Cycloanthranilylproline-Derived Constituents from a Myxomycete *Fuligo candida*

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Cycloanthranilylproline (1) and its derivatives (2—4) were isolated from field-collected fruit-bodies of a myxomycete *Fuligo candida* and their structures were elucidated by spectral data. Compound 4, which was contained in the water-soluble fraction of the extract of this myxomycete, was unstable and quite susceptible to decarboxylation to yield compound 2, which was a major constituent of the EtOAc-soluble fraction of this extract.

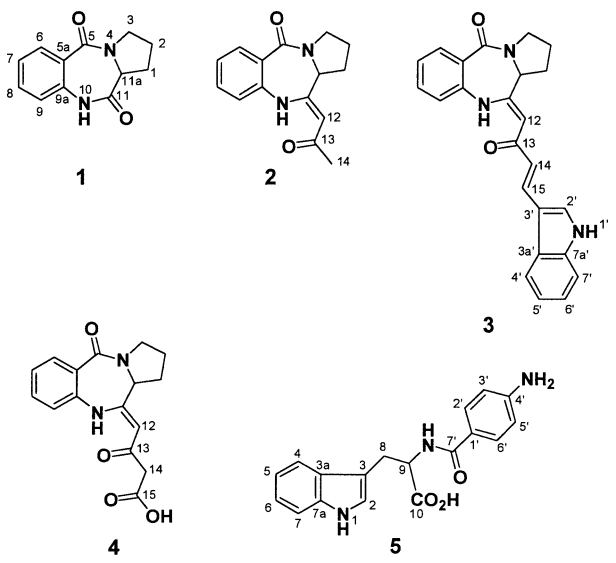
Key words myxomycete; *Fuligo candida*; cycloanthranilylproline

During our studies on search for new secondary metabolites from the myxomycetes (true slime molds),[1)] we have isolated bioactive naphthoquinones[2,3)] and bisindole-derivatives[4)] from field-collected fruit bodies of several myxomycetes. We recently investigated the extract of fruit bodies of *Fuligo candida*, and here we describe isolation and structure elucidation of cycloanthranilylproline (**1**) and its derivatives (**2**—**4**).

The fruit bodies of *Fuligo candida*, collected in Kochi Prefecture, Japan, were extracted with 90% MeOH and 90% acetone. The combined extracts were partitioned between EtOAc and water, and the EtOAc-soluble fraction was then subjected to chromatographies on silica gel, ODS, and Sephadex LH-20 to give cycloanthranilylproline (**1**) and its derivatives (**2**, **3**). From the water-soluble fraction, separation by ODS and silica gel flash chromatographies along with reversed-phase HPLC (Develosil C30-UG-5) afforded an unstable polar cycloanthranilylproline-derivative (**4**), together with 4-aminobenzoyltryptophan (**5**). Compound **1** (=cycloanthranilylproline)[5)] was previously known, and was shown to contain L-proline from the comparison of the sign of optical rotation of **1** in the literature, which was previously obtained from a Cruciferous plant *Isatis indigotica*.[5)]

Compound **2**, which was the major constituent of the EtOAc-soluble fraction and was positive on the Ehlrich-reagent test on TLC, was obtained as colorless plates, and shown to have the molecular formula $C_{15}H_{16}O_2N_2$ by the high resolution (HR)-FAB-MS data (*m/z* 257.1289, $[M+H]^+$, Δ −0.1 mmu). The ^{13}C-NMR spectrum of **2** (Table 1) showed signals for two carbonyls (δ_C 198.2, 165.5), eight sp^2 olefinic or aromatic carbons, one nitrogen-bearing sp^3 methine (δ_C 55.2), and three sp^3 methylenes (δ_C 47.0, 23.3, 26.9), one of which was suggested to be attached to a nitrogen atom from its chemical shift (δ_C 47.0). The ^{1}H-NMR spectrum of **2** showed signals due to four aromatic protons (δ_H 7.02—7.96), and sp^3 methine and methylene protons (δ_H 2.1—3.8). These NMR spectral data were similar to those of cycloanthranilylproline (**1**), and the ^{1}H–^{1}H COSY (H-6/H-7, H-7/H-8, and H-8/H-9; H-11a/H_2-1 and H_2-2/H_2-3) and heteronuclear multiple bond connectivity (HMBC) (H-6/C-5, H-6/C-9a, H-6/C-8, H-7/C-5a, H-7/C-9, H-8/C-6, H-8/C9a, H-9/5a, H-9/C-7, NH-10/C-9a, NH-10/C-5a, and NH-10/C-9; H-11a/C-1, H-11a/C-2, H-11a/C-3, H_2-1/C-11a, H_2-1/C-2, H_2-1/C-3, H_2-2/C-1, H_2-3/C-1, and H_2-3/C-2) spectra of **2** also suggested the presence of anthranilic acid and proline residues. Difference in spectral data of **2** from **1** was the observation of signals due to a conjugated methyl ketone [δ_H 2.19 (3H, s) and 5.29 (1H, s); δ_C 30.0, 198.2, 91.0, and 158.7], which was deduced to be attached to the C-11 position from the HMBC correlations (H_3-14/C-13, H_3-14/C-12, H-12/C-13, H-12/C-11, H-12/C-11a; H-11a/C-11, H-11a/C-12; NH-10/C-12, and NH-10/C-11a). Nuclear Overhauser effect (NOE) correlation was observed between H-12 and one of H_2-1, implying the 11*Z*-configuration. Thus, structure of compound **2** corresponded to that derived from condensation of acetone with compound **1** at C-11 position.

