J. Microbiol. Biotechnol. (2007), **17**(7), 1217–1220

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Antifungal Cyclopeptolide from Fungal Saprophytic Antagonist *Ulocladium atrum*

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Received: January 2, 2007 Accepted: February 19, 2007

Abstract The saprophytic fungus *Ulocladium atrum* Preuss is a promising biological control agent for *Botrytis cinerea* in greenhouse- and field-grown crops. However, despite its known potent antifungal activity, no antifungal substance has yet been reported. In an effort to characterize the antifungal substance from *U. atrum*, we isolated an antibiotic peptide. Based on extensive spectroscopic analyses, its structure was established as a cyclopeptolide with a high portion of *N*-methylated amino acids, and its ^{1}H and ^{13}C chemical shifts were completely assigned based on extensive 1D and 2D NMR experiments. Compound **1** exhibited potent antifungal activity against the plant pathogenic fungus *Botrytis cinerea* and moderate activity against *Alternaria alternate* and *Magnaporthe grisea*.

Keywords: Cyclopeptolide, *Ulocladium atrum*, antifungal substance, biocontrol agent

Botrytis cinerea Pers. is a ubiquitous fungi that causes economically significant diseases in field and greenhouse vegetables, fruits, ornamentals, and other crops throughout the world [1, 5]. The saprophytic fungus *Ulocladium atrum* Preuss is a promising biological control agent for *B. cinerea* in greenhouse- and field-grown crops [2, 3, 4, 7, 9, 13]. The biocontrol effect of *U. atrum* against *B. cinerea* is based on both species interacting and effective suppression of the pathogen's sporulation in necrotic plant tissue [8, 10–12]. It has also been proposed that the production of extracellular hydrolytic enzymes could assist in the biocontrol of *B. cinerea* in necrotic plant tissues by *U. atrum* [2]. Despite its known significant antifungal activity, no antifungal substance has yet been reported. Accordingly, this research investigated the antifungal substance from the fermentation broth of the fungus *U. atrum* and isolated a unique cyclopeptolide **1**, which contained a high portion of *N*-methylated amino acids. In an extensive literature search, **1** was identified as an antifungal substance against yeasts and yeast-like fungi, but not against filamentous fungi [6]. Differently from the previous report, however, **1** exhibited potent antifungal activity against plant pathogenic filamentous fungi. In this paper, we report the isolation, structure determination, complete ^{1}H and ^{13}C assignments, and antifungal activity of **1**.

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Fermentation of *Ulocladium atrum*

The fungal saprophytic antagonist, *U. atrum* (CNU 9055 isolate), isolated earlier from tomato field soil, was used in the present study. Three pieces of an actively growing fungal mycelial mat (approx. 10×10 mm) were transferred from a potato dextrose agar plate to 500-ml Erlenmeyer flasks (totaling 4 l) containing 100 ml of sucrose 5.0%, KH_2PO_4 0.5%, KNO_3 1.0%, $MgSO_4 \cdot 7H_2O$ 0.25%, and $FeCl_3$ 0.002%, and the flasks were shaken on a rotary shaker (150 rpm) at 25°C for 10 days.

Isolation of Antifungal Substance

By using antifungal activity-guided fractionation, **1** was isolated from the fermentation broth of the fungus *U. atrum*. The fermentation broth (4 l) was filtrated to eliminate the mycelium, and then the filtrate was extracted twice with two liters of ethyl acetate. The ethyl acetate-soluble portion was concentrated under reduced pressure and subjected to a column of silica gel eluting with CH_2Cl_2/MeOH (97:3, v/v). An active fraction (850 mg) was rechromatographed on a column of silica gel eluting with CH_2Cl_2:MeOH (93:7, v/v), followed by Sephadex LH-20 column chromatography eluting with CH_2Cl_2/MeOH (1:1, v/v) to afford **1** (85.5 mg).

