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TWO DIKETOPIPERAZINE CYCLO(PRO-PHE) ISOMERS FROM MARINE BACTERIA *Bacillus subtilis* sp. 13-2

Guanghua Wang,[1,2] Shikun Dai,[1,2] Minjie Chen,[1,2] Houbuo Wu,[1] Lianwu Xie,[1] Xiongming Luo,[1,2] and Xiang Li[1*]

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*Two bioactive diketopiperazines, cyclo(*S-*Pro*-S-*Phe) and cyclo(*R-*Pro*-S-*Phe), against* Bacillus subtilis *(terrestrial),* Staphylococcus aureus, *and* Escherichia coli *DH5α were isolated from marine surface sediment bacteria* Bacillus subtilis *sp.13-2. To our knowledge, this was the first time that cyclo(*S-*Pro*-S-*Phe) and cyclo(*R-*Pro*-S-*Phe) were isolated from* Bacillus subtilis*, and the first time that cyclo(*R-*Pro*-S-*Phe) was isolated from a natural source. These two compounds were established on the basis of ESI-MS spectral data, as well as NMR 1D and 2D (COSY, HMQC, and HMBC) spectral data.*

Keywords: *Bacillus subtilis* sp.13-2*,* cyclo(*S*-Pro-*S*-Phe), cyclo(*R*-Pro-*S*-Phe).

Drug discovery from marine natural products has enjoyed a renaissance in the past few years. Ziconotide and trabectedin were approved in the United States and the European Union in 2004 and 2007, respectively [1]. Marine bacteria is a large treasury for natural products [2–4] research. During our search for new bioactive natural products from marine sediment bacteria from the South China Sea, the strain *Bacillus subtilis* sp.13-2 (100% 16S rDNA similarity with model *Bacillus subtilis* strain) was found to be bioactive against *Bacillus subtilis* (terrestrial), *Staphylococcus aureus,* and *Escherichia coli* DH5α. *Bacillus subtilis* sp.13-2 can grow well in both fresh and marine water culture medium. Two bioactive diketopiperazine (DKP) isomers cyclo(*S*-Pro-*S*-Phe) (A), 50 mg, and cyclo(*R*-Pro-*S*-Phe) (B), 4.7 mg, were obtained by chromatographic purification in addition to bioactivity tracing against *Bacillus subtilis* from 30 liters 2216E marine water culture broth of *Bacillus subtilis* sp.13-2. Compound B was eluted just before A by HPLC using a C-18 reverse-phase column with 30:70 methane–water. This was the first time that cyclo(*S*-Pro-*S*-Phe) and cyclo(*R*-Pro-*S*-Phe) were isolated from marine *Bacillus subtilis*, and the first time that cyclo(*R*-Pro-*S*-Phe) was isolated from a natural source.

The molecular formula for both compounds **1** and **2** was determined to be $C_{14}H_{16}O_2N_2$ by analysis of their NMR spectroscopic data (Table 1) and ESI-MS measurement of molecular ions *m/z* $[M + H]^+$ at 245.2, $[M + Na]^+$ at 267.2, and $[2M + H]^+$ at 489.5.

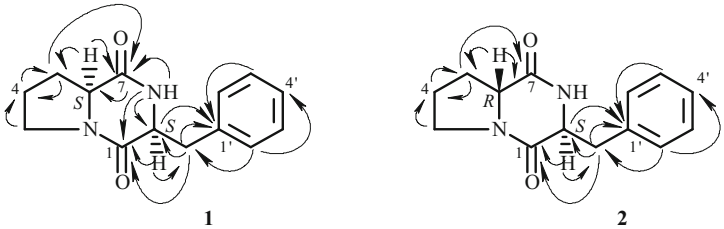

Fig. 1. Two bioactive diketopiperazines from *Bacillus subtilis* sp. 13-2 and their main HMBC correlations (H→C).

1) Key Laboratory of Marine Bio-resources Sustainable Utilization, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, 510301, P. R. China, e-mail: Xianglixiang@yahoo.com; 2) Graduate University of Chinese Academy of Sciences, Beijing, 100049, P. R. China. Published in Khimiya Prirodnykh Soedinenii, No. 4, pp. 491–493, July–August, 2010. Original article submitted March 3, 2009.

