Metabolites from an Antarctic Sponge-Associated Bacterium, *Pseudomonas aeruginosa*

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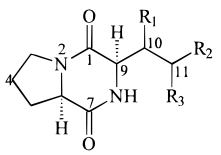
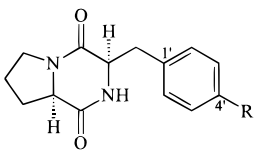
In an ongoing survey of the bioactive potential of microorganisms associated with marine invertebrates, the culture media of a sponge-associated bacterial strain of *Pseudomonas aeruginosa* was found to contain metabolites which inhibit the growth of several Gram-positive microorganisms. A series of diketopiperazines (**1**−**6**) including a new natural product (**6**) and two known phenazine alkaloid antibiotics (**7** and **8**) were isolated from the culture broth of this bacterium.

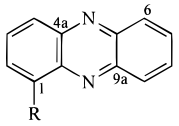
There is a great deal of interest in marine microorganisms as a new source of bioactive substances, and a number of novel compounds with potent biological activity have been discovered recently through cultivation of marine bacteria.[3] Microorganisms associated with marine invertebrates are considered to be of particular importance since metabolites previously thought to arise from the invertebrates may be biosynthesized by their endobionts.[4−7] *Isodictya setifera* Topsent (family Esperiopsidae) is a bioactive antarctic sponge[8] from which we have isolated microorganisms for evaluation of their bioactivity. One of the associated bacteria, a strain of *Pseudomonas aeruginosa* (Pseudomonadaceae), exhibited strong antibacterial activity.[9] Fractionation of the culture broth has uncovered a new diketopiperazine (DKP), *cyclo*-(L-proline-L-methionine) (**6**), accompanied by five known DKP's (**1**−**5**) and two known phenazine alkaloids (**7** and **8**). Although the chemistry of the sponge genus *Isodictya* is currently unknown, the nature of the bioactivity is currently being investigated in our laboratory. In this paper, we describe the isolation and characterization of the bioactive *P. aeruginosa* compounds.





(**1**) R_1=CH_3; R_2=R_3=H: *cyclo*-(L-Pro-L-Val)
(**2**) R_1=H; R_2=R_3=CH_3: *cyclo*-(L-Pro-L-Leu)
(**3**) R_1= R_2= CH_3; R_3=H: *cyclo*-(L-Pro-L-Ile)
(**6**) R_1= R_2= H; R_3=SCH_3: *cyclo*-(L-Pro-L-Met)

(**4**) R = H: *cyclo*-(L-Pro-L-Phe)
(**5**) R = OH: *cyclo*-(L-Pro-L-Tyr)



(**7**) R = -CO_2H
(**8**) R = -$CONH_2$

Antimicrobial activity against *Bacillus subtilis, Staphylococcus aureus, and Micrococcus luteus* was observed in culture broth from *I. setifera*-derived *P. aeruginosa* grown in LB media.[9] The culture filtrate was adsorbed onto Amberlite XAD-2 and eluted with water containing increasing concentrations of MeOH, and fractions were assayed for antimicrobial activity. The 80% MeOH fraction contained most of the activity and was further fractionated by silica gel column chromatography and reversed-phase HPLC to give DKP's (**1**−**6**) and bioactive phenazine alkaloids (**7** and **8**).

The presence of the DKP ring system in compounds **1**−**6** was evident from the characteristic ^{13}C NMR chemical shifts of two CONH groups (δ_C 165−170) and ^{1}H NMR shifts of the two α-methine residues (δ_H 3.9−4.3); proline was found in all isolated *P. aeruginosa* DKP's on the basis of the presence of three broad 2H multiplets (δ_H 1.8−3.7). From ^{1}H and ^{13}C NMR chemical shifts and ^{1}H−^{1}H COSY correlations, valine, leucine, isoleucine, phenylalanine, and tyrosine were identified as the second amino acid residue in compounds **1**−**5**, respectively. The ^{1}H NMR data of DKP's **1** and **2**[10] and ^{1}H and ^{13}C NMR data of **3** and **4**[11] were in agreement with those found in the literature. NMR data for the tyrosine-containing compound **5** is not available in the literature, but the optical rotation was in agreement with the published value.[12]

In addition to the chemical shifts of the proline moiety, the ^{1}H NMR and COSY spectra of compound **6** displayed a spin system consisting of a two proton triplet at δ_H 2.69, a methyl singlet (δ_H 2.14), and two one-proton multiplets (δ_H 2.05 and 2.38), corresponding to a −$CH_2CH_2SCH_3$ moiety of methionine. The molecular formula of $C_{10}H_{16}O_2N_2S$ was determined by HRFABMS, securing the identity of **6** as *cyclo*-(prolinemethionine), which has not been reported from nature.

