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SECONDARY METABOLITES FROM *MICROMONOSPORA ECTRINOSPORA* G017

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Abstract

Eight compounds, cyclo-(Pro-Tryp) (**1**), *N*-[2-(1H-indol-3-yl)-2-oxo-ethyl] acetamide (**2**), cyclo-(Pro-Tyr) (**3**), cyclo-(Pro-Phe) (**4**), cyclo-*trans*-4-OH-(Pro-Phe) (**5**), cyclo-(Pro-Leu) (**6**), cyclo-(Pro-Val) (**7**), and uracil (**8**) were isolated from the culture broth of the marine *Micromonospora ectrinospora* G017 strain. The structures of the isolated compounds were established on the basis of their spectral data, including mass spectrometry and NMR.

Keywords. *Micromonospora ectrinospora* G017 strain, cyclo-(Pro-Tryp), N-[2-(1H-Indol-3-yl)-2-oxo-ethyl] acetamide, cyclo-(Pro-Tyr), cyclo-(Pro-Phe), cyclo-*trans*-4-OH-(Pro-Phe), cyclo-(Pro-Leu), cyclo-(Pro-Val), uracil.

1. INTRODUCTION

Marine bacteria are considered to play a central role as symbionts of most marine invertebrates and also represent one of the most novel biomedicial resources remaining to be explored [1].

In search of bioactive metabolite from marine bacteria, we examined the extract of the culture broth of the marine *Micromonospora ectrinospora* G017 strain. During our screening program, the EtOAc extract of this strain exhibited an inhibition against *Mycobacterium tuberculosis*. Herein, we described the isolation and structural determination of eight compounds (**1-8**) from the EtOAc extract of the culture broth of *M. ectrinospora* G017.

2. EXPERIMENTAL

2.1. General Experiment procedures

^{1}H, ^{13}C-NMR and 2D NMR spectra were recorded on a Brucker 400 and 600 MHz spectrometer with TMS as internal standard. High resolution ESI mass spectra were measured an a FT-ICR MS VARIAN 910 spectrometer. Thin-layer chromatography (TLC) used TLC silica gel Merck 60 F_{254}. Column chromatography (CC) were carried out on silica gel 40-63 μm. For preparative HPLC purifications using a C_{18} (21.2x150 mm) column, for analytic HPLC using a C_6 phenyl 4.6 column.

2.2. Bacteria isolation and fermentation

The marine sediment was collected in Ha Long Bay – Quang Ninh on July 2012. 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 up to 3 weeks at 28 °C. The isolated discrete colonies were observed and used for identification. The fermentation was carried out in 3 L flask using a modification of the published method [2].

2.3. Extraction and Isolation

Culture broth (10 L) of *M. ectrinospora* G017 strain was filtered to remove the bacteria, and then extracted with ethyl acetate (7 L × 5 times). The extract solution was concentrated under diminished pressure to give 1.7 g. This residue was applied to a silica gel column chromatography (CC), eluted with CH_2Cl_2/MeOH mixture (from 0 to 100 % of MeOH in CH_2Cl_2) to give 16 fractions. Fraction 8 (82 mg) was purified by preparative HPLC using acetonitrile/water mixture (10-30 % ACN, 5 % formic acid in ACN) to yield 1.0 mg of **1** (t_R 32 min), 3.3 mg of **4** (t_R 24.4 min), 4.5 mg of **6** (t_R 22.3 min) and 10 mg of **7** (t_R 13.7 min). Fraction 13 (59 mg) was separated by preparative HPLC using acetonitrile/water mixture (5-40 % ACN, 5 % formic acid in ACN) to obtained 1.5 mg of **2** (t_R 24.5 min), 11 mg of **3** (t_R 17.6 min) and 4.5 mg of **5** (t_R 20.5 min). Fraction 15 (34 mg) was purified by preparative TLC (CH_2Cl_2/MeOH: 9.5/0.5) to afford **8** (6 mg).

Cyclo-(Pro-Tryp) (1): White solid; HRESI-MS: *m/z* 284.1409 $[M+H]^+$ (Calcd. 284.1399 for $C_{16}H_{18}N_3O_2$); ^{1}H-NMR (600 MHz, CD_3OD): δ_H (ppm) 0.95 (1H, m, H_a-5), 1.49 (1H, m, H_a-4), 1.71 (1H, m, H_b-4), 1.97 (1H, m, H_b-5); 3.29 (1H, m, H_a-3), 3.50 (1H, m, H_b-3), 3.45 (2H, m, H-10), 4.01 (1H, m, H-6), 4.42 (1H, m, H-9), 7.10 (1H, s, H-2'), 7.02-7.58 (4H, H-aromatic); ^{13}C-NMR (150 MHz, CD_3OD): δ_C (ppm) 22.5 (C-4), 29.1 (C-10), 29.2 (C-5), 45.9 (C-3), 57.2 (C-9), 60.7 (C-6), 109.5-138.0 (C-aromatic), 125.6 (C-2'), 167.4 (C=O); 170.3 (C=O).