Compound **3** was a yellow pigment, having an absorption maxium at λ_{max} 415 nm, and compound **3** also contained the cycloanthranilylproline moiety, which was revealed from its ^{1}H- and ^{13}C-NMR data (Table 1). In place of the methyl ketone group which was embraced by compound **2**, compound **3** was shown to have an indole moiety as well as cross-conjugated ketone group [δ_C 160.4 (C-11), 93.4 (C-12), 190.2 (C-13), 123.8 (C-14), and 134.9 (C-15); δ_H 5.81 (1H, s; H-12), 7.02 (1H, d, *J*=15.2 Hz, H-14), and 7.92 (1H, d, *J*=15.2 Hz, H-15)] by its ^{1}H- and ^{13}C-NMR data (Table 1) aided by the HMBC spectrum (indole moiety: H-2′/C-3′, H-2′/C-3′a, H-



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2′/C-7′a, H-5′/C-3′a, H-5′/C-7′, H-6′/C-4′, H-6′/C-7′a, H-2′/C-3′, H-7′/C-5′, and H-7′/C-3′a; cross-conjugated ketone group: H-12/C-13, H-14/C-13, and H-15/C-13). The HMBC spectrum also indicated that the cross-conjugated ketone group was attached to C-11 of the cycloanthranilylproline moiety and C-3′ of the indole moiety (H-12/C-11, H-12/C-11a, and H-11a/C-11; H-15/C-2′, and H-15/C-3a′). Therefore, compound **3** was considered to be derived from condensation of a cycloanthranilic acid, an acetone, and an indole-3-carbaldehyde.

Compound **4**, which was positive on the Ehlrich- and Fast Red B-reagent tests on TLC, was isolated from the water-soluble fraction of the extract of this myxomycete. This compound was unstable and proved to be easily converted into compound **2**. The water-soluble fraction of this extract did not initially contain compound **2**. However, after ODS flash chromatography of the water-soluble fraction, compound **2** was obtained substantially. Isolation of compound **4** was carried out carefully by HPLC separation using Develosil C30-UG-5 eluted with 30% MeOH. In DMSO-d_6 solution, compound **4** was almost totally changed into compound **2** during the ^{1}H-NMR experiment. Since compound **4** was not dissolved in chloroform or acetone, NMR studies of compound **4** were carried out in CD_3OD solution, in which conversion from **4** to **2** was slow and not significantly observed. The ^{1}H- and ^{13}C-NMR spectra of compound **4** (Table 1) were almost similar to those of compound **2**, but no signal due to a methyl group was observed for **4**. The electron impact-mass spectra (EI-MS) analysis of compound **4** showed an prominent ion peak at *m/z* 256, which correspond to the molecular ion of compound **2**, thus implying that compound **4** was quite susceptible to fragmentation to yield compound **2** during the EI-MS measurement. In the ^{13}C-NMR spectrum of **4**, a signal of low-field resonance (δ_C 175.8) was observed, which was assignable to a carboxyl group (C-15). The presence of a carboxyl group may be consistent with the fact that compound **4** was polar and contained in the water-soluble fraction. The HMBC spectrum of compound **4** in CD_3OD showed connectivities from δ_H 3.30 to δ_C 92.6 (C-12), 198.1 (C-13), and 175.8 (C-15), suggesting that the signal at δ_H 3.30 was assignable to the hydrogens on C-14 methylene group and compound **4** possesses an acetic acid moiety (CH_2COOH) in place of the methyl group of compound **2**. It was therefore reasonably accounted for that compound **4** possessing a β-keto carboxylic acid moiety may be easily subject to decarboxylation to afford compound **2** possessing a methyl group. This explanation was further corroborated by the liquid chromatography-mass spectrometry (LC-MS) study. A crude fraction mainly containing compound **4** was subjected to LC-MS analysis (Develosil C30-UG-5, 30% MeOH, 1.8 ml/min; t_R 6.8 min; Waters ZQ 2000), which clearly showed intense quasi-molecular ions for **4** at *m/z* 301 $(M+H)^+$ and 323 $(M+Na)^+$.