Verification of the structure of **6** was achieved by synthesis. The linear dipeptide *t*-BOC-L-proline-L-methionine methyl ester was prepared by DCC-mediated coupling of the appropriately protected amino acids. Cyclization of the dipeptide by treatment with formic acid,[13] followed by heating in butanol and toluene, resulted in a product whose physical and spectroscopic properties matched those of the natural product.

The absolute stereochemistry of the DKP's was determined by analysis of DKP hydrosylates on chiral Macherey-Nagel TLC plates.[14,15] The separations were achieved in MeOH, H_2O, and CH_3CN mixtures (see Experimental Section). Results indicated that all resulting amino acids were of the L-configuration.

Many terrestrial yeast, lichens, and fungi produce diketopiperazines in culture.[16] Their presence in a few

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marine bacterial culture broths has also been reported. For example, *cyclo*-(L-Pro-L-Val), *cyclo*-(L-Pro-L-Leu) and *cyclo*-(L-Pro-L-Ala) have been isolated from symbiotic marine bacterium *Micrococcus* sp.,[4] and *cyclo*-(L-Pro-L-Ile), *cyclo*-(L-Pro-L-Tyr), *cyclo*-(L-Pro-L-Val), and *cyclo*-(L-Pro-L-Leu) were among three other DKP's detected in culture media of *Vibrio parahaemolyticus* isolated from the toxic mucus of the box fish *Ostracion cubicus*.[17] Cyclic dipeptides, however, occur in numerous marine sponges, e.g., *Dysidea herbacea*,[18] *Tedania ignis*,[10] *Geodia baretti*,[19] *Leucophloeus fenestrata*,[20] *Jaspidae* sp.,[21] *Dysidea fragilis*,[22] and *Calyx* cf. *podatypa*.[11] *Cyclo*-(L-Pro-Gly) has been reported from the sea star *Luidia clatharata*.[23] In our assays, compounds **1**−**6** were inactive as antibiotics or cytotoxins.

In addition to the DKP's, two yellow phenazine alkaloids, **7** and **8**, contained in the XAD 80% MeOH fraction, were purified by repeated silica gel column chromatography. Structural assignment of the compounds was achieved by interpretation of MS and NMR data, including two-dimensional COSY, HMQC, and HMBC and comparison of their physiochemical characteristics to those found for **7** and **8** in the literature.[24,25] Acid hydrolysis converted **8** to **7**, confirming the presence of an amide group in the former. All compounds of the phenazine class exhibit a variety of antibacterial properties especially against plant pathogenic Gram-positive bacteria and fungi.[26,27] Antibacterial tests with two phenazines isolated in this study showed these pigments to be active against *Bacillus cereus* (MIC by disk assay < 0.5 μg/mL) with carboxylate **7** being more potent than carboxamide **8**. The compounds were less active against *M. luteus* and *S. aureus* (MIC > 5 μg/mL).

We investigated the sponge, *I. setifera*, from which the bacterium was isolated, for the presence of phenazines and cyclic dipeptides. Neither type of metabolite was evident in extracts of the sponge. This result suggests that either the bacterium was not producing these secondary metabolites in the sponge or that a significant volume of microbe in the sponge may be required to detect the metabolites of this associated bacterium.

