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NOTE

Two new cyclic tetrapeptides from deep-sea bacterium *Bacillus amyloliquefaciens* GAS 00152

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Marine microorganisms continue to be a productive focus for much of marine natural products investigation.[1–3] These organisms flourishing in diverse marine environments, have produced a wide variety of structurally unique and biologically active secondary metabolites many of which have attracted considerable attention.[1,4] In the last decades, the number of reported secondary metabolites from marine bacteria has steadily increased.[2] Medium ring-sized peptides containing the amino acids leucine, isoleucine, phenylalanine, valine and proline have been reported from diverse marine sources, including microorganisms.[5,6] The genus *Bacillus* has produced biologically active compounds.[7,8] In our search for bioactive natural products from the bacterium *B. amyloliquefaciens* collected from South China Sea deep-sea sediment, the culture broth contained two new cyclic tetrapeptides, cyclo-(Leu-Pro-Ile-Pro) (**1**) and cyclo-(Tyr-Pro-Phe-Gly) (**2**) (Figure 1).

The bacterial strain GAS 00152 was isolated from underwater sediment (depth 2476 m) collected at South China Sea (112°21′E, 18°24′N), in March, 2011. For taxonomic identification, blast search results at EzTaxon.org Server showed 16S ribosomal RNA gene sequence of the strain GAS 00152 has the highest similarity (100%) with *Bacillus amyloliquefaciens* strain CA812^T. A reference specimen was deposited in the Guangxi Key Laboratory of Marine Environmental Science, Guangxi Academy of Sciences, China.

B. amyloliquefaciens GAS 00152 was incubated on a rotary shaker (150 r.p.m.) at 28 °C for 7 days, maintained in 500 ml × 600 conical flasks containing the liquid medium (150 ml per flask) composed of peptone (10 g l^{-1}) and yeast extract (5 g l^{-1}), and seawater after adjusting its pH to 7.4. The culture broth (90 l) of *B. amyloliquefaciens* GAS 00152 was extracted with EtOAc. The EtOAc extract was concentrated *in vacuo* to afford 15.37 g of residue, which was subjected to column chromatography on silica gel, using $CHCl_3$-Me_2CO (10:0, 50:1, 25:1,10:1, and 3:1, v-v) as eluent, giving seven fractions (A–G). Fraction C subjected to a second column chromatography, using $CHCl_3$-MeOH (1:1, v-v) as eluent, was then separated by HPLC, using a mixture of MeOH-H_2O (55:45, v-v) to yield **1** (5.7 mg). Fraction E subjected to a second column chromatography using $CHCl_3$-MeOH (1:1, v-v) as eluent, was then separated by HPLC, using a mixture of MeOH-H_2O (25:75, v-v) to yield **2** (7.9 mg).

Compound **1** was obtained as a colorless oil and showed a molecular ion peak at *m/z* 421.2813 $[M+H]^+$ (calcd for $C_{22}H_{37}N_4O_4$, 421.2815) by HR-ESI-MS consistent with the molecular formula $C_{22}H_{36}N_4O_4$. The ^{1}H NMR (Table 1) chemical shifts of four α protons at δ_H 4.25 (dd, J = 9.6, 6.6 Hz), 4.23 (dd, J = 9.6, 6.3 Hz), 3.84 (dd, J = 5.0, 2.1 Hz) and 3.68 (d, J = 5.5 Hz); and ^{13}C NMR spectrum (Table 1) chemical shifts of four carbonyl carbons at δ_C 171.6, 171.6, 169.1 and 167.9, supported the presence of peptide fragments. The fact that compound **1** was negative to the ninhydrin test, suggested a cyclic or an N-terminus-blocked peptide.[9] The identification of each amino acid unit was accomplished by detailed interpretation of the ^{1}H–^{1}H COSY data (Figure 2). Beginning with signals of the methine protons at δ_H 3.84 and 3.68, proton correlations including the upfield methyl signals readily revealed the presence of Leu and Ile residues. Also illustrated were two series of similar proton correlations containing signals of the methine protons at δ_H 4.25 and 4.23 in the ^{1}H NMR spectrum, which were interpreted to be two Pro residues. Confirmation of the spectral interpretation as well as the stereo-chemical assignment of each amino acid residue was established by Marfey's methods.[4] Acid-catalyzed hydrolysis of **1** followed by treatment with L-Marfey's reagent and HPLC analysis showed that Leu and Pro had the L-configuration. The stereochemistry at the side chain of the Ile residue was also determined to be 2*S*, 3*S* on the basis of HPLC coinjection with derivatives prepared from both Ile and *allo*-Ile. Thus, compound **1** consisted of all L-forms of Ile, Leu, Pro (I) and Pro (II).