Structure Determination of Antifungal Substance

Compound **1** was isolated as a colorless amorphous crystalline, and its positive electron spray ionization (ESI)

mass provided quasi-molecular ion peaks at *m/z* 1126.7 $[M+H]^+$ and *m/z* 1148.7 $[M+Na]^+$, suggesting a molecular mass of 1,125 daltons. The molecular formula of **1** was established as $C_{57}H_{91}N_9O_{14}$ by high-resolution ESI mass measurement (*m/z* 1148.6545 $[M+Na]^+$, −3.8 mmu) in combination with ^1H and ^{13}C NMR data. Its UV absorption at 277 nm implied the presence of aromatic functionality. The above physicochemical properties, lipophilicity, and the ^1H and ^{13}C NMR spectra suggested that **1** was a cyclic peptide, which was also supported by the positive reaction of its hydrolysate (6 N HCl, 110°C, 24 h) to a ninhydrin reagent.

The structure of **1** was determined by extensive one- and two-dimensional NMR analyses. In the ^1H NMR spectrum measured at 700 MHz in $CDCl_3$, aromatic methine protons assignable to a 1,4-disubstituted benzene moiety at δ 7.00 (d, *J*=8.4 Hz) and 6.71 (d, *J*=8.4 Hz); three exchangeable amide protons at δ 7.50, 7.15, and 7.00 that were collapsed on shaking with D_2O; 10 α-methine protons between δ 3.0 to 6.4; a methoxyl methyl singlet at δ 3.79; five *N*-methyl singlets at δ 3.45, 3.00, 2.90, 2.79, and 2.48; and several methylene and 11 methyl signals between δ 0.6 to 2.3 were observed. Interestingly, although **1** is a peptidic compound with a relatively large molecular mass, only three amide protons were evident whereas several *N*-methyl signals were observed. The ^{13}C NMR spectrum measured at 176 MHz revealed the presence of 59 well-resolved carbons containing two overlapped sp^2 methines from a 1,4-disubstituted benzene moiety. With the aid of the HMQC spectrum, these peaks were established as 11 carbonyl carbons, one oxygenated

Table 1. ^1H and ^{13}C NMR data of compound **1** in CD_3OD.[a]

No.	δC	δH	No.	δC	δH
2-Hydroxypropanoic acid			α	56.6	5.30 (1H, d, *J*=11.1)
1	172.9		β	32.7	2.22 (1H, m)
2	67.1	5.47 (1H, q, *J*=6.9)[b]	γ (CH_3)	15.2	0.95 (3H, d, *J*=6.6)
3	18.0	1.41 (1H, d, *J*=6.9)	(CH_2)	24.4	1.33 (1H, m)
homoPro					1.08 (1H, m)
α	46.4	5.67 (1H, t, *J*=7.1)	δ	10.0	0.92 (3H, t, *J*=7.5)
β	27.7	1.89 (1H, m)	CO	170.8	
		1.77 (1H, m)	*N*(Me)Iso (2)		
γ	18.6	2.21 (1H, m)	NMe	41.4	3.45 (3H, s)
		1.66 (1H, m)	α	74.6	3.12 (1H, d, *J*=11.6)
δ	25.0	1.94 (1H, m)	β	33.8	2.72 (1H, m)
		1.49 (1H, m)	γ (CH_3)	17.7	1.08 (3H, d, *J*=6.5)
ε	43.3	4.29 (1H, m)	(CH_2)	26.0	1.52 (1H, m)
		3.65 (1H, m)			1.07 (1H, m)
CO	171.3		δ	10.9	0.93 (3H, t, *J*=7.1)
N(Me)Val (1)			CO	170.2	
NMe	28.7	2.79 (3H, s)	Gly		
α	67.0	4.33 (1H, d, *J*=10.5)	NH		7.15 (1H, dd, *J*=5.8, 3.2)
β	26.0	2.45 (1H, m)	α	41.0	4.05 (1H, dd, *J*=17.4, 5.8)
γ	19.5	1.06 (3H, d, *J*=6.4)			3.47 (1H, dd, *J*=17.4, 3.2)
	19.1	0.89 (3H, d, *J*=6.7)	CO	168.4	
CO	167.8		*N*(Me)Val (2)		
Val			NMe	28.4	2.48 (3H, s)
NH		7.00 (1H, d, *J*=10.3)	α	61.5	4.75 (1H, d, *J*=11.0)
α	54.7	4.54 (1H, t, *J*=10.3)	β	26.6	2.15 (1H, m)
β	29.1	2.10 (1H, m)	γ	18.7	0.87 (3H, d, *J*=6.7)
γ	20.1	0.89 (3H, d, *J*=6.7)		18.5	0.69 (3H, d, *J*=6.7)
	18.0	0.81 (3H, d, *J*=6.7)	CO	168.8	
CO	170.7		O(Me)Tyr		
N(Me)Asp			NH		7.50 (1H, d, *J*=9.9)
NMe	30.5	2.90 (3H, s)	α	51.1	5.21 (1H, m)
α	52.1	6.36 (1H, dd, *J*=11.6, 5.4)	β	33.7	3.26 (2H, m)
β	35.1	3.12 (1H, t, *J*=11.6)	γ	129.8	
		2.89 (1H, dd, *J*=11.6, 5.4)	δ	130.2	7.00 (2H, d, *J*=8.4)
γ	172.0		ε	113.1	6.71 (2H, d, *J*=8.4)
CO	169.8		ζ	158.1	
N(Me)Iso (1)			OCH_3	55.3	3.79 (3H, s)
NMe	29.9	3.00 (3H, s)	CO	170.5	