***N*-[2-(1H-Indol-3-yl)-2-oxo-ethyl] acetamide (2):** White solid; HRESI-MS: *m/z* 217.0976 $[M+H]^+$ (Calcd. 217.0977 for $C_{12}H_{13}N_2O_2$); ^{1}H-NMR (600 MHz, CD_3OD): δ_H (ppm) 2.08 (3H, s, CO<u>CH</u>$_3$), 4.60 (2H, s, -CH_2-), 7.25-8.22 (4H, m, H-aromatic), 8.24 (1H, s, H-2); ^{13}C-NMR (150 MHz, CD_3OD): δ_C (ppm) 22.5 (CO<u>C</u>H$_3$), 44.8 (-CO<u>CH</u>$_2$N-), 112.9-138.3 (C-aromatic, C-2), 173.7 (C=O), 191.9 (C=O).

Cyclo-(Pro-Tyr) (3): White solid; HRESI-MS: *m/z* 261.1224 $[M+H]^+$ (Calcd. 261.1239 for $C_{16}H_{17}N_2O_3$); ^{1}H-NMR (400 MHz, CD_3OD): δ_H (ppm) 1.25 (1H, m, H_a-4), 1.81 (2H, m, H_b-4, H_a-5), 2.13 (1H, m, H_b-5), 3.07 (2H, m, H-10), 3.40 (1H, m, H_a-3), 3.57 (1H, m, H_b-3), 4.06 (1H, m, H-6), 4.38 (1H, m, H-9), 6.73 (2H, d, *J* = 8.5 Hz, H-2', 6'), 7.05 (2H, d, *J* = 8.5 Hz, H-3', 5'); ^{13}C-NMR (100 MHz, CD_3OD): δ_C (ppm) 21.3 (C-4), 28.0 (C-5), 36.3 (C-10), 44.5 (C-3), 56.5 (C-9), 58.7 (C-6), 126.4 (C-1'), 130.7 (C-2', 6'), 114.8 (C-3', 5'), 156.3 (C-4'), 165.6 (C=O), 169.4 (C=O).

Cyclo-(Pro-Phe) (4): White solid; HRESI-MS: *m/z* 245.1316 $[M+H]^+$ (Calcd. 245.1290 for $C_{14}H_{17}N_2O_2$); ^{1}H-NMR (400 MHz, CD_3OD): δ_H (ppm) 1.60 (2H, m, H_a-4, H_a-5), 1.91-2.07 (3H, m, H_b-4, H_b- 5, H_a-10), 2.64 (1H, m, H_b-10), 3.02 (1H, dd, *J* = 4.8, 13.6 Hz, H_a-3), 3.22 (1H, dd, *J* = 4.4, 13.6 Hz, H_b-3), 3.59 (1H, m, H-6), 4.22 (1H, t, *J* = 4.8 Hz, H-9), 7.20-7.33 (5H, aromatic); ^{13}C-NMR (100 MHz, CD_3OD): δ_C (ppm) 21.1 (C-4), 28.4 (C-5), 39.6 (C-10), 44.7 (C-3), 57.7 (C-9), 58.4 (C-6), 127.1-129.9 (CH- aromatic), 135.3 (C-1'), 166.0 (C=O), 170.0 (C=O).

Cyclo-*trans*-4-OH-(Pro-Phe) (5): White solid; HRESI-MS: *m/z* 261.1231 $[M+H]^+$ (Calcd. 261.1239 for $C_{14}H_{17}N_2O_3$); ^{1}H-NMR (400 MHz, CD_3OD): δ_H (ppm) 1.40 (1H, ddd, *J* = 4.8, 12.0, 12.8 Hz, H_a-5), 2.10 (1H, dd, *J* = 4.8, 13.2 Hz, H_b-5), 3.20 (2H, m, H-10), 3.35 (1H, m, H_a-3), 3.74 (1H, dd, *J* = 4.8, 12.8 Hz, H_b-3), 4.30 (1H, t, *J* = 4.8 Hz, H-4), 4.39 (1H, m, H-6), 4.50 (1H, m, H-9), 7.28 (5H, H-aromatic); ^{13}C-NMR (100 MHz, CD_3OD): δ_C (ppm) 36.6 (C-10), 37.5 (C-5), 53.8 (C-3), 56.2 (C-9), 56.9 (C-6), 67.1 (C-4), 126.7 (C-4'), 128.1 (C-3', 5'), 129.6 (C-2', 6'), 136.0 (C-1'), 165.7 (C=O), 169.9 (C=O).

