Two new cyclopeptides from the co-culture broth of two marine mangrove fungi and their antifungal activity

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ABSTRACT

Background: The strategy that co-cultivation two microorganisms in a single confined environment were recently developed to generate new active natural products. In the study, two new cyclic tetrapeptides, cyclo (D-Pro-L-Tyr-L-Pro-L-Tyr) (1) and cyclo (Gly-L-Phe-L-Pro-L-Tyr) (2) were isolated from the co-culture broth of two mangrove fungi Phomopsis sp. K38 and Alternaria sp. E33. Their antifungal activity against Candida albicans, Gaeumannomyces graminis, Rhizoctonia cerealis, Helminthosporium sativum and Fusarium graminearum was evaluated.

Materials and Methods: Different column chromatographic techniques with different solvent systems were used to separate the constituents of the n-butyl alcohol extract of the culture broth. The structures of compounds 1 and 2 were identified by analysis of spectroscopic data (one-dimensional, two-dimensional - nuclear magnetic resonance, mass spectrometry) and Marfey’s analytic method. Dilution method was used for the evaluation of antifungal activity. Results: Compounds 1 and 2 were identified as cyclo (D-Pro-L-Tyr-L-Pro-L-Tyr) and cyclo (Gly-L-Phe-L-Pro-L-Tyr), respectively. Compounds 1 and 2 showed moderate to high antifungal activities as compared with the positive control.

Conclusions: Compounds 1 and 2 are new cyclopeptides with moderate antifungal activity being worthy of consideration for the development and research of antifungal agents.

Key words: Antifungal activity, co-culture, cyclopeptide, mangrove fungi

INTRODUCTION

Natural products (NPs) obtained from microorganisms are historical source of valuable medicinal lead compounds,[1] but the difficulties involved in working with complex mixtures and the rediscovery of the same bioactive chemical structures diminish the attractiveness of such NPs.[2,3] It suggests that it is necessary to develop more efficient approaches to activate the cryptic biosynthetic pathways of microorganisms. In recent years, a strategy that co-cultivation two microorganisms in a single confined environment was developed to generate new NPs through possible interspecies crosstalk, which resulted in the biosynthesis of highly diverse NPs due to the activation of complex regulatory mechanisms.[4] Moreover, these stress-induced molecules exhibited specific antimicrobial,[5-9] and anticancer activities.[10-12]

During our continuing search for structurally new bioactive compounds from mangrove fungi, we investigated the chemical constituents of the metabolites of Phomopsis sp. K38 and Alternaria sp. E33 that were both isolated from the South China sea coast, respectively.[13-15] Then, we investigated the secondary metabolites produced by their co-cultivation, yielded three new molecules, including an antifungal xanthone,[8] a new cytotoxic nonadride,[12] and an antifungal polysubstituted benzaldehyde.[9] In this study, we describe the isolation and identification of another two new cyclopeptides from the co-culture broth of the fungi described above, cyclo (D-Pro-L-Tyr-L-Pro-L-Tyr) and cyclo (Gly-L-Phe-L-Pro-L-Tyr). Their antifungal effects against one human-derived fungus and four plant pathogenic fungi were measured in vitro.

MATERIALS AND METHODS

General

Nuclear magnetic resonance (NMR) data were recorded on a Bruker AVANCE 400 spectrometer (Bruker BioSpin GmbH company, Rheinstetten, Germany),
using deuterated dimethyl sulfoxide (DMSO-d$_6$) as solvent and tetramethylsilane as internal standard and coupling constants (J) are in Hz. Electrospray ionization mass (ESIMS) and high resolution ESIMS (HRESIMS) were operated on LCQ-DECA-XP (Thermo USA), and LCMS-IT-TOF (Shimadzu, Japan) mass spectrometers, respectively. The purification of the compounds was carried out on Agilent 1100 HPLC system (Agilent Technologies Co., Ltd, Santa Clara, USA) equipped with a quaternary pump, a diode array detector, and a reverse phase silica gel column (Phenomenex Syngery Hydro-RP, 250 × 100 mm, 5 μm) at a flow rate of 1.0 mL/min. Prefractionation of the extract was carried out using silica gel column (200-300 mesh; Qingdao Haiyang Chemicals Co., Limited, Qingdao, China) and Supelclean™ C-18 solid phase extraction (SPE) tubes. Thin-layer chromatography was performed on precoated silica gel 60 F254 plates (Merck, Germany). All solvents were HPLC grade and used without further purification.

