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Diketopiperazines from Costa Rican endolichenic fungus *Colpoma* sp. CR1465A

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ABSTRACT

Three new diketopiperazines (**1**–**3**), cyclo(L-Pro-D-*trans*-Hyp) (**1**), cyclo(L-Pro-D-Glu) (**2**), and cyclo(D-Pro-D-Glu) (**3**) and five known diketopiperazines (**4**–**8**) were isolated from the endolichenic fungus *Colpoma* sp. CR1465A identified from the Costa Rican plant *Henriettea tuberculosa* (Melatomataceae). The structures of the new compounds **1**–**3** were elucidated using a combination of extensive spectroscopic analyses, including 2D NMR and HR-MS, and their absolute configurations were determined by a combination of NOESY analysis and Marfey's method. Cyclo(L-Pro-D-*allo*-Thr) (**4**) was recently isolated from a South China Sea marine sponge *Callyspongia* sp., but its NMR spectroscopic data were not reported, and cyclo(L-Pro-L-Asp) (**5**) was previously reported but only as a synthetic product. The NMR data assignments of compounds **4** and **5** are reported for the first time. All of the isolated compounds were tested for antifungal and antimicrobial properties.

Fungi have long been recognized as one of the largest natural sources for a wide variety of drugs, including antibiotic, immunosuppressant and anti-cancer agents.[1,2] Despite their potential application in drug discovery, fungi remain relatively unexplored, as only a small portion of the world's estimated 1.5 million fungal species has contributed pharmaceutically significant compounds. Only ~5% of the world's fungal species have been studied and even fewer have been explored for their chemical potential.[3–5] Among them, endosymbiotic fungi, which live inside vascular plants and other organisms such as lichens, are some of the most under explored.

In our ongoing effort to search for biologically active natural products, we investigated endosymbiotic fungi from Costa Rica's tropical rainforests as part of an International Cooperative Biodiversity Group program.[6–9] In this study, we focused on an endolichenic fungal species, designated as CR1465A, which was isolated from *Henriettea tuberculosa* (Melatomataceae) collected at Área de Conservación Cordillera Volcánica Central in Costa Rica. CR1465A closest relative, based on DNA sequencing, is a *Colpoma* sp. and was subjected to large-scale fermentation and secondary metabolite analyses. The chemical investigation of CR1465A led to the isolation of three new diketopiperazines (**1**–**3**), together with five known diketopiperazines (**4**–**8**) (Fig. 1).[10] The structures of the new compounds were elucidated by extensive spectroscopic analyses, including 1D, 2D NMR and HR-MS, and their absolute configurations were determined by a combination of NOESY analysis and Marfey's method. Compound **4** was recently isolated from South China Sea marine sponge *Callyspongia* sp. without NMR data, and compound **5** was previously reported but only as a synthetic product. The NMR data assignments of compounds **4** and **5** are reported for the first time. This Letter describes the isolation and structural elucidation of compounds **1**–**8**, as well as their antimicrobial activities.

Compound **1** was isolated as a white amorphous powder with a negative specific rotation value, $[\alpha]_D^{25}$ −30.8 (*c* 0.05, H_2O). Its molecular formula was determined to be $C_{10}H_{14}N_2O_3$ from the $[M+H]^+$ peak at *m*/*z* 211.1087 (calcd. for $C_{10}H_{15}N_2O_3$, 211.1083) in the positive-ion HR-ESI-MS spectrum. The IR spectrum of **1** showed a broad hydroxy band at 3387 cm^{-1} and a carbonyl absorption band at 1670 cm^{-1}. The ^1H NMR spectrum (Table 1) of **1** showed signals for three down-field shifted methine protons at δ_H 4.18 (dd, *J* = 7.5, 7.0 Hz), 4.52 (dd, *J* = 4.5, 4.0 Hz), and 4.58

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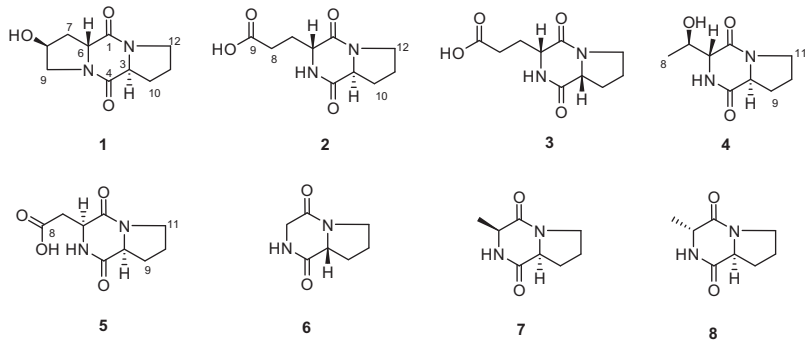

Figure 1. Chemical structures of compounds **1**–**8**.

