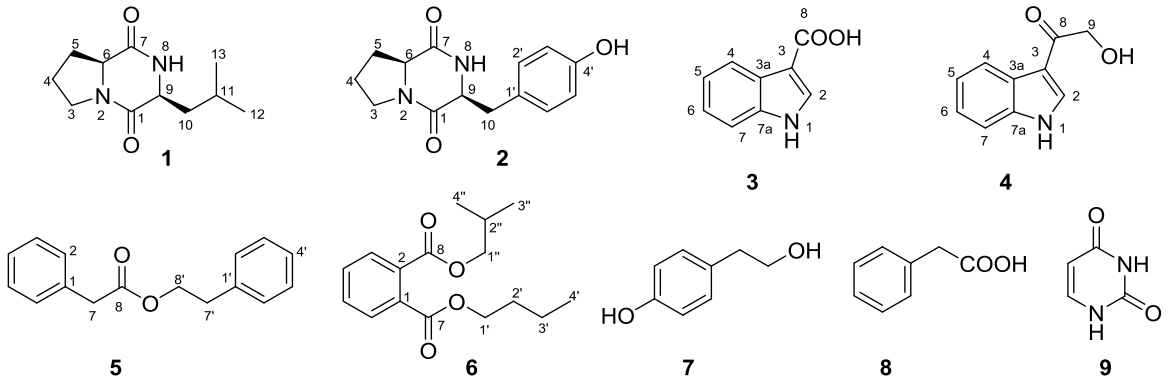

Figure 1: Structures of **1-9** from *Streptomyces* sp. G039

Compound **4** was isolated as white solids. The 1D NMR spectra of **4** displayed the signals close to those of **3** in which four aromatic protons of 1,2-disubstituted benzene ring [δ_H 8.28 (m, H-4), 7.45 (m, H-7), 7.33 (m, H-5) and 7.35 (m, H-6)] and a proton at δ_H 7.92 (*J* = 4.0 Hz, H-2) were observed. Comparison of ^{13}C NMR and DEPT spectra of **3** and **4** indicated that the carboxylic carbon signal of **3** was replaced by signals of a ketone at δ_C 193.2 and an oxymethylene at δ_C 65.4. Analysis of 2D NMR spectra, especially HMBC spectrum established the structure of **4** as shown in figure 1. This compound was reported from several marine bacteria and found to be active against parasite *Trypanosoma cruzi* [9].

Compound **5** was obtained as yellow oil. The ESI-MS indicated the pseudo-molecular ion peak at *m/z* 241 $[M+H]^+$. The ^{1}H NMR spectrum of **5** displayed signals for 10 aromatic protons at δ_H 7.16-7.35 and three methylenes at δ_H 2.94 (t, *J* = 7.0 Hz, CH_2-7'), 3.62 (s, CH_2-7), 4.33 (t, *J* = 7.0 Hz, CH_2-8'). Analysis of the ^{13}C NMR and DEPT spectra revealed the presence of the groups observed in the

^{1}H-NMR spectrum (two phenyl rings and three methylenes), with additional signals of a carboxylate carbon at δ_C 171.4 (C-8) and two aromatic quaternary carbons at δ_C 137.7 (C-1') and 133.9 (C-1). Analysis of the HMBC spectrum of **5** allowed determining the structure of **5** as phenethyl-2-phenyl acetate [10].

ESI-MS spectrum of **6** presented the protonated molecular ion at *m/z* 279 $[M+H]^+$. The ^{1}H-NMR spectrum showed the signals of four aromatic protons and eighteen protons in the aliphatic region. Its ^{13}C NMR and DEPT spectra indicated signals of two carboxylate carbons at δ_C 167.6 and 167.7 (C-7 and C-8), six aromatic carbons, a sp^3 methine, four methyenes and three methyls. The chemical shifts of the two methylenes at δ_C 65.6 and 71.8 suggested their linkage to oxygens. The NMR data of **6** were consistent with those of *n*-butyl–isobutyl phthalate [11, 12].

By analysis of the NMR spectra, compounds **7**, **8** and **9** were determined to be uracil [3, 13], 4-(2-hydroxyethyl) phenol [14] and 2-phenylacetic acid [15], respectively. Their NMR data were identical with those reported in the literature.

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