A β-Amino Acid Containing Tripeptide from a *Pseudomonas*−*Alteromonas* Bacterium Associated with a Black Sea Sponge

Salvatore De Rosa,*[,†] Alfonso De Giulio,[†] Giuseppina Tommonaro,[†] Simeon Popov,[‡] and Athanas Kujumgiev[§]

Istituto per la Chimica di Molecole di Interesse Biologico, CNR, Via Toiano 6, 80072 Arco Felice, Napoli, Italy, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria, and Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria

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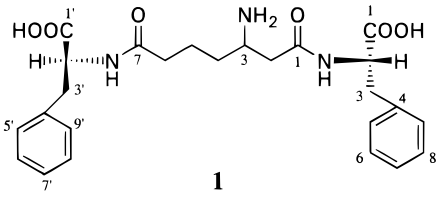
A novel tripeptide, **1**, was isolated from the extracellular extract of a *Pseudomonas*−*Alteromonas* bacterium that was associated with the Black Sea sponge *Dysidea fragilis*. Compound **1** contains the novel β-aminopimelic acid described for the first time from a natural product. The structure of **1** is suggested on the basis of the analysis of spectroscopic data and chemical degradation.

It is well-known that marine sponges are conglomerates of various organisms, and in some cases about 40−60% of their wet weight is due to the presence of symbiotic and/or parasitic organisms.[1,2] We are interested in discovering which organism may be the true producer of certain metabolites isolated from the whole sponge extracts.

Marine microorganisms are of considerable current interest as a new and promising source of biologically active compounds. They produce a variety of metabolites, some of which can be used for drug development.[2,3] It is also known that some bioactive compounds isolated from invertebrates originate from symbiotic microorganisms (e.g., tetrodotoxin, saxitoxin, okadaic acid, surugatoxins, etc.).[3−5]

Because of the unusual environment in the Black Sea (salinity twice lower than in the oceans, the presence of hydrogen sulfide 100 m below the surface, significant pollution, etc.), the metabolism of the inhabiting organisms sometimes changes and unusual compounds have been identified in algae and invertebrates.[6−8] Preliminary investigations on some Black Sea bacteria showed that they possess unusual lipid compositions.[6,7]

Recently, we reported the isolation of a bacterium, identified as *Pseudomonas* or *Alteromonas* (DF-1), from *Dysidea fragilis*, the most common sponge in the Black Sea.[9] In this paper, we describe the isolation and structure elucidation of an extracellular tripeptide (**1**), containing a β-amino acid, from the cultures of microorganism DF-1.



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The bacterium *Pseudomonas* or *Alteromonas* was grown in Bacto broth with a salt concentration of 16% (salt concentration of Black Sea) at 37 °C for 48 h. The *n*-butanol extract of the culture (7 L), after removal of bacteria, was purified repeatedly by ODS and Sephadex LH-20 (MeOH/H_2O, 7:3) columns, followed by a lobar C-18 column to give **1** as a yellow amorphous powder.

Compound **1** had $[\alpha]_D$ −27.5 and showed a pseudomolecular ion peak at *m/z* 470.2279 ($M + H^+$, calcd 470.2291) in the HRFABMS (positive ion) spectrum, consistent with a molecular formula $C_{25}H_{31}N_3O_6$. TLC analysis of the acid hydrolysate of **1** revealed the presence of Phe, a common amino acid, and of a new amino acid with R_f similar to α-aminopimelic acid (α-Apa). Examination of the ^{1}H NMR spectrum immediately suggested a peptide with aromatic and aliphatic residues. The integration of the ^{1}H NMR spectrum showed that Phe and the new amino acid are at a ratio of 2:1. Both the ^{1}H and ^{13}C NMR spectra included signals for a monosubstituted benzene [δ 7.26 (m), 7.20 (m); 137.0 (s), 130.3 (d), 129.9 (d) and 128.3 (d)] which were identified as a phenylalanine residue by COSY-45, HMQC, and HMBC spectra. The remaining signals in the ^{13}C NMR spectrum were due to two carbonyl groups (δ 172.5 and 166.7), one methine (δ 59.6), and four methylenes (δ 46.5, 46.3, 28.9, and 22.8). The COSY-45 spectrum indicated that the methine proton at δ 4.15 (H-3) was coupled to a diastereotopic proton at δ 4.02 [(H-2) dd, J = 17.1 and 2.4 Hz; the other diastereotopic proton was at δ 3.72 (d, J = 17.1 Hz)] and was coupled to a nonequivalent methylene at δ 2.22, 1.83 (H-4). The latter two protons were coupled to a second nonequivalent methylene H-5 (δ 1.94, 1.83), which, in turn, were coupled to a methylene proton at δ 3.42 (H-6). HMBC correlations observed between the H-2 methylene protons and the carbonyl at δ 166.7, and H-5 methylene protons and the carbonyl at δ 172.5, suggested the presence of a β-aminopimelic acid (β-Apa) residue. HMBC correlation between the α-proton of Phe and both the carbonyl groups of the β-Apa residue defined the amino acid sequence.

The stereochemistry of the standard amino acid was determined by HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent,[10] which allowed us to assign the L configuration for Phe residues.