Table 1
^{1}H and ^{13}C NMR data of compounds **1**–**3** in D_2O[a]

Position	1		2		3	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		173.7		175.0		175.7
2						
3	4.18 dd (7.5, 7.0)	61.5	4.20 dd (7.5, 7.0)	61.7	4.55 dd (9.5, 6.0)	59.3
4		172.3		169.8		172.4
5						
6	4.58 dd (11.5, 6.5)	61.9	4.25 dd (5.0, 4.5)	57.5	4.22 dd (5.0, 4.5)	57.6
7	2.22 m; 2.05 m	38.8	2.06 m	28.3	2.05 m	28.2
8	4.52 dd (4.5, 4.0)	71.0	2.32 m	34.4	2.34 m	34.2
9	3.58 dd (13.0, 4.5) 3.38 br d (13.0)	54.9		183.6		183.4
10	2.20 m; 1.83 m	31.7	2.21 m; 1.82 m	30.6	2.46 m; 2.08 m	31.8
11	1.95 m; 1.88 m	25.0	1.96 m; 1.84 m	24.7	1.88 m; 1.80 m	24.8
12	3.45 dd (11.5, 8.5); 3.33 m	47.8	3.43 m; 3.41 m	48.0	3.57 m; 3.48 m	48.5

[a] ^{1}H and ^{13}C NMR data were recorded at 600 and 150 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

(dd, J = 11.5, 6.5 Hz). The ^{13}C NMR data (Table 1) of **1**, which were assigned by HSQC and HMBC analyses, displayed signals for two carbonyl resonances at δ_C 173.7 and 172.3. Analyses of the ^{1}H–^{1}H COSY, HSQC, and HMBC data obtained for **1** revealed the presence of proline and 4-hydroxyproline (Fig. 2). The HMBC correlations of the α-protons of two amino acid residues at δ_H 4.18 and 4.58 with two carbonyl carbon resonances at δ_C 173.7 and 172.3 indicated that compound **1** was a diketopiperazine composed of the amino acids proline and 4-hydroxyproline (Fig. 2). The full NMR assignments of **1** were determined by ^{1}H–^{1}H COSY, HSQC, and HMBC analyses of the spectroscopic data (Table 1). The stereochemistry of **1** was determined using a combination of NOESY analysis, degradative reaction, and derivatization. The relative configuration of the three stereogenic centers in **1** was established as 3*S**, 6*R**, and 8*S** by NOESY spectral analysis combined with molecular modeling (Fig. 3). The absolute configuration of the proline residue was determined by Marfey's method.[11–13] A small sample of **1** (0.2 mg) was hydrolyzed with 6 N HCl (1 mL) at 110 °C for 15 h and derivatized with Marfey's reagent (N_α-(2,4-dinitro-5-fluorophenyl)-L-alaninamide; L-FDAA). An LC–MS comparison of the Marfey's derivative derived from **1** with Marfey's derivatives prepared from D- or L-proline showed that compound **1** contained L-proline, indicating the absolute configuration of the proline residue α-position as 3*S*. This analysis simultaneously determined the 6*R* and 8*S* configurations based on the relative stereochemistry previously assigned by NOESY. Thus, the structure of **1** was established as a cyclic dipeptide called cyclo(L-Pro-D-*trans*-Hyp).[14]

Compound **2** was isolated as a white amorphous powder with a negative specific rotation value, $[\alpha]_D^{25}$ −5.0 (*c* 0.11, H_2O) and its molecular formula was determined as $C_{10}H_{14}N_2O_4$ from the $[M+H]^+$ peak at *m*/*z* 227.1034 (calcd. for $C_{10}H_{15}N_2O_4$, 227.1032) in the positive-ion HR-ESI-MS spectrum. The ^{1}H NMR spectrum (Table 1) of **2** showed signals for two down-field shifted methine protons at δ_H 4.20 (dd, J = 7.5, 7.0 Hz) and 4.25 (dd, J = 5.0, 4.5 Hz). The ^{13}C NMR data (Table 1) of **1** exhibited signals for two carbonyl resonances at δ_C 175.0 and 169.8. An analysis of the 2D NMR data (COSY, HSQC, and HMBC data) of **2** revealed the presence of glutamic acid and proline (Fig. 2), and the HMBC correlations between the α-protons of the two amino acid residues at δ_H 4.20 and 4.25 and the two carbonyl carbons at δ_C 175.0 and 169.8 enabled us to establish connectivity to a diketopiperazine consisting of glutamic acid and proline (Fig. 2). On the other hand, the ^{1}H and ^{13}C NMR data (Table 1) of **3** were very similar to those of **2**, with apparent slight differences in the chemical shifts and coupling constants for H-3/C-3 and C-4 in **1** compared to those in **2**, suggesting that compound **3** was a stereoisomer of compound **2**. The connectivity of glutamic acid and proline in **3** was also