Cyclo-(Pro-Leu) (6): White solid; HRESI-MS: *m/z* 211.1432 $[M+H]^+$ (Calcd. 211.1447 for $C_{11}H_{19}N_2O_2$); ^{1}H-NMR (400 MHz, CD_3OD): δ_H (ppm) 0.97 (3H, d, *J* = 6.4 Hz, CH_3-12), 0.98 (3H, d, *J* = 6.4 Hz, CH_3-13), 1.55 (1H, m, H-11), 1.90-2.05 (5H, m, CH_2-4, 5, H-10), 2.31 (1H, m, H-10), 3.52 (2H, m, H-3), 4.15 (1H, m, H-6), 4.30 (1H, m, H-9); ^{13}C-NMR (100 MHz, CD_3OD): δ_C (ppm) 20.8 (C-12), 21.9 (C-13), 22.2 (C-5), 24.3 (C-11), 27.7 (C-4), 38.0 (C-10), 45.0 (C-3), 53.2 (C-9), 58,9 (C-6), 167.5 (C=O), 171.4 (C=O).

Cyclo-(Pro-Val) (7): White solid; HRESI-MS: *m/z* 197.1262 $[M+H]^+$ (Calcd. 197.1290 for $C_{10}H_{17}N_2O_2$); ^{1}H-NMR (400 MHz, CD_3OD): δ_H (ppm) 0.95 (3H, d, *J* = 6.8 Hz, CH_3-11), 1.11 (3H, d, *J* = 6.8 Hz, CH_3-12), 1.94-2.05 (3H, m, CH_2-4, H_a-5), 2.33 (1H, m, H_b-5), 2.50 (1H, m, H-10), 3.54 (2H, m, H-3), 4.05 (1H, m, H-6), 4.22 (1H, m, H-9); ^{13}C-NMR (100 MHz, CD_3OD): δ_C (ppm) 15.2 (C-11), 17.4 (C-12), 21.8 (C-4), 28.1 (C-5), 28.5 (C-10), 44.8 (C-3), 58.6 (C-9), 60.1 (C-6), 166.2 (C=O), 171.2 (C=O).

Uracil (8): White solid; HRESI-MS: *m/z* 135.0170 $[M+Na]^+$ (Calcd. 135.0170 for $C_4H_4N_2NaO_2$); ^{1}H-NMR (400 MHz, CD_3OD): δ_H (ppm) 5.61 (1H, d, *J* = 7.6 Hz, H-5), 7.39 (1H, d, *J* = 7.6 Hz, H-6); ^{13}C-NMR (100 MHz, CD_3OD): δ_C (ppm) 100.3 (C-5), 142.1 (C-6), 152.1 (C-1), 165.9 (C-4).

3. RESULTS AND DISCUSSION

Compound **1** was isolated as a white solid. In its positive HRESI mass spectrum, the pseudo-molecular ion was observed at *m/z* 284.1409 $[M+H]^+$, suggesting a molecular formula of $C_{16}H_{19}N_3O_2$. The ^{1}H-NMR spectrum of **1** indicated the presence of five aromatic protons at δ_H 7.01-7.58 and ten protons in the aliphatic region. Analyses of the ^{13}C-NMR and DEPT spectra with the aid of the HSQC of **1** indicated the presence of 16 carbons, including four sp^3 methylenes, two sp^3 methines, eight aromatic carbons (five methines and three quaternary carbons) and two carbonyl carbons at δ_C 167.4 and 170.3. Analysis of COSY spectrum revealed the presence of three spin – spin coupling systems: CH_2-3/CH_2-4/CH_2-5/CH-6; CH-9/CH_2-10 and H-4'/H-5'/H-6'/H-7'. Detailed analysis of 2D NMR spectra, especially HMBC spectrum allowed determining the structure of **1** as cyclo-(Pro-Tryp). This cyclodipeptide was previously described [3, 4].

Compound **2** was obtained as a white amorphous solid. The HRESI mass spectrum of **2** presented a base peak at *m/z* 217.0976 $[M+H]^+$, consistent with the molecular formula $C_{12}H_{12}N_2O_2$. In the ^{1}H-NMR spectrum, the signals of five protons at the aromatic region of **2** was close to those of **1**. However, at the aliphatic region in the ^{1}H NMR of **2**, only signals of an acetyl at δ_H 2.08 and a methylene at δ_H 4.60 were observed. The ^{13}C-NMR and DEPT spectra of **2** showed signals of the groups observed above with additional signals of a ketone (δ_C 191.9), a carboxyl group (δ_C 173.7) and three quaternary aromatic carbons. The chemical shifts of the methylene (δ_C 44.8, δ_H 4.60) suggested its connection to nitrogen. Analysis of the HMBC spectrum revealed the presence of indole moiety as in the structure of **1**. Finally, the structure of **2** was established by cross-peaks of the methylene protons at δ_H 4.60 to the carbons at δ_C 191.9 (ketone), 173.7 (acetyl) and C-3 of the indole ring (δ_C 115.8). Comparison of the NMR data with those reported in the literature determined the structure of **2** as *N*-[2-(1H-indol-3-yl)-2-oxo-ethyl] acetamide [5-7].