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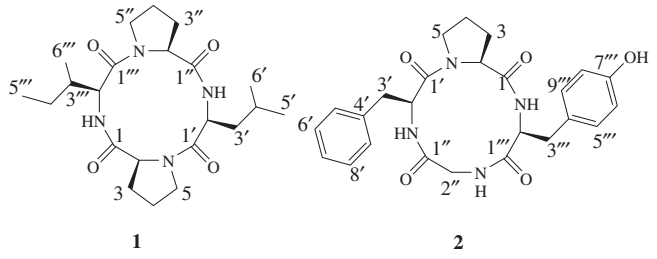
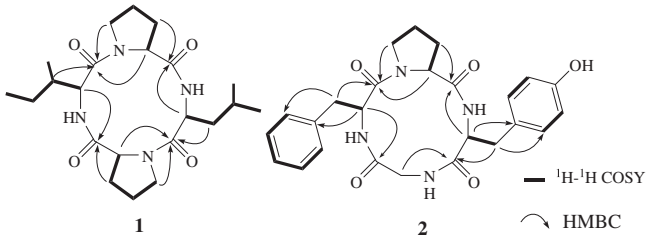
Further structural assignment and sequencing of the amino acid residues were accomplished with the aid of HMBC experiments (Figure 2). HMBC correlations of C-1′ with H-2, H-5 and H-3′ placed a peptide linkage between Leu and Pro (I). Correlations of C-1″ with H-2′ and H-3″ also denoted a peptide linkage between Leu and Pro (II). The placement of a peptide linkage between Ile and Pro (I) was secured by HMBC correlations of C-1 with protons H-2‴ and H-3. The placement of a peptide linkage between Ile and Pro (II) was confirmed by HMBC correlations of C-1‴ with H-2″, H-5″ and H-3‴. A literature survey revealed that fenestin A,[10] a cyclic tetrapeptide from the marine sponge *Leucophloeus fenestrata*, had the same amino acid composition but differed from **1** in the sequence of amino acid residues. In the MS, fragment ion at *m/z* 210 is consistent with Pro-Leu or Pro-Ile fragments. These data are consistent with cyclo-(Leu-Pro-Ile-Pro) or with cyclo-(Leu-Ile-Pro-Pro), however, by analogy with fenestin A, the latter structure would be expected to show a fragment at *m/z* 194 for Pro-Pro in the MS. The combination of these data confirmed the structure of **1** as cyclo-(Leu-Pro-Ile-Pro).

Compound **2** was obtained as a white amorphous solid and had a molecular ion peak at *m/z* 465.2138 $[M+H]^+$ (calcd for $C_{25}H_{29}N_4O_5$, 465.2136) by HR-ESI-MS consistent with the molecular formula $C_{25}H_{28}N_4O_5$. In ^{13}C NMR spectrum (Table 2), in addition to signals corresponding to the side chains of the above amino acids, four carbonyl signals at δ_C 168.3, 167.2, 165.7 and 164.9 could be assigned as the amide carbons of Pro, Gly, Tyr and Phe, respectively,



1 **2**

Figure 1 The structures of compounds **1** and **2**.



1 **2**

Figure 2 Key ^{1}H–^{1}HCOSY correlations and HMBC correlations of **1** and **2**.

Table 1 ^{1}H and ^{13}C NMR data for 1 (600 and 150 MHz, CD_3OD, δ in p.p.m.)

Residue	*Position*	δ_H *(mult., J in Hz)*	δ_C *(mult)*
Pro (I)	1		171.6, C
	2	4.25, dd (9.6, 6.6)	59.3, CH
	3	2.35, m	30.3, CH_2
	3	1.93, m	
	4α	2.00, m	23.1, CH_2
	4β	1.95, m	
	5α	3.55, m	46.8, CH_2
	5β	3.48, m	
Leu	1′		169.1, C
	2′	3.84, dd (5.0, 2.1)	57.1, CH
	3′α	1.68, m	43.6, CH_2
	3′β	1.57, m	
	4′	1.77, m	25.5, CH
	5′	0.99, d (5.5)	23.3, CH_3
	6′	0.99, d (5.5)	21.9, CH_3
Pro (II)	1″		171.6, C
	2″	4.23, dd (9.6, 6.3)	59.7, CH
	3″α	2.33, m	29.9, CH_2
	3″β	1.91, m	
	4″α	2.02, m	23.3, CH_2
	4″β	1.93, m	
	5″α	3.51, m	46.7, CH_2
	5″β	3.49, m	
Ile	1‴		167.9, C
	2‴	3.68, d (5.5)	63.4, CH
	3‴	1.90, m	41.0, CH
	4‴	1.59, m	26.0, CH_2
	5‴	0.96, t (7.0)	11.6, CH_3
	6‴	1.00, d (7.3)	16.7, CH_3

Table 2 ^{1}H and ^{13}C NMR data for 2 (600 and 150 MHz, DMSO-d_6, δ in p.p.m.)