[a]NMR data were measured at 700 MHz for proton and at 176 MHz for carbon.
[b]Proton resonance integral, multiplicity, and coupling constant (*J*=Hz) are in parentheses.

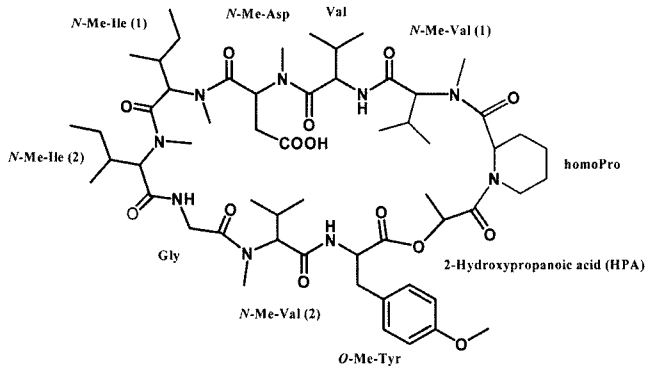

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Fig. 1. Structure of compound **1**.

sp^2 quaternary carbon (δ 158.1), one sp^2 quaternary carbon (δ 129.8), four sp^2 methines for 1,4-disubstituted benzene, nine oxygen- or nitrogen-bearing methine carbons between δ 40–75, two nitrogen-bearing methylene carbons (δ 43.3 and 41.0), one methoxyl methyl carbon at δ 55.3, five nitrogen-bearing methyl carbons (δ 41.4, 30.5, 29.9, 28.7, and 28.4), seven methylene and five methine carbons between δ 18–36, and 11 methyl carbons between δ 10–20. The ^1H and ^{13}C NMR spectral data are summarized in Table 1. Alkyl groups revealing the identity of component amino acid residues were assigned from a combination of the TOCSY and ^1H-^1H COSY spectra. Namely, the partial fragments shown in Fig. 2 suggested the presence of one mole of Ala (finally assigned as 2-hydroxypropanoic acid), homoPro (or Lys), Asp (or Asn), Gly, and Tyr moieties; two moles of Ile moiety; and three moles of Val moiety. HomoPro, but not Lys, was determined by the HMBC correlation from the α-methine proton at δ 5.67 to the ε-methylene carbon at δ 43.3. Methyltyrosine was established by the long-range correlations from the β-protons at δ 3.26 to the carbons at δ 129.8 and 130.2, and from the methoxyl

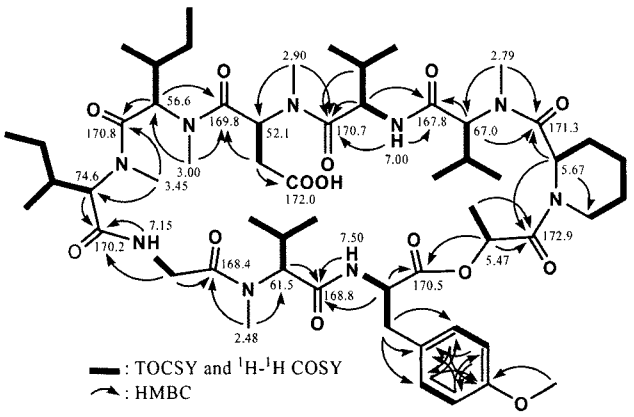

Fig. 2. ^1H-^1H COSY, TOCSY, and HMBC correlations for compound **1**.