**Fungus and cell material**

The *Phomopsis* sp. K38 and *Alternaria* sp. E33 strains were isolated from Leizhou Peninsula, Guangdong Province, China. Stock cultures were maintained on slant solid commeal seawater agar. Plugs of agar supporting mycelia growth were cut and transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium (glucose 10 g/L, Peptone 2 g/L, yeast extract 1 g/L, NaCl 30 g/L). The flask was incubated at 30°C on a rotary shaker for 5-7 days. The mycelium was aseptically transferred to 500 mL Erlenmeyer flasks containing culture liquid (200 mL). The flasks were then incubated at 30°C for 25 days.

The human fungi (*Candida albicans*) was obtained from Microbial Culture Collection Center of Guangdong Institute of Microbiology of China, and four crop-threatening fungi (*Gaenumannomyces graminis*, *Rhizoctonia cerealis*, *Helminthosporium sativum* and *Fusarium graminearum*) were obtained from College of Science, South China Agricultural University. Microplate reader (TECAN, Inc.). Flat-bottom microtiter plates, 96 well were from Falcon, NJ, USA.

**Extraction and isolation**

The cultures (30 L) were filtered through cheesecloth. The filtrate was concentrated to 3 L, in vacuo below 50°C and extracted 5 times by shaking with an equal volume of ethyl acetate and n-butyl alcohol in sequence, respectively. The combined n-butyl alcohol extract (11.8 g) was subjected to silica gel column chromatography and eluted with petroleum ether–ethyl acetate–methanol (70:30:0, 50:50:0, 0:100:0, 0:90:10, 0:70:30, 0:50:50, and 0:0:100, respectively) to afford fractions 1–7 (0.011, 0.021, 0.032, 0.35, 0.67, 2.8, 5.5 g, respectively). Fraction five (0.67 g) was fractionated using C-18 SPE cartridges eluted with a stepwise gradient of 20%, 40%, 50%, 60%, 70%, 80%, and 100% of methanol in water to yield eight fractions (SubF1-SubF8). Fractions SubF1 to SubF8 were analyzed by NMR spectroscopy and fraction SubF1 (eluted with 20% of methanol in water) showed the presence of the signals of polypeptides in its 1hydrogen-1-NMR (H-NMR) spectrum. Therefore, fraction SubF1 (0.019 g) was purified by reverse phase HPLC (Synergi Hydro 250 × 10 mm column, isocratic elution of 30% methanol-70% distilled water, ultraviolet detector at 254 nm, flow of 1.0 mL/min) to afford 2.1 mg of cyclo (D-Pro-L-Tyro-L-Pro-L-Tyro) (1) and 2.6 mg of cyclo (Gly-L-Phe-L-Pro-L-Tyro) (2).

**1-fluoro-2,4-dinitrophenyl-5-l-alanineamide (Marfey’s reagent) derivatization and absolute stereochemistry**

Peptides (0.5 μmol) were hydrolyzed using 1 mL of 6M HCl in a sealed reaction vial at 100°C for 20 h. Traces of HCl was removed under vacuum. The resulting hydrolysate and amino acid standards were derivatized with 200 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-l-alanineamide (FDAA) in acetone and 40 μL of 1M NaHCO$_3$. The mixtures were heated at 40°C for 1 h. The solution was cooled to room temperature, neutralized with 20 μL of 2M HCl, and evaporated to dryness. Liquid chromatography-mass spectrometry analysis of FDAA-derivatized hydrolysates and standard FDAA-derivatized amino acids were carried out using C8 (4.6 × 150 mm column) with a linear gradient of H$_2$O (0.05% HCOOH)-MeOH (0.05% HCOOH) 60:40-10:90 in 75 min at 0.6 mL/min. The absolute configuration of amino acid residues was determined by comparing the retention time with the standard FDAA-derivatized amino acids.