Compound **1** contains β-Apa, which is reported here for the first time from a natural source. The related diaminopimelic acid (Dap) was earlier reported as a constituent of a peptidoglycan that exhibited strong antiviral activity.[11] Furthermore, Dap is the direct biosynthetic precursor to lysine in bacteria and higher plants.[12]

Experimental Section

General Experimental Procedures. Optical rotation was measured on a JASCO DIP 370 polarimeter, using a 10-cm microcell. FABSMS were obtained on a VG-ZAB instrument

* To whom correspondence should be addressed. Tel.: +39 081 8534172. Fax: +39 081 8041770. E-mail: sderosa@icmib.na.cnr.it.
† Istituto per la Chimica di Molecole di Interesse Biologico, CNR.
‡ Institute of Organic Chemistry with Centre of Phytochemistry, BAN.
§ Institute of Microbiology, BAN.

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equipped with a FAB source, using glycerol as a matrix. ESMS were performed by using a BioQ triple quadrupole mass spectrometer; aliquots of the peptide solution were injected in the ion source at a flow of 5 μL/min, and scanning was performed from *m/z* 50 to *m/z* 1000 at 10 s/scan, using a cone voltage of 40 V; masses are reported as average mass. ^{1}H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively, on a Bruker AMX-500 spectrometer in CD_3OD/D_2O, using the residual CD_3OD resonance at 3.48 and 49.0 ppm as internal references, respectively. TLC was carried out on precoated Merck F_{254} silica gel plates and chromatographic solvents (A) *n*-BuOH−HOAc−H_2O (12:3:5) and (B) *i*-PrOH−H_2O−NH_3 (20:2:1).

Cultivation and Isolation. The bacterium *Pseudomonas*−*Alteromonas* was isolated from the sponge *Dysidea fragilis* collected in the Black Sea, as already described.[9] Cultures of DF-1 (a voucher specimen of the strain is maintained in the Institute of Microbiology BAN collection) were grown in Bacto broth (NaCl 1.6%, PH 7). Cultures (7 L) were incubated at 37 °C for 48 h, under shaking. The cells were isolated by centrifugation at 5000 rpm, for 10 min. The culture, after elimination of bacteria, was extracted with *n*-butanol (500 mL × 3) and evaporated under reduced pressure. The extract was chromatographed on a reversed-phase C_{18} column with H_2O−MeOH gradient. The fraction that eluted with 50% of MeOH was subjected to a Sephadex LH-20 column (4 × 100 cm) using MeOH−H_2O, 7:3. The fraction eluting from 100 to 150 mL was subjected to chromatography on a lobar C_{18} column with MeOH−H_2O, 7:3, and then MeOH, to give **1** (20 mg), with R_f's on TLC of 0.5 and 0.38 in solvent A and B, respectively.

Compound 1: yellow amorphous powder; mp ∼190 °C dec; $[\alpha]_D$ −27.5° (c = 0.18 H_2O); NMR data are reported in Table 1; HRFABMS *m/z* 470.2279 $(M + H)^+$ (calcd for $C_{25}H_{31}N_3O_6$, 470.2291); ESMS *m/z* (%) $[M]^+$ 469 (2), $[M - H_2O]^+$ 451 (2), $[M - C_7H_7]^+$ 378 (4), $[M - Phe]^+$ 320 (3), $[M - Phe - Apa]^+$ 149 (20), 141 (8), 120 (100).

Amino Acid Analysis. Compound **1** (100 μg) was hydrolyzed with 6 N HCl (500 μL) at 110 °C for 16 h. The acid hydrolysate was dried under N_2 and then dissolved in 500 μL of H_2O. This solution was analyzed by TLC, using Phe and α-Apa as reference compounds. The compounds were detected with ninhydrin. Two spots were observed with R_f's of 0.51 (Phe) and 0.27 (Apa) in solvent A and of 0.35 (Phe) and 0.12 (Apa) in solvent B.

Marfey Analysis of 1. Compound **1** (100 μg) was hydrolyzed with 6 N HCl (500 μL) at 110 °C for 16 h. The acid hydrolysate was dried under N_2 and to it were added 50 μL of a 10% L-FDAA (1-fluoro-2,4-dinitropnenyl-5-L-alanine amide) solution in acetone and 40 μL of 1 N $NaHCO_3$ followed by heating at 50 °C for 2 h. After cooling to room temperature, the reaction mixture was neutralized with 2 N HCl (40 μL), dried under N_2, and then dissolved in 500 μL of DMSO. This solution was analyzed by reversed-phase HPLC (Spherisorb S5ODS2; 35% MeCN + 0.1% TFA; flow 1.5 mL/min), and the FDAA derivative was detected by UV at 340 nm. L-Phe (retention time 14.4 min) was identified by co-injection with standard FDAA amino acid derivatives. L- and D-Phe were derivatized as described above.

Table 1. NMR Spectral Data of Compound **1** in CD_3OD/D_2O Solution[a]

C no.	^{13}C	^{1}H	HMBC (J_{C-H} = 10 Hz)
Phe			
1	176.1		H-2, H-3
2	57.4	3.70 dd (7.9, 4.9)	H-3
3	38.5	3.12 dd (14.3, 4.9), 2.90 dd (14.3, 7.9)	H-5/H-9, H-2
4	137.0		H-6/H-8, H-2, H-3
5/9	130.3	7.20 m	H-7, H-3
6/8	129.9	7.26 m	
7	128.3	7.20 m	H-5/H-9
β-Apa			
1	166.7		H-2, H-2Phe
2	46.5	4.02 dd (17.1, 2.4), 3.72 bd (17.1)	H-4
3	59.6	4.15 m	H-4, H-5
4	28.9	2.22 m, 1.83 m	H-3, H-5, H-6
5	22.8	1.94 m, 1.83 m	H-6, H-4
6	46.3	3.42 m	
7	172.5		H-5, H-2′Phe

[a] Chemical shifts are referred to residual CD_3OD resonance. Coupling constants (Hz) are in parentheses.

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