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CYCLO(PRO-TYR) FROM AN ENDOPHYTIC RHIZOBIUM ISOLATED FROM *Glycyrrhiza uralensis*

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Endophytes have been recognized as important sources of a variety of structurally novel and biologically active secondary metabolites, including terpenoids, steroids, alkaloids, and isocoumarin derivatives. However, secondary metabolites produced by terrestrial endophytic bacteria have not been well studied; much attention has mainly been focused on the regulatory factors of the interactions between rhizobia and their hosts [1]. Recently, we reported a new antibacterial stearolactone, rhizobialide, isolated from an endophytic rhizobium, *Mesorhizobium* sp. CCNWGX022, inside roots of *Glycyrrhiza uralensis* [2].

Rhizobia N003, a mutualistic endophytic bacterium, was isolated from a well-known traditional Chinese medicine *Glycyrrhiza uralensis* Fisch., also known as licorice, distributed in Bachu County, Xinjiang Uygur Autonomous Region in the arid and semi-arid regions of Northwest China. Based on physiological, biochemical, and morphological characteristics, this strain was identified as *Mesorhizobium* by Prof. G.-H. Wei at the Research Center of Microbiology, Northwest A & F University, Yangling, Shaanxi, P. R. China. A voucher specimen of this strain, *Mesorhizobium* sp. CCNWGX003, has been deposited at the Culture Collection of Northwest A & F University.

The phytochemical constituents of this endophytic strain, rhizobia N003 from *G. uralensis*, have not been reported so far.

Fermentation and Isolation of Metabolite. This endophytic rhizobium was inoculated into Erlenmeyer flasks (150 mL), each containing 50 mL TY culture medium (5.0 g tryptone, 3.0 g yeast extract, and 0.57 g $CaCl_2$ in every 1000 mL distilled water). The flasks were shaken at 28°C on a rotary shaker at 140 rpm.

A suspension (1 mL) of the activated strain was transferred aseptically to a 500 mL Erlenmeyer flask containing YMB culture medium (300 mL), made of mannitol (10 g/L), yeast extract (3 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), and NaCl (0.1 g/L). The flasks were incubated at 28°C in darkness for 96 h on a rotary shaker at 140 rpm. Biomass was removed by filtration and 100 L of fermentation broth was harvested.

After *in vacuo* evaporation of the water at 40°C, the concentrated cultures were successively extracted with petroleum ether and ethyl acetate. The ethyl acetate extract (14.2 g) was applied to a silica gel chromatography column (CC) with increasing polarities of a mixture of $CHCl_3$–MeOH (100:0 to 0:100) to give seven fractions (Fr. 1–7). Fraction 4 (1.70 g) was chromatographed on a silica gel CC with petroleum ether–acetone (30:1, 20:1 and 0:100) to provide four fractions (Fr.4.1–Fr.4.4). Fraction 4.2 (14 mg) was subjected to repeated CC (silica gel, $CHCl_3$—acetone, 50:50; Sephadex LH-20, $CHCl_3$–MeOH, 50:50; silica gel, $CHCl_3$–MeOH, 98:2) to afford pure compound **1** (7 mg).

Cyclo(L-pro-L-tyr) (1). White powder. $C_{14}H_{16}N_2O_3$. $[\alpha]_D^{23}$ –93° (*c* 0.16, acetone). IR (KBr, ν_{max}, cm^{-1}): 3418, 1704, 1600, 1513, 1444, 1370. ^1H NMR (500 MHz, C_5D_5N, δ, ppm, J/Hz): Tyrosine: 8.58 (1H, s, CONH), 4.54 (1H, m, H-1), 3.60 (1H, m, Ha-2), 3.35 (1H, m, Hb-2), 7.42 (2H, d, J = 8.5, H-2′, 6′), 7.12 (2H, d, J = 8.5, H-3′, 5′); Proline: 4.14 (1H, m, H-1″), 2.16 (1H, m, H-2a″), 1.80 (1H, m, H-2b″), 1.58 (2H, m, H-4″), 3.60 (1H, m, H-5a″), 3.35 (1H, m, H-5b″). ^{13}C NMR (125 MHz, C_5D_5N, δ) Tyrosine: 166.2 (s, CO), 57.1 (d, C-1), 36.3 (t, C-2), 127.8 (s, C-1′), 131.4 (d, C-2′, 6′), 116.3 (d, C-3′, 5′), 157.9 (s, C-4′); Proline: 170.0 (s, CO), 59.5 (d, C-1″), 28.8 (t, C-2″), 22.6 (t, C-3″), 45.4 (t, C-4″); Positive FAB-MS *m/z*: 261 $[M + H]^+$ (base peak).