Compound **5** was another constituent isolated from water-soluble fraction. The molecular formula of compound **5** was revealed as $C_{18}H_{17}N_3O_3$ from the HR-FAB-MS data (*m/z* 324.1349, $[M+H]^+$, Δ −2.4 mmu). In the ^{1}H- and ^{13}C-NMR spectra of **5** signals due to a tryptophan residue and a *p*-substituted benzoic acid residue were observed. The ^{13}C-NMR chemical shift of the C-4′ position (δ_C 153.2) implied that an

Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1**—**4**

	1 ($CDCl_3$)		**2** ($CDCl_3$)		**3** (CD_3COCD_3)		**4** (CD_3OD)	
	δ_H/Hz	δ_C	δ_H/Hz	δ_C	δ_H/Hz	δ_C	δ_H/Hz	δ_C
1	2.00—2.03 m	26.2	2.11—2.16 m	26.9	2.23—2.35 m	29.0	2.06—2.22 m	27.9
	2.75—2.78 m		2.38—2.41 m		2.55—2.63 m	24.1	2.49—2.51 m	
2	2.00—2.03[a] m	23.5	2.10—2.60[a] m	23.3	2.05—2.15 m	47.4	2.06—2.22 m	24.3
3	3.57—3.62 m	47.3	3.61—3.68 m	47.0	3.65—3.73 m	47.4	3.54—3.61 m	48.1
	3.78—3.82 m		3.78—3.84 m		3.55—3.62 m		3.72—3.77 m	
5		165.4		165.5		165.8		167.8
5a		127.1		127.0		128.2		128.1
6	7.99 dd 7.7, 1.4	131.1	7.96 dd 7.8, 1.4	131.1	7.89 dd 7.8, 1.7	131.4	7.85 dd 7.8, 1.5	131.7
7	7.26 td 7.7, 1.0	125.1	7.20 dt 7.8, 1.4	124.2	7.19—7.23 m	124.3	7.16 dt 7.8, 0.9	125.4
8	7.46 td 7.7, 1.4	132.4	7.44 dt 7.8, 1.4	132.3	7.49—7.54 m	133.1	7.44 ddd 7.8, 7.6, 1.5	134.0
9	7.02 d 7.7	121.0	7.02 dd 7.8, 1.4	121.9	7.12 dd 8.1, 1.2	122.6	7.03 d 7.6	123.2
9a		135.2		136.9		138.6		138.7
10	8.68 br s		12.6 br s		10.8[c] br s			
11		171.2		158.7		160.4		160.9
11a	4.07 d 6.1	56.7	4.29 dd 7.8, 2.2	55.2	4.45 dd 8.0, 1.6	56.1	4.39 d 7.6	57.2
12			5.29 s	91.0	5.81 s	93.4	5.65 s	92.6
13				198.2		190.2		198.1
14			2.19[b] s	30.0	7.02 d 15.2	123.8	3.30	54.0[d]
15					7.92 d 15.2	134.9		175.8
1′					13.4[c] br s			
2′					7.83 d 2.7	131.5		
3′						114.4		
3a′						126.4		
4′					8.03 d 6.9	121.2		
5′					7.19—7.23 m	121.6		
6′					7.19—7.23 m	123.4		
7′					7.49—7.54 m	113.0		
7a′						138.6		

a) 2H. *b*) 3H. *c*) Interchangeable signals. *d*) Assignment was based on a weak HMQC correlation.