Experimental Section

General Experimental Procedures. All solvents were distilled from glass. NMR spectral analyses were performed on an 8.46-T NMR instrument operating at 360 MHz for ^{1}H and 90 MHz for ^{13}C. One-bond heteronuclear ^{1}H−^{13}C connectivities were determined by HMQC; two- and three-bond ^{1}H−^{13}C connectivities were determined by HMBC optimized for 7 Hz couplings; chemical shifts are reported in ppm with the chemical shift of residual solvent nuclides used as internal standards. HRFABMS were measured on a Finnigan MAT 95Q spectrophotometer at the University of Florida and EIMS on Finnigan GC−MS 3000 at the San Diego State University. Optical rotations were determined on a JASCO DIP-360 digital polarimeter. Silica gel 60 (230−400 mesh) and Amberlite XAD-2 were used for column chromatography. A Waters 401 HPLC system with a Waters 490E UV detector and YMC ODS-AQ (10 × 250 mm, 5 μm) column were used for HPLC. Macherey-Nagel chiral TLC plates were purchased from Alltech. Stock cultures of the *P. aeruginosa* used in this study are preserved at Florida Tech at −70 °C. The microbial test organisms *B. subtilis*, *S. aureus*, and *M. luteus* were purchased from Presque Isle cultures.

Bacterial Source and Culture Conditions. *I. setifera* was collected using scuba between 30 and 40 m depth from Hut Point and Danger Slopes on Ross Island, Antarctica. The microbes were collected by swabbing the interior and exterior of the sponge, and initial growth of microorganisms was achieved on marine agar (Difco). The bacterium used in this study was identified (Analytical Services, Inc.) as *P. aeruginosa*. Single colonies of the bacterium were subcultured at 25 °C in LB media until they reached stationary phase. To provide cultures for metabolite isolation, 10 mL of a fresh stationary culture was used to inoculate a total of 10 × 1 L cultures in LB media. These cultures were grown with aeration for 7 days at 25 °C. A detailed description of the bacterial isolation and growth properties is found elsewhere.[9]

Isolation of Metabolites. The cells were removed from the cultures by centrifugation and filtration through a 0.2 μm filter, and the filtrate (5 L) was passed through an Amberlite XAD-2 column (40 × 8 cm). The column was eluted batchwise with increasing amounts of MeOH in H_2O. The fraction eluted with 80% MeOH showed antibacterial properties and was purified by column chromatography on silica gel using mixtures of $CHCl_3$ and MeOH. Yellow pigments eluted in $CHCl_3$ were further purified on silica gel using $CHCl_3$ to obtain **7** and **8**. DKP's were present in silica gel column fractions eluted with 2−10% MeOH in $CHCl_3$. Final purification of DKP's (**1**−**6**) was achieved by repeated RP HPLC using 50% aqueous MeOH.

Bioassays. Column fractions or purified compounds were applied to 5 mm filter paper disks and dried. Disks containing the same volume of the appropriate solvent were used as a control. Disks were then placed on LB agar plates that had been spread with 0.1 mL of a fresh stationary culture of tester strains. Plates were incubated for 2 days at 25 °C to allow tester strain growth. Zones of inhibition were measured as excess diameter.

***cyclo*-(L-Pro-L-Val) (1).** *cyclo*-(L-Pro-L-Val) was obtained as a colorless amorphous solid (6.8 mg): $[\alpha]^{20}_D$ −139.4° (c = 0.16, EtOH); ^{13}C NMR ($CDCl_3$) δ 170.19 (s, C-7), 165.1 (s, C-1), 60.58 (d, C-9), 59.03 (d, C-6), 45.36 (t, C-3), 28.74 (t, C-5), 28.58 (d, C-10), 22.57 (t, C-4), 16.26 (2 × q, 10-Me).

***cyclo*-(L-Pro-L-Leu) (2).** *cyclo*-(L-Pro-L-Leu) was obtained as a colorless amorphous solid (6.5 mg): $[\alpha]^{20}_D$ −136.0° (c = 0.12, EtOH); ^{13}C NMR ($CDCl_3$) δ 59.2 (d, C-6), 53.6 (d, C-9), 45.72 (t, C-3), 38.87 (t, C-10), 28.33 (t, C-5), 24.96 (d, C-11), 23.5 (t, C-4), 22.95 (q, 11-Me), 21.4 (q, 11-Me).

***cyclo*-(L-Pro-L-Ile) (3).** *cyclo*-(L-Pro-L-Ile) was obtained as a colorless amorphous solid (6.0 mg): $[\alpha]^{20}_D$ −173.5° (c = 0.16, EtOH).