**Antifungal activity**

Antifungal activity were tested *in vitro* by dilution method,[16,17] against one human fungus (*C. albicans*), and four crop-threatening fungi (*G. graminis*, *R. cerealis*, *H. sativum* and *F. graminearum*). Tested compounds (dissolved in DMSO) were serially diluted using 20% DMSO in sterile water and transferred in duplicate to 96-well flat-bottom microplates. The inocula were prepared by picking 1-3 colonies from agar plates and resuspending in 5 ml sterile water, and filtering through Miracloth (Calbiochem, La Jolla, CA). The optical density at 630 nm of the suspensions was compared to the 0.5 McFarland standard. The microorganisms were diluted in Sabouraud dextrose culture broth to afford final target inocula of 1⁶ CFU/ml. The fungal inocula were added to the samples to achieve a final volume of 200 μl and final sample concentrations from 500 to 20 μg/ml. Positive (Ketoconazole or Triadimefon) and blank (media only) controls were included on each test plate. All organisms were read at 630 nm using the EL-340 Biokinetics reader (Bio-Tek Instruments, Vermont) prior to and after incubation (28°C for 72 h). The minimum
inhibitory concentration was defined as the lowest test concentration that allowed no detectable growth.

RESULTS

Cyclo (D-Pro-L-Tyr-L-Pro-L-Tyr) (1)
White powders, $[\alpha]^2_{D} = 70.5^\circ$ (c 0.01, CH$_3$OH). $^1$H-NMR (400 MHz, DMSO-d$_6$), $^{13}$carbon-13 NMR (C-NMR) (100 MHz, DMSO-d$_6$) are listed in Table 1. ESIMS m/z 521 [M + H]+. HR-ESI-MS m/z 521.2391 ([M + H]+, calcd. 521.2394).

Cyclo (Gly-L-Phe-L-Pro-L-Tyr) (2)
White powders, $[\alpha]^2_{D} - 26.5^\circ$ (c 0.01, CH$_3$OH). $^1$H-NMR (400 MHz, DMSO-d$_6$), $^{13}$C-NMR (100 MHz, DMSO-d$_6$) are listed in Table 1. ESIMS m/z 465 [M + H]+. HR-ESI-MS m/z 465.2134 ([M + H]+, calcd. 465.2132).

DISCUSSION

Cyclo (D-Pro-L-Tyr-L-Pro-L-Tyr) (1) has a molecular formula of C$_{28}$H$_{32}$N$_4$O$_6$ as determined by HRESIMS m/z 521.2391 ([M + H]+, calcd. 521.2394), indicating 15° of unsaturation. The peptide nature of this compound was suggested by the molecular formula, the signals of the $\alpha$-protons with chemical shifts between $\delta$ 3.50 and 4.50 in the $^1$H-NMR spectrum, and four amide carbonyls with chemical shifts at $\delta$ 168.7, 165.5, 165.3 and 168.8 in the $^{13}$C-NMR spectrum. Detailed analyses of the $^1$H- and $^{13}$C-NMR spectral data for one [Table 1],