Compound **3** was isolated as a white solid. In its positive HRESI mass spectrum, the pseudo-molecular ion was observed at *m/z* 261.1224 $[M+H]^+$, suggesting a molecular formula $C_{14}H_{16}N_2O_3$. In the ^{1}H-NMR spectrum, the signals at the aliphatic region of **3** was close to those of **1**. The differences between **3** and **1** were signals at aromatic region: the signals of the five aromatic protons in **1** were replaced by those of an A_2B_2 system [δ_H 6.73 (2H, d, *J* = 8.5 Hz, H-2', 6'), 7.05 (2H, d, *J* = 8.5 Hz, H-3', 5')] in **3**. Detailed analysis of the ^{13}C-NMR and DEPT spectra with the aid of the 2D NMR spectra indicated the structure of **3** which was a cyclodipeptide, forming from a proline and a 4-hydroxy-phenylalanine units. The NMR data of **3** were in agreement with those reported in the literature for cyclo-(Pro-Tyr) [8].

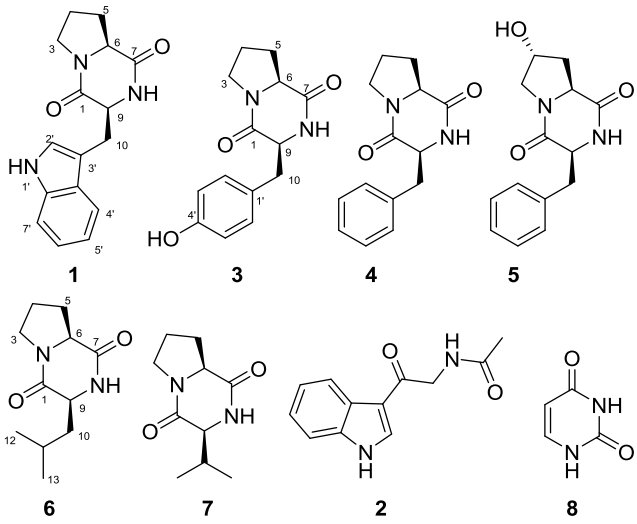

Figure 1: Secondary metabolites from the marine bacterium *M. ectrinospora* G017

Compound **4** was isolated as a white solid. In its positive HRESI mass spectrum, the pseudo-molecular ion was observed at *m/z* 245.1316 $[M+H]^+$, consistent with the molecular formula $C_{14}H_{16}N_2O_2$. The 1D-NMR spectra (^{1}H and ^{13}C) of compound **4** were close to those of **3**, except for the presence of a phenyl ring instead of the A_2B_2 system. This data strongly suggested that the 4-hydroxy-phenylalanine fragment in **3** was replaced by a phenylalanine moiety in **4**. Comparison of NMR data revealed the structure of **4** which was identical to cyclo-(Pro-Phe) [9].

1D-NMR spectra of compound **5** were similar to those of **4**, except for the presence of an oxymethine group at δ_H 4.30 (1H, t, *J* = 4.8 Hz, H-4) and δ_C 67.1 (C-4) instead of a methylene group of **4**. This observation suggested that **5** should be a hydroxylated derivative of **4**. Analyses of the 2D-NMR spectra indicated **5** being cyclo-*trans*-4-OH-(Pro-Phe) [10].

Compound **6** was isolated as a white amorphous solid. The HRESI mass spectrum of **6** presented a base peak at *m/z* 211.1432 $[M+H]^+$ suggesting a molecular formula $C_{11}H_{18}N_2O_2$. Considering the molecular formula and the NMR data, the structure of compound **6** could be a cyclodipeptide. Analyses of the 2D-NMR spectra demonstrated the presence of two amino acid moieties: proline and leucine.

Thus, compound **5** was established to be cyclo-(Pro-Leu) [10, 11].

HRESI mass spectrum of **7** presented a base peak at *m/z* 197.1262 $[M+H]^+$ suggesting a molecular formula $C_{10}H_{16}N_2O_2$. The 1D NMR (^1H, ^{13}C NMR) spectra of compound **7** revealed structural similarity to **6**, except for the absence of a methylene group in the molecule of **7**. Complete analysis of the NMR spectra and comparison with reported NMR data indicated that compound **7** was cyclo-(Pro-Val) [10, 11].

Compound **8** was obtained as a white amorphous solid and determined to be uracil. Its NMR data were consistent with those reported in the literature [11].

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