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TABLE 1. ^{1}H and ^{13}C NMR Data (500 MHz, $CDCl_3$, δ, ppm, J/Hz) of Compounds **1** and **2**

C atom	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
1	164.99		164.80	
3	45.28	3.49–3.53 (1H, m)	45.14	3.36–3.41 (1H, m)
		3.58–3.64 (1H, m)		3.59–3.65 (1H, m)
4	22.38	1.8–2.00 (2H, m)	21.67	1.64–1.85 (1H, m)
				1.90–1.96 (1H, m)
5	28.22	1.8–2.00 (1H, m)	28.92	1.64–1.85 (1H, m)
		2.23–2.30 (1H, m)		2.15–2.20 (1H, m)
6	59.00	4.03 (1H, t, J = 7.5)	57.74	2.91–2.94 (1H, dd, J = 6.5,10.5)
7	169.40		169.29	
NH		6.04 (s)		6.49 (1H, br.s)
9	56.18	4.26 (1H, dd, J = 3.5, 10)	59.04	4.22 (1H, m)
10	36.69	3.49–3.64 (1H, m)	40.48	3.06 (1H, dd, J = 4, 13.5)
		2.81 (1H, dd, J = 10, 14.5)		3.15 (1H, dd, J = 6.5, 13.5)
Ar				
1′	135.93	7.18–7.32 (5H, m)	135.26	7.20–7.33 (5H, m)
2′	129.16		129.90	
3′	129.05		128.80	
4′	127.9		127.33	

The ^{1}H NMR spectrum of compound **1** showed a singlet at 6.04 ppm, which was assigned to the NH proton. Signals from 1.8 to 4.26 ppm were attributed to CH_2 and CH groups, and those from 7.18 to 7.32 ppm to aromatic protons.

The ^{13}C NMR spectrum displayed signals of two amide carbonyls at 164.99 and 169.4 ppm, and those in the aliphatic region were assigned by an HSQC experiment to four CH_2 (22.38, 28.22, 36.69, and 45.28 ppm) and two CH (56.18 and 59 ppm).

The 2- and 3- bond correlations in the HMBC spectrum between H-6 and C-7, H-6 and C-5, H-5 and C-7, H-4 and C-5, H-5 and C-4, and H-3 and C-4 in addition to the chemical shift for C-3 (45.28 ppm) showed that the half moiety linkage should be $R_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CHR\text{-}CONR_2$. The correlations between H-10 and C-1′, H-2′ and C-10, H-9 and C-10, H-9 and C-1, H-10 and C-1, H-3′ and C-1′, and H-2′ and C-4′ indicated another half moiety linkage at $Ar\text{-}CH_2\text{-}CHR\text{-}CO\text{-}NR_2$. There were also HMBC correlations from H-8 to C-9, C-1, C-7, and C-6. So, the final skeleton of this compound should be cyclo (Pro-Phe) (Fig.1).

Compound **2** was also determined as cyclo (Pro-Phe) too. Compound **2** had similar ^{13}C and ^{1}H NMR data to compound **1** (Table 1). The most obvious difference was the ^{1}H chemical shift at 4.03 ppm moving upfield to 2.91–2.94 ppm, and that of the NH (6.04 ppm) proton moving downfield to 0.5 ppm. The HSQC and HMBC spectrum also indicated the ^{13}C chemical shift exchange between C-6 (57.74 ppm) and C-9 (59.04 ppm), but there was no HMBC correlations found from H-8 (Fig. 1).

The absolute configuration of these two DKPs was based on their optical rotation trends from the literature. The sign of $[\alpha]_D$ in the DKPs was either negative or positive depending only on the Pro absolute configuration being *S* or *R*, respectively [5]. Compound **1** had an $[\alpha]_D$ of –70° at 20°C in methanol, and +67° for that of compound **2**, so the absolute configuration of Pro should be *S* and *R* in compound **1** and **2**, respectively. The chemical shift of H-6 was evidence of *cis/trans* H6/9 relationships, which around 4.08 ppm was a *cis* configuration, and that upfield to nearby 2.85 ppm was a *trans* configuration due to the shielding effects of the aromatic ring in its folded conformation [5, 6]. So, the absolute configuration of compounds **1** and **2** should be cyclo(*S*-Pro-*S*-Phe) and cyclo(*R*-Pro-*S*-Phe), respectively. There has been only a chemical synthesis report about cyclo(*R*-Pro-*S*-Phe) [7], giving no natural origin and no NMR data.