| Position | $\delta$$_H$ (J in Hz) | $\delta$$_C$ | Position | $\delta$$_H$ (J in Hz) | $\delta$$_C$ |
|----------|------------------------|---------|----------|------------------------|---------|
| 1 (D-Pro1) | 168.7 | 3.23 (m, 2H) | 5 (L-Pro1) | 165.3 | 1 (L-Pro1) | 166.1 |
| 2 (D-Pro1) | 2.85 (dd, 2.4, 10.4 Hz, 1H) | 2 (Gly) | 2 (L-Pro1) | 166.5 |
| 3 (D-Pro1) | a1.59 (m, 1H) | NH (Gly) | 5 (L-Pro1) | 165.5 |
| 4 (D-Pro1) | b1.93 (m, 1H) | 1 (L-Tyr) | 2 (L-Pro1) | 7.85 (br s, 1H) |
| 5 (D-Pro1) | 3.23 (m, 2H) | NH (L-Tyr) | 3 (L-Pro1) | 7.85 (br s, 1H) |
| 1 (L-Tyr1) | 7.85 (br s, 1H) | 3 (L-Tyr) | 2 (L-Pro2) | 58.8 |
| 2 (L-Tyr1) | 4.25 (m, 1H) | 5 (L-Tyr) | 168.8 | 4.02 (dd, 7.2, 8.0 Hz, 1H) |
| 3 (L-Tyr1) | 2.92 (m, 2H) | 5 (L-Tyr) | 168.0 | 1.38 (m, 1H) |
| 4 (L-Tyr1) | 126.2 | 5 (L-Tyr) | 5.80 | 2.01 (m, 1H) |
| 5 (L-Tyr1) | 7.04 (d, 8.4 Hz, 1H) | 6 (L-Tyr) | 6.53 | 1.73 (m, 2H) |
| 6 (L-Tyr1) | 6.85 (d, 8.4 Hz, 1H) | 7 (L-Tyr) | 7.89 (m, 1H) |
| 7 (L-Tyr1) | 9.39 (s, 1H) | 6 (L-Pro) | 169.3 | 1.38 (m, 1H) |
| 1 (L-Pro2) | 168.8 | 6 (L-Pro) | 136.4 | 1.38 (m, 1H) |
| 2 (L-Pro2) | 4.05 (dd, 7.2, 8.0 Hz, 1H) | 7 (L-Pro) | 130.5 | 2.01 (m, 1H) |
| 3 (L-Pro2) | a1.39 (m, 1H) | 6 (L-Pro) | 130.5 | 2.01 (m, 1H) |
| 4 (L-Pro2) | b2.00 (m, 1H) | 6 (L-Pro) | 130.5 | 2.01 (m, 1H) |
| 5 (L-Pro2) | 3.43 (m, 2H) | 6 (L-Pro) | 130.5 | 2.01 (m, 1H) |
| 1 (L-Tyr2) | 165.3 | 6 (L-Pro) | 166.7 |
| NH (L-Tyr2) | 8.13 (br s, 1H) | 3 (L-Phe) | 167.6 |
| 2 (L-Tyr2) | 3.91, (q, 4.8 Hz, 1H) | 2 (L-Phe) | 56.0 | 4.05 (q, 4.8 Hz, 1H) |
| 3 (L-Tyr2) | a2.75 (dd, 4.8, 13.6 Hz, 1H) | 6 (L-Phe) | 39.5 | b2.10 (m, 1H) |
| 4 (L-Tyr2) | b2.90 (m, 1H) | 6 (L-Phe) | 39.5 | b2.10 (m, 1H) |
| 5 (L-Tyr2) | 6.89 (d, 8.4 Hz, 1H) | 5' (L-Phe) | 130.5 | 7.17 (dd, 8.4, 2.4 Hz, 1H) |
| 6 (L-Tyr2) | 6.89 (d, 8.4 Hz, 1H) | 5' (L-Phe) | 130.5 | 7.17 (dd, 8.4, 2.4 Hz, 1H) |
| 6' (L-Tyr2) | 6.68 (d, 8.4 Hz, 1H) | 5' (L-Phe) | 130.5 | 7.17 (dd, 8.4, 2.4 Hz, 1H) |
| 7 (L-Tyr2) | 6.68 (d, 8.4 Hz, 1H) | 5' (L-Phe) | 130.5 | 7.17 (dd, 8.4, 2.4 Hz, 1H) |
| 6 (L-Tyr2) | 6.68 (d, 8.4 Hz, 1H) | 5' (L-Phe) | 130.5 | 7.17 (dd, 8.4, 2.4 Hz, 1H) |
| 7 (L-Tyr2) | OH, 9.25 (br s, 1H) | 5' (L-Phe) | 130.5 | 7.17 (dd, 8.4, 2.4 Hz, 1H) |

NMR: Nuclear magnetic resonance
with the aid of correlated spectroscopy (COSY),
heteronuclear multiple quantum correlation (HSQC), and
heteronuclear multiple bond correlation (HMBC) spectra,
established the presence of two proline and two tyrosine
residues. The amino acids composition accounted for 14
out of the 15° of unsaturation, requiring that one is a cyclic
tetrapeptide. The amino acid sequence of compound 1 was
deduced by an interpretation of the HMBC spectrum. The
signals at lowest field (δ 168.7 and 168.8) in the 13C-NMR
spectrum were assigned to the carbonyls of the proline
units, while the remaining signals (δ 165.5 and 165.3)
to the two tyrosine units. HMBC correlations from the
H-2 (δ 2.85) of D-proline 1 (D-Pro 1) to the carbonyls
at δ 168.7 (D-Pro 1 C-1) and δ 165.5 (L-Tyr1 C-1), the
H-2 (δ 3.91) and -NH group (δ 8.13) of L-tyrosine
2 (L-Tyr 2) to the carbonyls at δ 168.7 (D-Pro1 C-1)
and δ 165.3 (L-Tyr2 C-1), the H-2 (δ 4.05) of L-proline
2 (L-Pro 2) to the carbonyls at δ 168.8 (L-Pro2 C-1)
and δ 165.3 (L-Tyr 2 C-1), the H-2 (δ 4.25) and -NH
group (δ 7.85) of L-tyrosine 1 (L-Tyr 1) to the carbonyls
at δ 168.8 (L-Pro2 C-1) and δ 165.5 (L-Tyr 1 C-1), defined
the amino acid sequence. The planar structure of one
was the same as the known cyclopeptide reported in the
patent, but their H-NMR spectra were quite different.
The H-NMR spectrum of compound 1 contained signals
of two different prolines and two different tyrosines, while
the latter only had signals of one proline and one tyrosine.
Thus, compound 1 was a dissymmetrical cyclic tetrapeptide
whose stereochemistry was different from the known
cyclopeptide with the symmetrical structure. The absolute
configurations of the amino acid residues were established
by acid hydrolysis of the peptide, derivation with FDAA,
Marfey’s reagent, and HPLC analysis of the derivatives. Compared
the retention times for FDAA derivatives of standard D-proline,
L-proline, D-tyrosine, and L-tyrosine, which were observed as 52.15,
50.86, 58.43 and 55.33 min, respectively, showed the two
tyrosine residues both have the L-configuration, while the
two proline residues were L-configured and D-configured,
respectively. Hence, the structure of compound 1 was
established as cyclo (D-Pro-L-Tyr-L-Pro-L-Tyr) [Figure 1].