amino group was attached to this position. The ^1H–^1H COSY and other spectral data were also suggested the structure of compound **5** to be 4-aminobenzoyltryptophan, which was first isolated as a natural product and its full characterization was first described here, although it was previously reported as a reaction product.[6)]

Cycloanthranilylproline derivatives (**2**—**4**) may be considered to contain L-proline since compound **1**, which was coisolated from the same organism here, had L-proline residue. We could not exclude the possibility that compound **2** was an artificial product. Although we used acetone for extraction, it may be likely that compound **2** was not produced through condensation of acetone with compound **1** but it was produced through decarboxylation from compound **4**. Treatment of compound **1** with acetone did not afford compound **2**. Compounds **1** and **2** were cytotoxic against murine leukemia P388 cells *in vitro* with IC_{50} values of 2.9 μg/ml and 13.0 μg/ml, respectively, while compounds **4** and **5** were inactive (IC_{50} >25 μg/ml).[7)]

Experimental

General Procedures UV spectra were obtained on a Hitachi U-3400 spectrometer. NMR spectra were recorded on JEOL JNM ecp600 spectrometers. HR-FAB-MS were acquired on a JMS HX-110 mass spectrometer.

Organism The fruit bodies of *Fuligo candida* were collected at Motoyama-machi in Kochi Prefecture, Japan, in August 2001 and 2002. Voucher specimens (#23060, 23446, and 23522) are maintained by Y. Y. (Ohtsu-ko, Kochi).

Extraction and Isolation The air-dried fruit bodies of *Fuligo candida* collected in 2001 (22.8 g) were extracted with 90% MeOH (500 ml×2) and 90% acetone (500 ml×1). The fruit bodies of *F. candida* collected in 2002 (111.5 g) were extracted with 90% MeOH (500 ml×2) and 90% acetone (500 ml×1). The combined MeOH and acetone extract (11.75 g) was partitioned between EtOAc (200 ml×3) and 8% MeOH in water (216 ml).

The EtOAc-soluble fraction was evaporated under reduce pressure to give a residue (3.96 g), which was subjected to silica gel column chromatography (column A; 3.5×27 cm) eluted with 0—100% acetone in hexane. A fraction (780 mg) of column A eluted with 16—33% acetone/hexane was further separated on an ODS column (column B; 2.5×27 cm) eluted with 33—100% MeOH in water to give compound **2** (385 mg). The fraction (65 mg) of column A eluted with 50—100% acetone/hexane was separated by a Sephadex LH-20 column (column C; 15×53 cm) eluted with methanol to give compound **1** (12.1 mg). Another fraction of column C (6.4 mg) was further purified by HPLC (Develosil ODS-UG-5; 10×250 mm; 50—80% MeOH and 70% CH_3CN) to afford compound **3** (1.5 mg).

The water-soluble fraction (8.22 g) was subjected to separation by an ODS flash chromatography (column D; 4.0×13 cm) eluted with 13—100% MeOH in water. A fraction (553 mg) of column D eluted with 25% aqueous MeOH was further separated by silica gel column chromatography (column E; 2.5×25 cm) eluted with EtOAc/MeOH/H_2O (40 : 3 : 2 to 24 : 4 : 3) to give compound **5** (53.7 mg). Another fraction of column E (195 mg) was further separated by Sephadex LH-20 (1.5×53 cm) followed by fractionation with ODS Sep Pak column to afford compound **2** (39.4 mg) in the fraction eluted with 20—50% aqueous MeOH. A fraction of column D (939 mg) eluted with 13% MeOH was partially (131 mg) purified with HPLC (Develosil C30-UG-5; 10×250 mm; 30% MeOH) to afford compound **4** (26.5 mg).

Compound **1** (Cycloanthranilylproline[5)]): Pale yellow powder; $[\alpha]_D^{23}$ +416° (*c*=1.2, MeOH), lit.[5)] $[\alpha]_D^{20}$ +505° (*c*=0.1, MeOH); ^1H- and ^{13}C-NMR (Table 1); EI-MS *m/z* 216 (M^+).

Compound **2**: Colorless plates; mp 140—144 °C; $[\alpha]_D^{26}$ +657° (*c*=1.40, MeOH); UV λ_{max} (MeOH) 338 nm (ε 60000); CD (MeOH) 229 ($\Delta\varepsilon$ −3.0), 259 (−9.2), 273 (−0.2), 303 (−11.8), and 340 nm (+21.5); ^1H- and ^{13}C-NMR (Table 1); EI-MS *m/z* 256 (M^+); HR-FAB-MS *m/z* 257.1289 [calcd for $C_{15}H_{17}O_2N_2$, (M+H) 257.1290].