In contrast to the stability of acyclic dipeptides, DKPs are susceptible to rapid epimerization in both acid- and base-catalyzed conditions [8]. In Pro-containing DKPs, epimerization occurs preferentially at H-6, and the equilibrium mixture consists of an 80–95% *trans* and 20–5% *cis* mixture of epimers [5]. The relative yields of *trans* and *cis* compounds in this study is about 1/10 (4.7 mg/50 mg), so the *trans* isomer would not be artifacts from epimerization reactions occurring during isolation work.

Cyclo(Pro-Phe) DKPs are ubiquitous in nature and are most commonly isolated from terrestrial *Penicillium bilaii* [9], *Streptomyces* [10], sponge [5], sponge-associated bacteria [11], *Pecton maximus* associated bacteria [7], and so on. They exhibit broad spectrum antimicrobial effects. Cyclo(*R*-Phe-*R*-Pro), cyclo(*R*-Pro-*S*-Phe), and cyclo(*S*-Pro-*R*-Phe) were active

against *Vibrio anguillarum* with MIC of 0.03, 0.10, and 0.13 μg/mL, respectively [7]. Cyclo(*S*-Phe-*S*-Pro) and cyclo(*S*-Leu-*S*-Pro) had synergistic effects in inhibiting the growth of vancomycin-resistant enterococci and pathogenic yeasts, in addition to being effective against *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus*, *Candida albicans,* and *Cryptococcus neoformans* with MIC values of 0.25–0.5 mg/L. They also exerted antimutagenic activity against *Salmonella typhimurium* TA98 and TA100 strains in a *Salmonella* mutation assay [12].

EXPERIMENTAL

General. Column chromatography: silica gel (200–300 mesh, Qingdao Marine Chemical Factory, Qingdao, P. R. China); Sephadex LH-20 (Amersham, England). ^{1}H and ^{13}C NMR spectra: AVANCE 500 (Bruker Biospin international AG), at 500 MHz in $CDCl_3$, δ in ppm refers to Me_4Si. ESI-MS: with API 2000 mass spectrometer in MeOH. Optical rotation: Perkin–Elmer polarimeter, Model 341, with 589 nm at 20°C in MeOH. HPLC: Hitachi, L-2300, C-18 reverse-phase column. MeOH: Honeywell, Burdick & Jackson ACS/HPLC, U.S.A.

Description of Producer Strain. *Bacillus subtilis* sp. 13-2 strain was isolated from marine surface sediments of South China Sea, which had 100% 16S rDNA similarity with model *Bacillus subtilis* strain and can grow on both marine and freshwater solid 2216E culture medium with active metabolites against *Bacillus subtilis*, *Staphylococcus aureus,* and *Escherichia coli* DH5α.

Compound Extraction and Purification. Thirty liters of *Bacillus subtilis* sp.13-2 marine liquid culture medium was extracted twice with equal volumes of acetoacetate at room temperature after incubation at 37°C for 7 days. The organic solution was collected by filtration and removed under vacuum at 45°C to yield crude extract. The crude extract was dissolved in petroleum ether and applied to a silica gel column packed in petroleum ether, using petroleum ether, chloroform–methanol 98:2, chloroform–methanol 19:1, chloroform–methanol 9:1, chloroform–methanol 8:2, chloroform–methanol 7:3, and methanol for the development of the column, each three column volumes. Fractions were monitored by *Bacillus subtilis* bioassay. Active fractions were mixed together and applied to a Sephadex column in methanol as the mobile phase for three column volumes. Fractions were also monitored by *Bacillus subtilis* bioassay. The active fractions were collected together and applied to HPLC with a C18 reverse-phase column in methanol–water 30:70 at 218 nm. Compounds **2** (4.7 mg) and **1** (50 mg) were eluted in line and identified by *Bacillus subtilis* bioassay.

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