Cyclo (Gly-L-Phe-L-Pro-L-Tyr) (2) showed a
pseudomolecular ion [M + H] + at m/z 465.2134
Corresponding to a molecular formula of C25H28N2O5 (calcd.
for C25H28N2O5, 465.2132). By detailed evaluation of COSY,
HSQC, and HMBC spectra, four different amino acid
residues were identified: Glycine, phenylalanine, proline,
and tyrosine. Furthermore, the molecular formula required
the peptide to be cyclic. The sequence of amino acids
in compound 2 was determined by HMBC from-NH
group and amino acid α-protons to the carbonyl carbon
of the preceding residue as follows: HMBC correlations
from the H-2 (δ 2.74, 3.33) and -NH group (δ 7.89)
of glycine (Gly) to the carbonyls at δ 166.1 (Gly-C-1)
and δ 165.5 (L-Tyr C-1), the H-2 (δ 4.24) and -NH
group (δ 7.85) of L-tyrosine (L-Tyr) to the carbonyls at δ
165.5 (L-Tyr C-1) and δ 169.3 (L-Pro C-1), the H-2 (δ 4.02)
of L-proline (L-Pro) to the carbonyls at δ 169.3 (L-Pro
C-1) and δ 167.6 (L-Phe C-1), the H-2 (δ 4.05) and -NH
group (δ 8.16) of L-phenylalanine (L-Phe) to the
 carbonyls at δ 167.6 (L-Phe C-1) and δ 166.1 (Gly-C-1).
The absolute configuration of the amino acids was
determined by Marfey’s analysis (the same method used
for compound 1). Compared with the retention times
for FDAA derivatives of standard D-proline (52.15 min),
L-proline (50.86 min), D-tyrosine (58.43 min),
L-tyrosine (55.33 min), D-phenylalanine (69.10 min),
and L-phenylalanine (62.46 min) indicated the
presence of L-phenylalanine, L-proline, and L-tyrosine
in compound 2. Therefore, the structure of compound 2 was
elucidated as cyclo (Gly-L-Phe-L-Pro-L-Tyr) [Figure 2].

Compounds 1 and 2 were evaluated for in vitro antifungal
activity against one human fungus (C. albicans), and four
crop-threatening fungi (G. graminis, R. cerealis, H. sativum

**Figure 1:** The structure of compound 1

**Figure 2:** The structure of compound 2
Table 2: Antifungal activity of compound 1 and 2 (MIC, μg/mL)

| Fungi                  | Samples | Compound 1 | Compound 2 | Positive control |
|-----------------------|---------|------------|------------|------------------|
| *Candida albican*     |         | 35         | 25         | 2*               |
| *Gaeumannomyces graminis* |       | 300        | 200        | 150*             |
| *Rhizoctonia cerealis* |        | 250        | 150        | 100*             |
| *Helminthosporium sativum* |     | 350        | 200        | 120*             |
| *Fusarium graminearum* |        | 400        | 250        | 150*             |

*Ketoconazole used as positive control; *Triadimefon used as positive control.  
MIC: Minimum inhibitory concentration

and *F. graminearum*. The results are summarized in Table 2. Both of them showed moderate to high antifungal activities as compared with the positive control, while compound 2 was more active than compound 1 [Table 2]. Thus, the result suggests that these two tetracyclopeptides are worthy of consideration for the development and research of antifungal agents.

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