Compound **3**: Yellow pigment; $[\alpha]_D^{26}$ +149° (*c*=0.6, MeOH); UV λ_{max} (MeOH) 415 nm (ε 8700); CD (MeOH) 232 ($\Delta\varepsilon$ −0.1), 238 (+0.6), 252 (−0.6), 268 (+0.5), 316 (−2.3), and 413 nm (+1.8); IR (KBr) 3420, 1590, and 1560 cm^{-1}; ^1H- and ^{13}C-NMR (Table 1); electrospray ionization mass spectroscopy (ESI-MS) *m/z* 384 $(M+H)^+$ and 406 $(M+Na)^+$.

Compound **4**: Colorless amorphous solid; $[\alpha]_D^{26}$ +466° (*c*=0.99, MeOH); UV λ_{max} (MeOH) 340 nm (ε 19000); CD (MeOH) 230 ($\Delta\varepsilon$ −2.0), 248 (−1.0), 260 (−5.6), 273 (−0.1), 304 (−8.6), and 343 nm (+16.2); IR (KBr) 3460, 1620, and 1560 cm^{-1}; ^1H- and ^{13}C-NMR (Table 1); ESI-MS *m/z* 301 $(M+H)^+$, 323 $(M+Na)^+$, and 623 $(2M+Na)^+$; HR-FAB-MS *m/z* 301.1187 [calcd for $C_{16}H_{17}O_4N_2$, (M+H) 301.1188].

Compound **5**: $[\alpha]_D^{23}$ −6.8° (*c*=0.76, MeOH); UV λ_{max} (MeOH) 282 nm (ε 12000); IR (KBr) 3340, 1605, and 1510 cm^{-1}; ^1H-NMR (CD_3OD) δ_H 7.11 (1H, s, H-2), 7.57 (1H, d, *J*=8.0 Hz, H-4), 6.99 (1H, t, *J*=8.0 Hz, H-5), 7.06 (1H, t, *J*=8.0 Hz, H-6), 7.30 (1H, t, *J*=8.0 Hz, H-7), 3.44 (1H, dd, *J*=14.6, 5.1 Hz, H-8), 3.30 (1H, m, H′-8), 4.86—4.92 (1H, m, H-9), 7.48 (2H, d, *J*=8.5 Hz, H-2′, H-3′), and 6.59 (2H, d, *J*=8.5 Hz, H-3′, H-5′); ^{13}C-NMR (CD_3OD) δ_C 124.4 (C-2), 111.2 (C-3), 128.9 (C-3a), 119.3 (C-4), 119.8 (C-5), 122.4 (C-6), 112.3 (C-7), 138.0 (C-7a), 28.4 (C-8), 55.2 (C-9), 175.8 (C-10), 122.7 (C-1′), 130.0 (C-2′, C-6′), 114.7 (C-3′, C-5′), 153.2 (C-4′), and 170.1 (C-7′); FAB-MS *m/z* 324 $(M+H)^+$; HR-FAB-MS *m/z* 324.1349 [calcd for $C_{18}H_{17}N_3O_3$, (M+H) 324.1373].

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References

1) Ishibashi M., *J. Synth. Org. Chem. Jpn.*, **61**, 152—163 (2003).
2) Naoe A., Ishibashi M., Yamamoto Y., *Tetrahedron*, **59**, 3433—3435 (2003).
3) Iwata D., Ishibashi M., Yamamoto Y., *J. Nat. Prod.*, **66**, 1611—1612 (2003).
4) Nakatani S., Naoe A., Yamamoto Y., Yamauchi T., Yamaguchi N., Ishibshi M., *Bioorg. Med. Chem. Lett.*, **13**, 2879—2881 (2003).
5) Wu X., Liu Y., Sheng W., Sun J., Qin G., *Planta Medica*, **63**, 55—57 (1997).
6) Brown T. B., Stevens M. F. G., *J. Chem. Soc.*, *Perkin Trans. 1*, **1975**, 2357—2360 (1975).
7) Rho M.-C., Hayashi M., Fukami A., Obata R., Sunazuka T., Tomoda H., Komiyama K., Ōmura S., *J. Antibiot.*, **53**, 1201—1206 (2000).