***cyclo*-(L-Pro-L-Phe) (4).** *cyclo*-(L-Pro-L-Phe) was obtained as a colorless amorphous solid (8.5 mg): $[\alpha]^{20}_D$ −93.9° (c = 0.36, EtOH).

***cyclo*-(L-Pro-L-Tyr) (5).** *cyclo*-(L-Pro-L-Tyr) was obtained as a colorless amorphous solid (11.5 mg): $[\alpha]^{20}_D$ −126.1° (c = 0.26, EtOH); ^{1}H NMR ($CDCl_3$) δ 7.03 (2H, d, J = 8.3 Hz, H-2′ and H-6′), 6.77 (2H, d, J = 8.3 Hz, H-3′ and H-5′), 6.11 (1H, s, NH), 4.22 (1H, dd, H-9, J =

2.9, 9.6 Hz), 4.07 (1H, t, H-6, J = 7.6 Hz), 3.57 (2H, m, H-3), 3.42 (1H, dd, H-10, J = 11.0, 14.0 Hz), 2.78 (1H, dd, H-10, J = 9.6, 14.4 Hz), 2.32 (1H, m, H-5a), 1.96 (1H, m, H-5b), 1.85 (2H, m, H-4); ^{13}C NMR ($CDCl_3$) δ 169.95 (s, C-7), 165.48 (s, C-1), 156.05 (s, C-4′), 130.58 (d, C-2′ and C-6′), 126.93 (s, C-1′), 116.32 (d, C-3′ and C-5′), 59.36 (d, C-6), 56.51 (d, C-9), 45.65 (t, C-3), 36.17 (t, C-10), 28.54 (t, C-5), 22.64 (t, C-4).

***cyclo*-(L-Pro-L-Met) (6).** *cyclo*-(L-Pro-L-Met) was obtained as a colorless amorphous solid (4.3 mg): $[\alpha]^{20}_D$ −82.2° (c = 0.14, EtOH); ^{1}H NMR ($CDCl_3$) δ 6.55 (1H, bs, NH), 4.24 (1H, t, H-9, J = 5.2 Hz), 4.12 (1H, t, H-6, J = 7.6 Hz), 3.57 (2H, m, H-3), 2.69 (2H, t, H-11, J = 7.0 Hz), 2.43 (1H, m, H-5a), 2.38 (1H, m, H-10a), 2.14 (3H, s, S-Me), 2.05 (1H, m, H-10b), 1.92 (1H, m, H-5b); ^{13}C NMR (CD_3OD) δ 170.35 (s, C-7), 165.57 (s, C-1), 59.24 (d, C-6), 54.92 (d, C-9), 45.7 (t, C-3), 30.54 (t, C-10), 29.08 (t, C-11), 28.42 (t, C-5), 22.88 (t, C-4) 15.52 (q, S-Me); HRFABMS m/z $[M + H]^+$ 229.1018 ($C_{10}H_{17}O_2N_2S$ requires 229.1010).

Stereochemical Analysis of DKP's. In a typical experiment a solution of DKP (0.5−1.0 mg) in 6 N HCl (1.0 mL) was heated at 110 °C in a sealed tube for 18−24 h. The freeze-dried solution dissolved in H_2O (0.5 mL) was analyzed on chiral TLC plates (activated at 100 °C for 15 min prior to use) with the solvent system MeOH:H_2O:CH_3CN in the ratio of 1:1:4 or 1:1:0.6. Spots were visualized with 0.1% ninhydrin spray reagent.

***N*-(*tert*-Butyloxycarbonyl)-L-proline-L-methionine Methyl Ester**. To a solution of *N*-(*tert*-butyloxycarbonyl)-L-proline (0.5 g) and *N*,*N*'-dicyclohexylcarbodiimide (0.48 g) in dry CH_2Cl_2 was added L-methionine methyl ester (0.38 g) and the reaction mixture stirred for 4 h at room temperature (20 °C). The precipitate was removed and the filtrate evaporated under vacuum. The crude product was purified by chromatography on silica gel. The fractions eluted with EtOAc gave the linear dipeptide as a white amorphous solid (0.66 g, 79%): $[\alpha]_D$ −54.5° (c = 0.98, EtOH); ^{1}H NMR ($CDCl_3$) δ 1.44 (9H, s, C(CH_3)$_3$), 1.87 (2H, m, proline-α-CH_2), 1.94 (2H, m, methionine-β-CH_2), 2.05 (3H, s, SCH_3), 2.11 (2H, m, proline-β-CH_2), 2.45 (2H, t, S-CH_2, J = 7.5 Hz), 3.45 (2H, m, proline-γ-CH_2), 3.72 (3H, s, CO_2CH_3), 4.28 (1H, m, methionine-α-CH), 4.63 (1H, m, proline-α-CH), 6.78 (1H, bs, CONH), 7.42 (1H, bs, CONH); HRFABMS m/z $[M + H]^+$ 361.1788 ($C_{16}H_{29}O_5N_2S$ requires 361.1797).