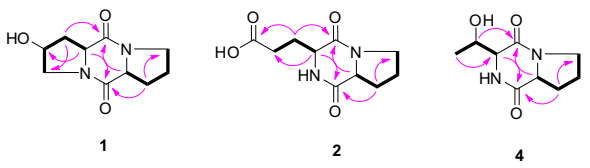

Figure 2. Key ^{1}H–^{1}H COSY (bold) and HMBCs (→) of **1**, **2**, and **4**.

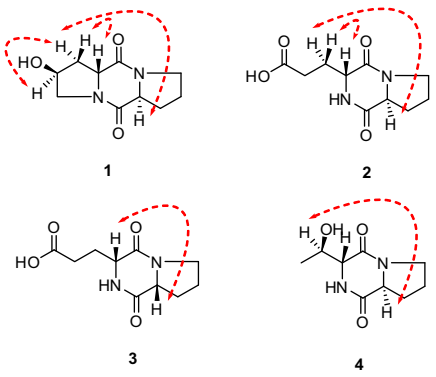

Figure 3. Key NOESY correlations of **1**–**4**.

Table 2
^{1}H and ^{13}C NMR data of compounds **4** and **5** in D_2O[a]

Position	4		5	
	δ_H	δ_C	δ_H	δ_C
1		168.6		171.9
2				
3	4.16 dd (7.5, 7.0)	61.7	4.22 dd (7.5, 7.0)	61.7
4		172.1		170.5
5				
6	4.01 br s	62.8	4.38 dd (5.0, 4.5)	51.8
7	4.32 qd (6.5, 1.5)	68.7	2.72 dd (19.5, 5.0) 2.77 dd (19.5, 4.5)	38.2
8	1.19 d (6.5)	21.3		177.3
9	2.21 m; 1.78 m	31.5	2.21 m; 1.82 m	30.8
10	1.93 m; 1.84 m	24.4	1.96 m; 1.83 m	24.7
11	3.51 m; 3.37 m	48.1	3.43 m; 3.41 m	48.2

[a] ^{1}H and ^{13}C NMR data were recorded at 600 and 150 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

elucidated by the HMBC correlations of each α proton with the amide carbonyl carbon. The stereochemistry of **2** and **3** was established based on Marfey's method and a NOESY analysis.[11–13] The relative configuration of the two stereogenic centers in **2** was assigned 3*S** and 6*R** by the NOESY correlations combined with molecular modeling, whereas the two stereogenic centers of **3** were deduced to be 3*R** and 6*R** in the relative configuration based on the NOESY correlations (Fig. 3). An LC–MS analysis of the Marfey's derivative derived from **2** allowed us to determine that compound **2** possessed L-proline, indicating the absolute configuration of the proline α-position as 3*S*. This result determined a 6*R* configuration of **2** based on the relative stereochemistry previously established in **2**. On the other hand, the LC–MS analysis of the Marfey's derivative derived from **3** allowed determination of the absolute configuration of its α-carbon as 3*R*, which also determined a 6*R* configuration based on the assigned relative configuration of **3**. Thus, the structure of **2** was characterized as a cyclic dipeptide called cyclo(L-Pro-D-Glu)[15] and the structure of **3** was a cyclic dipeptide called cyclo(D-Pro-D-Glu).[16]