methyl protons at δ 3.79 to the quaternary carbon at δ 158.1. Aspartic acid, but not asparagine, was also assigned by the process of elimination. The amino acid sequence of **1** was determined from HMBC correlations of the amide proton and *N*-methyl protons of each amino acid with the carbonyl carbon of an adjacent amino acid; namely, from the α-methine proton of homoPro at δ 5.67 to the carbonyl carbon of HPA at δ 172.9 that was, in turn, correlated with the methine proton of HPA at δ 5.47; from the *N*-methyl protons of *N*-Me-Val (1) at δ 2.79 to the α-methine carbon of *N*-Me-Val (1) and the carbonyl carbon of homoPro at δ 171.3; from the amide proton at δ 7.00 to the carbonyl carbon of *N*-Me-Val (1) at δ 167.8; from the *N*-methyl protons of *N*-Me-Asp at δ 2.90 to the α-methine carbon of *N*-Me-Asp and the carbonyl carbon of Val at δ 170.7; from the *N*-methyl protons of *N*-Me-Ile (1) at δ 3.00 to the α-methine carbon of *N*-Me-Ile (1) and the carbonyl carbon of *N*-Me-Asp at δ 169.8; from the *N*-methyl protons of *N*-Me-Ile (2) at δ 3.45 to the α-methine carbon of *N*-Me-Ile (2) and the carbonyl carbon of *N*-Me-Ile (1) at δ 170.8; from the amide proton of Gly at δ 7.15 to the carbonyl carbon of *N*-Me-Ile (2) at δ 170.2; from the *N*-methyl protons of *N*-Me-Val (2) at δ 2.48 to the α-methine carbon of *N*-Me-Val (2) and the carbonyl carbon of Gly at δ 168.4; and from the amide proton of *O*-Me-Tyr at δ 7.50 to the carbonyl carbon of *N*-Me-Val (2) at δ 168.8, as shown in Fig. 2. From the above results, the sequence of compound **1** was established as HPA-homoPro-*N*MeVal(1)-Val-*N*MeAsp-*N*MeIle(1)-*N*MeIle(2)-Gly-*N*MeVal(1)-*O*MeTyr. Finally, the methine proton of HPA at δ 5.47 was correlated with the carbonyl carbon of *O*-Me-Tyr at δ 170.5, suggesting the planar structure of **1** as shown. In an extensive literature survey, **1** was identified as a cyclopeptolide, which was isolated as an antifungal substance from the fungus *Septoria* sp. NRRL15761 [6]. Although its derivatives were previously synthesized, the isolation, structure determination, and complete ^1H and ^{13}C chemical shift assignments of **1** are reported for the first time in this paper. Compound **1** is a unique cyclopeptolide with a high portion of *N*-methylated lipophilic amino acid residues, which may contribute to its extraordinary lipophilicity.

Antifungal Activity

Compound **1** has been reported as an antifungal substance against yeasts and yeast-like fungi, but not against filamentous fungi [6]. However, we found that **1** exhibited potent antifungal activity against plant pathogenic filamentous fungi. In particular, **1** exhibited specific and potent antifungal activity against the plant pathogenic fungi *Botrytis cinerea*, *B. elliptica*, *Alternaria alternate*, and *Magnaporthe grisea* with an MIC of 33.3 μM, and moderate activity against *Colletotrichum acutatum* and *C. gloeosporioides* with an MIC of 100 μM. Compound **1** also inhibited the conidial germination and growth of the germ tube of *B. cinerea*, in a

dose-dependent manner. Compound **1**, however, showed no activity up to 100 μM against *Fusarium oxysporum*, *Phytophthora infestans*, and *Rhizoctonia solani*.

Acknowledgment

This work was supported by a grant from the BioGreen 21 Program of the Rural Development Administration, Republic of Korea.

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