Synthesis of *cyclo*-(L-Pro-L-Met) (6). The protected linear dipeptide (0.1 g) was stirred with formic acid (20 mL, 98%) at room temperature for 2 h. After removal of the excess formic acid in vacuuo, the gummy residue was refluxed in toluene (15 mL) and butan-2-ol (10 mL) for 4 h. After the solution was concentrated and cooled, the crude product **6** (15 mg, 24%) was recovered by filtration. Preparative HPLC yielded a sample for analytical and spectroscopic measurements: HRFABMS m/z $[M + H]^+$ 229.1009 ($C_{10}H_{17}O_2N_2S$ requires 229.1010).

Phenazine-1-carboxylic Acid (7). Phenazine-1-carboxylic acid was isolated as yellow needles (EtOAc/hexane) (5.0 mg): mp 240−242 °C (lit.[24] mp 239−240 °C); UV (MeOH) λ_{max} (log ϵ) 368 (3.48); ^{1}H NMR ($CDCl_3$) δ 15.62 (1H, s, COOH), 9.01 (1H, dd, J = 9.1, 1.0 Hz, H-2), 8.42 (1H, dd, J = 8.6, 1.0 Hz, H-4), 8.28 (1H, m, H-9), 8.23 (1H, m, H-6), 7.97 (1H, t, H-3, J = 6.5 Hz), 7.9 (2H, m, H-7 and H-8); ^{13}C NMR ($CDCl_3$) δ 166.1 (s, *C*OOH), 143.72 (s, C-4a), 143.36 (s, C-5a), 141.73 (s, C-9a), 141.03 (s, C-10a), 136.16 (d, C-2), 134.53 (d, C-4), 131.95 (d, C-8), 131.27 (d, C-7), 130.1 (s, C-1), 130.09 (d, C-3), 129.97 (d, C-9), 129.31 (d, C-6); EIMS m/z (%) $[M]^+$ 224 (3), 181 (12), 180 (100), 179 (45), 76 (28), 50 (35).

Phenazine-1-carboxamide (**8**). Phenazine-1-carboxamide was isolated as pale yellow needles (EtOAc/hexane) (9.2 mg): mp 242−244 °C (lit.[25] mp 241 °C); UV (EtOH) λ_{max} (log ϵ) 366 (3.44); ^{1}H NMR ($CDCl_3$) δ 8.98 (1H, d, J = 9.0 Hz, H-2), 8.54 (1H, d, J = 8.7 Hz, H-4), 8.36 (1H, d, J = 7.9 Hz, H-6), 8.29 (1H, d, J = 8.7 Hz, H-9), 8.0 (1H, t, H-3, J = 7.3 Hz), 7.9 (2H, m, H-7 and H-8); ^{13}C NMR ($CDCl_3$) δ 166.83 (s, *C*ONH_2), 143.33 (s, C-4a), 143.63 (s, C-5a), 140.3 (s, C-9a), 140.08 (s, C-10a), 137.63 (d, C-2), 135.32 (d, C-4), 133.41 (d, C-8 and C-9), 130.48 (s, C-1), 130.48 (d, C-3), 131.92 (d, C-7), 128.2 (d, C-6); HRMS found m/z 223.0745 (calcd for $C_{13}H_9ON_3$ $[M]^+$ 223.0745).

Conversion of Phenazine 8 to Phenazine 7. Phenazine **8** (2 mg) was refluxed in concentrated HCl (15 mL) for 5 h. After neutralization of the solution with $NaHCO_3$, the product **7** (1.9 mg, 95%) was extracted with $CHCl_3$.

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