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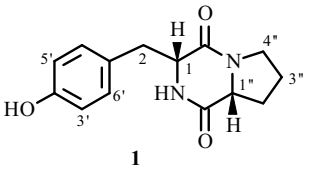
TABLE 1. Effects of Compound **1** on Allelopathic Effects on *Lactuca sativa*

Concentration, μg/mL	Germination rate, %	Seedling length, cm	Root length, cm//Inhibitory rate, %
100	80.7 ± 5.0	1.39 ± 0.08	1.76 ± 0.10[a]/27.0
50	81.3 ± 6.1	1.45 ± 0.24	2.06 ± 0.20[a]/14.5
25	80.7 ± 3.1	1.29 ± 0.12	2.31 ± 0.07
12.5	84.0 ± 5.3	1.20 ± 0.01	2.31 ± 0.03
6.25	76.7 ± 5.8	1.50 ± 0.29	2.21 ± 0.05
0[b]	83.3 ± 3.1	1.42 ± 0.21	2.41 ± 0.19

[a]Indicates a significant difference between control and treatment: *P < 0.05; [b]negative control.



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Compound **1** was identified as a cyclodipeptide, cyclo(L-pro-L-tyr), by IR, MS, and 1D and 2D NMR (COSY, HMBC) spectral analysis and through its physical-chemical properties as well as comparison with literature data [3–5].

Allelopathic Assay. The allelopathic bioassay was carried out against *Lactuca sativa* L. [6]. Their seeds were surface-sterilized by immersion for 40 min in 0.5% potassium permanganate solution, and then 50 of them were placed in each Petri dish (9 cm) with two layers of filter paper after washing with distilled water. The Petri dishes and filter paper were all heat-sterilized. A 3 mL acetone solution of compound **1** (100, 50, 25, 12.5, 6.25, and 0 μg/mL) was added to each Petri dish, and after the acetone evaporated, 3 mL of distilled water was added. After incubation at 22°C in darkness for 96 h, seed germination and seeding and root length were calculated. Each treatment was triplicated. Significance differences between treatment and control were examined by Duncan′s test.

Antibacterial Assay. The assay was carried out in 96-well culture plates using broth medium (Sigma, St. Louis, Mo) against *Escherichia coli*, *Staphylococcus aureus,* and *Bacillus subtilis* [7]. These bacteria were incubated in the medium for 12 h at 30°C at 190 rpm., and the spore concentration was diluted to approximatedly 1×10^5–1×10^6 CFU with broth medium. After incubation for 24 h at 30°C, the MICs were examined.

Allelopathic and Antibacterial Activity and Ecological Significance. The allelopathic and antibacterial activities of compound **1** were assayed. Compound **1** showed inhibitory effects on the root length of *Lactuca sativa* L. by 27.0% and 14.5% at concentrations of 100 and 50 μg/mL, respectively, as shown in Table 1. However, no inhibitory effect was observed on the germination rate or seedling length. It was found that compound **1** inhibited two bacteria, *Escherichia coli* and *Staphylococcus aureus,* at MIC of 250 μg/mL, and *Bacillus subtilis* at 500 μg/mL. Ampicillin (Sigma, St. Louis, MI) was used as the positive control (7.8 μg/mL).

To the best of our knowledge, the occurrence of this cyclodipeptide (**1**) in a species of terrestrial endophytic rhizobia in licorice is reported for the first time. Compound **1**, also known as maculosin, a phytotoxin first reported in the pathogenic fungus *Alternaria alternate* of spotted knapweed (*Centaurea maculosa*), can cause black necrotic lesions on the leaves of this plant [8]. Recently, compound **1** was also isolated from marine-derived actinomycete [9], marine bacterium *Pseudomonas* sp. [10], and an endophytic fungus *Penicillium* sp. of the mangrove plant *Acrostichum aureurm* [11], as well as the plant species *Phytolacca polyandra* [5]. Guo et al. [3] reported that compound **1**, which was isolated from the bacterium *Pseudomonas fluorescens* GcM5-1A carried by the pine wood nematode (*Bursaphelenchus xylophilus*), is toxic to suspension cells and seedlings of Japanese black pine. In our allelopathic assay, inhibitory effects on the roots of *Lactuca sativa* L. were observed. Besides, antibacterial activities of **1** against *Staphylococcus aureus* and methicillin-resistant *S. aureus* [11] as well as against several marine bacterial species [10] were also reported. These findings were consistent with the antibacterial results of compound **1** in the present study.

It has been reported that endophyte-infected plants can produce increased growth, reduce rhizosphere nematode population, and increase resistance to insect feeding, pathogens, and drought stress compared to endophyte-free plants [12–14]. The discovery of compound **1** from cultures of this endophytic rhizobium suggests that rhizobium also produce bioactive chemicals to protect their host plants from invasion by others.

This dipeptide, as a potential chemical defense, can not only cause diseases or toxicity in other plants, but can also protect the plant against pathogens. As a result, the endophyte rhizobium is presumably involved in the superior adaptability and competitiveness of *G. uralensis* in arid and semiarid regions. However, the actual function of the dipeptide produced by this rhizobium from *G. uralensis* needs further study.

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