Compound **4** was isolated as a white amorphous powder with a negative specific rotation value, $[\alpha]_D^{25}$ −11.0 (*c* 0.05, H_2O). Its molecular formula was established to be $C_9H_{14}N_2O_3$ from the $[M+H]^+$ peak at *m*/*z* 199.1091 (calcd. for $C_9H_{15}N_2O_3$, 199.1083) in the positive-ion HR-ESI-MS spectrum. The ^{1}H and ^{13}C NMR spectra (Table 2) of **4** and the detailed analysis of the 2D NMR data (COSY, HSQC, and HMBC data) of **4** suggested the presence of threonine and proline, whose diketopiperazine structure was further established by COSY and HMBC experiments (Fig. 2). The absolute configuration of **4** was determined by the modified Mosher's method (see Supplementary Data) and the Marfey's method in combination with a NOESY analysis.[11–13] Treating **4** with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] and DMAP in the pyridine produced the (*R*)-MTPA esters **4r**. Similarly, treating **4** with (*R*)-(-)-MTPA-Cl afforded the (*S*)-MTPA ester **4s**. Analysis of the ^{1}H NMR chemical shift differences ($\Delta\delta_{S-R}$) (see Supplementary Data) of the two MTPA esters allowed assignment of the absolute configuration of C-7 as 7*R*. In addition, the LC–MS analysis of the Marfey's derivative derived from **4** allowed assignment of the absolute configuration of C-3 as 3*S*. Finally, the NOESY correlation between H-3 and H-7, but the lack of a NOESY correlation between H-3 and H-6, determined a 6*R* configuration of **4** (Fig. 3). Thus, the structure of **4** was elucidated as a cyclic dipeptide called cyclo(L-Pro-D-*allo*-Thr).[17] A literature survey revealed that compound **4**, cyclo(L-Pro-D-*allo*-Thr), was recently isolated from the South China Sea marine sponge *Callyspongia* sp.,[18] but its NMR spectroscopic data were not reported. This is the first NMR data assignment of cyclo(L-Pro-D-*allo*-Thr).

The known compounds were identified as cyclo(L-Pro-L-Asp) (**5**),[19,20] cyclo(D-Pro-Gly) (**6**),[21] cyclo(L-Pro-L-Ala) (**7**),[22] and cyclo(L-Pro-D-Ala) (**8**),[23] by comparing their spectroscopic and physical data with reported values. Their absolute configurations were established using a combination of NOESY and Marfey's method. On the other hand, cyclo(L-Pro-L-Asp) (**5**) has been previously reported but only as a synthetic product.[20] The NMR data assignment of **5** as a natural product is reported here (Table 2).

The isolated diketopiperazines (**1**–**8**) were tested for antifungal and antimicrobial properties in triplicate against standardized bacterial and yeast strains from the American Type Culture Collection (ATCC) [Gram-negative bacteria; *Escherichia coli* (ATCC 25922), Gram-positive bacteria; *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (BAA-2313) and yeasts; *Saccharomyces cerevisiae* (ATCC 9763) and *Candida albicans* (ATCC 10231)]. In this study, the minimum inhibitory concentration and the minimum fungicidal concentration values of compounds **1**–**8** were determined using broth-dilution techniques. Unfortunately none of the isolates displayed antimicrobial activities at <200 μg/mL.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.03.115.

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10. The EtOH extract of liquid cultures was fractionated by the HP-20 column chromatography eluting with a step gradient of two column volumes of 100% H_2O, 20% MeOH/H_2O, 40% MeOH/H_2O, 60% MeOH/H_2O, 80% MeOH/H_2O, and 100% MeOH to give six fractions (fractions A–F). Fraction C was fractionated by preparative reversed-phase HPLC (C_{18} column, Phenomenex Luna, 250 × 21.2 mm, 5 μm) using 8% CH_3CN/H_2O (+0.1% formic acid) for 20 min, then to 100% CH_3CN (+0.1% formic acid) in the next 10 min, and 100% CH_3CN (+0.1% formic acid) for the next 5 min (flow rate: 10 mL/min) to give 35 fractions (fractions C1–C35). Fractions C11, C12, C13 and C14 were further purified using semi-preparative HPLC (Agilent 1200 Series HPLC system, Phenyl-hexyl column, Phenomenex Luna, 250 × 10.0 mm, 5 μm, flow rate: 2 mL/min). Fraction C11 was purified with 3.3% CH_3CN/H_2O (+0.1% formic acid) to give compounds **4** (0.5 mg, t_R 24.8 min), **5** (0.6 mg, t_R 25.5 min) and **6** (0.8 mg, t_R 21.2 min), and fraction C12 with 4.0% CH_3CN/H_2O (+0.1% formic acid) to give compounds **1** (0.5 mg, t_R 31.8 min) and **8** (0.4 mg, t_R 23.5 min). Fraction C13 was purified using 4.0% CH_3CN/H_2O (+0.1% formic acid) to give **7** (1.4 mg, t_R 30.3 min), and fraction C14 using 5.7% CH_3CN/H_2O (+0.1% formic acid) to give compounds **2** (1.1 mg, t_R 26.7 min) and **3** (0.7 mg, t_R 28.1 min)
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13. Small samples of compounds **1**–**4** (**1**: 0.2 mg; **2**: 0.1 mg; **3**: 0.1 mg; **4**: 0.1 mg) were individually hydrolyzed with 6 N HCl (1 mL) at 110 °C for 15 h. Each reaction was allowed to cool to room temperature, dried *in vacuo*. A solution of Marfey's reagent (20 μL, 10 mg/mL in acetone) was added, followed by 1 N aqueous $NaHCO_3$ (100 μL). Each reaction was heated to 80 °C for 10 min, cooled to room temperature, and acidified with 2 N HCl (50 μL). The reaction mixture was filtered and analyzed by LC–MS using the following gradient; 0–30 min, linear gradient from 10% to 70% CH_3CN/H_2O + 0.1% formic acid; 30–