Residue	*Position*	δ_H *(mult., J in Hz)*	δ_C *(mult)*
Pro	1		168.3, C
	2	4.06, dd (9.6, 6.3)	55.5, CH
	3α	1.98, m	28.6, CH_2
	3β	1.51, m	
	4α	1.78, m	21.3, CH_2
	4β	1.51, m	
	5α	3.36, m	44.6, CH_2
	5β	3.11, m	
Phe	1′		164.9, C
	2′	3.91,dd (5.5, 4.8)	58.1, CH
	3′α	3.01, dd (13.6, 4.8)	38.8, CH_2
	3′β	2.87, dd (13.6, 5.5)	
	4′		136.0, C
	5′/9′	7.20, d (7.2)	130.8, 2CH
	6′/8′	7.28, dd (7.2, 5.5)	128.1, 2CH
	7′	7.28, t (5.5)	126.8, CH
	NH	8.11, s	
Gly	1″		167.2, C
	2″α	3.34, d (11.0)	43.7, CH_2
	2″β	3.11, d (11.0)	
	NH	8.11, s	
Tyr	1‴		165.7, C
	2‴	2.85, dd (6.2, 3.4)	57.2, CH
	3‴α	3.00, dd (12.6, 6.2)	38.6, CH_2
	3‴β	2.77, dd (12.6, 3.4)	
	4‴		125.9, C
	5‴/9‴	6.66, d (7.0)	115.1, 2CH
	6‴/8‴	6.90, d (7.0)	135.9, 2CH
	7‴		156.3, C
	NH	7.88, s	

by the $^3J_{CH}$ correlation from their β protons (Figure 2). The ^{1}H NMR (Table 2) of **2** showed two α protons at δ_H 4.06 (dd, $J = 9.6$, 6.3 Hz) and 3.91 (dd, $J = 5.5$, 4.8 Hz) while a third α proton was observed upfield at δ_H 2.85 (dd, $J = 6.2$, 3.4 Hz). The side chain signals for the individual acids were determined by ^{1}H–^{1}H COSY data (Figure 2) and were fully consistent with the presence of Pro, Phe, Gly and Tyr residues. Confirmation of the spectral interpretation as well as the stereo-chemical assignment of each amino acid residue was established by Marfey methods.[4] Acid-catalyzed hydrolysis of **2** followed by treatment with L-Marfey's reagent and HPLC analysis showed that Phe and Pro had the L-configuration. The stereochemistry at the side chain of the Ile residue was also determined to be 2*S* on the basis of HPLC coinjection with derivatives prepared from both Tyr and Phe. Thus, compound **2** consisted of all L-forms of Pro, Tyr and Phe.

Further structural assignment and sequencing of the amino acid residues was accomplished with the aid of HMBC experiments (Figure 2). HMBC correlations of C-1′ with H-2, H-5 and H-3′ placed a peptide linkage between Phe and Pro. Correlations of C-1″ with H-2′, denoted a peptide linkage between Phe and Gly. The placement of a peptide linkage between Gly and Tyr was secured by HMBC correlations of C-1‴ with protons H_α-2″ and H_β-2″. The placement of a peptide linkage between Tyr and Pro was confirmed by HMBC correlations of C-1 with H-2‴ and H-3. In the MS, fragment ions at *m/z* 260, 244, 220 and 204 were consistent with Pro-Tyr, Pro-Phe, Tyr-Gly and Phe-Gly fragments, respectively, which were consistent with cyclo-(Phe-Pro-Tyr-Gly). Thus, the structure of **2** was determined to be cyclo-(Phe-Pro-Tyr-Gly).

The cytotoxicities of compounds **1** and **2** were assayed *in vitro* against the HepG2 and HeLa cell lines by MTT method.[11] Compound **1** exhibited cytotoxic activity with IC_{50} values of 26.6 μM and 34.7 μM, respectively. The values of compound **2** were 38.2 μM and 46.1 μM, respectively.

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