40 min, linear gradient from 70% to 100% CH_3CN/H_2O + 0.1% formic acid. Standards were prepared from the appropriate authentic D- or L-prolines (0.2 mg) by derivatizing them with Marfey's reagent using the above procedure. The retention times for Marfey's derivatives were as follows: proline (L-Pro, 15.95 min and D-Pro, 16.41 min). Samples prepared from compounds **1**–**4** was also co-injected with standards to confirm their assignment which proved that compounds **1**, **2**, and **4** contain L-proline (**1**: 15.98 min; **2**: 15.95 min; **4**: 15.96 min) while compound **3** contains D-proline (16.40 min). The other known compounds were analyzed using the same procedure.

14. Cyclo(L-Pro-D-*trans*-Hyp) (**1**): White amorphous powder; $[\alpha]_D^{25}$ −30.8 (*c* 0.05, H_2O); UV (MeOH) λ_{max} (log ε) 204 nm; IR (KBr) ν_{max} 3387, 2925, 1670, 1450, 1221, 1032 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR data, see Table 1; ESIMS (positive-ion mode) *m*/*z*: 211 $[M+H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 211.1087 $[M+H]^+$ (Calcd for $C_{10}H_{15}N_2O_3$, 211.1083).
15. Cyclo(L-Pro-D-Glu) (**2**): White amorphous powder; $[\alpha]_D^{25}$ −5.0 (*c* 0.11, H_2O); UV (MeOH) λ_{max} (log ε) 200 nm; IR (KBr) ν_{max} 3365, 2941, 1708, 1672, 1459, 1215, 1030 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR data, see Table 1; ESIMS (positive-ion mode) *m*/*z*: 227 $[M+H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 227.1034 $[M+H]^+$ (Calcd for $C_{10}H_{15}N_2O_4$, 227.1032).
16. Cyclo(D-Pro-D-Glu) (**3**): White amorphous powder; $[\alpha]_D^{25}$ +10.5 (*c* 0.07, H_2O); UV (MeOH) λ_{max} (log ε) 208 nm; IR (KBr) ν_{max} 3366, 2940, 1705, 1670, 1450, 1219, 1030 cm^{-1}; $_1H$ (600 MHz) and ^{13}C (150 MHz) NMR data, see Table 1; ESIMS (positive-ion mode) *m*/*z*: 227 $[M+H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 227.1041 $[M+H]^+$ (Calcd for $C_{10}H_{15}N_2O_4$, 227.1032).
17. Cyclo(L-Pro-D-*allo*-Thr) (**4**): White amorphous powder; $[\alpha]_D^{25}$ −11.0 (*c* = 0.05, H_2O); UV (MeOH) λ_{max} (log ε) 202 nm; IR (KBr) ν_{max} 3385, 2920, 1673, 1452, 1220, 1030 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR data, see Table 2; ESIMS (positive-ion mode) *m*/*z*: 199 $[M+H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 199.1091 $[M+H]^+$ (Calcd for $C_9H_{15}N_2O_3$, 199.1083).
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19. Cyclo(L-Pro-L-Asp) (**5**): White amorphous powder; $[\alpha]_D^{25}$ −25.5 (*c* 0.06, H_2O); UV (MeOH) λ_{max} (log ε) 205 nm; IR (KBr) ν_{max} 3358, 2948, 1710, 1670, 1456, 1222, 1032 cm^{-1}; ^1H (600 MHz) and ^{13}C (150 MHz) NMR data, see Table 2; ESIMS (positive-ion mode) *m*/*z*: 213 $[M+H]^+$. HR-ESIMS (positive-ion mode) *m*/*z*: 213.0872 $[M+H]^+$ (Calcd for $C_9H_{13}N_2O_4$, 213.0875).
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