WF-10129, A NOVEL ANGIOTENSIN CONVERTING ENZYME INHIBITOR PRODUCED BY A FUNGUS, *DORATOMYCES PUTREDINIS*

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WF-10129 is an angiotensin converting enzyme (ACE) inhibitor produced by *Doratomyces putredinis*. IC_{50} of the compound is 1.4×10^{-8} M for the ACE activity. WF-10129 was purified from cultured filtrate by successive ion exchange chromatography and HPLC. The chemical structure **1** was elucidated on the basis of spectroscopic and chemical evidence. The compound is a dipeptide composed of L-tyrosine and a novel amino acid. WF-10129 inhibits the pressor response of angiotensin I when administered intravenously at 0.3 mg/kg in rats.

Since the discovery and development of captopril as a clinically important anti-hypertensive drug, many research groups have screened for novel angiotensin converting enzyme (ACE) inhibitors from microbial products. Many compounds which belong to a variety of chemical entities have been reported[1~11]. We recently discovered WF-10129 during our screening program searching for novel angiotensin converting enzyme inhibitor from fungal products. This paper describes taxonomic studies on the producing strain, fermentation and isolation procedures. We also describe the structural elucidation of WF-10129 and biological characterization of the inhibitor.

Materials and Methods

Fermentation

Seed flasks (500 ml), containing 100 ml of the seed medium consisting of corn starch 3%, gluten meal 1%, cotton seed flour 1%, dried yeast 1%, corn steep liquor 1% and $CaCO_3$ 0.2% were inoculated with growth from a well-grown slant culture of *Doratomyces putredinis* F-10129. The flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) for 4 days at 25°C. The content of the flasks was used to inoculate 20 liters of fermentation medium in a 30-liter stainless steel fermentor. The composition of the production medium was as follows; corn starch 4%, gluten meal 3%, dried yeast 0.5%, $MgSO_4\cdot7H_2O$ 0.05% and $CaSO_4\cdot2H_2O$ 0.2%. The pH of the medium was adjusted to 7.0 before sterilization. Fermentation was allowed to proceed for 7 days at 25°C. At 3 days of cultivation, 400 g of glucose was added to the fermentator. The production of active compound in the fermentation broth was monitored by measuring its enzyme inhibitory activity.

ACE Assay

The ACE activity was measured fluorimetrically according to the method of CARMEL *et al.*[12] with slight modification. *o*-Aminobenzoylglycyl-*p*-nitro-L-phenylalanyl-L-proline (ABz-Gly-Phe(NO_2)-Pro) was used as a substrate and diluted guinea pig serum was used as an enzyme source. The substrate was synthesized in our laboratories. In the routine assay, 200 μl of substrate solution containing 0.58 mM of ABz-Gly-Phe(NO_2)-Pro, 1 M NaCl and 0.2 M Tris-HCl buffer, pH 8.2, was mixed with 10 μl of fermentation broth and 40 μl of diluted guinea pig serum. In the kinetic studies of Dixon plot or Lineweaver-Burk plot, 150 μl of substrate solution which contained several concentration of ABz-

Gly-Phe(NO_2)-Pro, 1 M NaCl and 0.2 M Tris-HCl buffer, pH 8.2, was mixed with 50 μl of inhibitor solution and 50 μl of diluted guinea pig serum.

The Pressor Response of Angiotensin I

A group of four male Sprague-Dowley rats (7 weeks old) was anesthetized with urethan (700 mg/kg, ip) and the mean arterial blood pressure was recorded from femoral artery *via* polyethylene catheter connected to the pressure tranducer which was coupled to a Biophysiograph 180 system (Nihondenki-San-Ei Instrument Co., Ltd.). Drug and angiotensin I were administered intravenously from the cannula which was introduced into the femoral vein. The inhibition of a pressure response provoked by 1 μg/kg iv angiotensin I was assessed.

Results

Identification of Strain F-10129

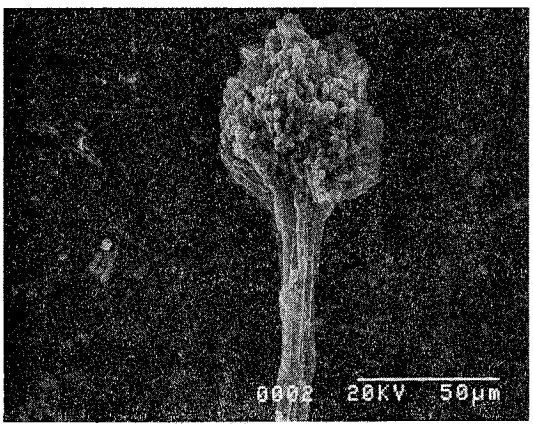
The strain F-10129 was originally isolated from a soil sample collected at Ayabe City, Kyoto Prefecture, Japan.

The strain F-10129 was a hyphomycete fungus, and formed mononematous and synnematous anamorphs on various culture media. The conidiogenesis was holoblastic, and the conidiogenous cell development was percurrent. Its mycological characteristics were as follows.

The synnemata, measuring 150~400×10~30 μm, consisted of a sterile stalk and a fertile head, and produced dry conidial columnars up to 800 μm in length (Fig. 1). The conidiophores were formed from aerial hyphae as right angle branches or at the upper quarter part of synnemata, and verticillately branched in penicillate fashion. They were hyaline, smooth, 70~150 μm long and 4~5 μm thick, and formed one to five annellophores at the tip of each branch. The annellophores were hyaline, smooth, lageniform to cylindrical, 7~15 μm long and 3~4 μm thick. The conidia were produced in long basipetal chains forming pale yellow columnars. They were hyaline, smooth, unicellular, ellipsoidal to ovoid, rounded at the apex and truncated at the base, 3.5~8 μm long and 2~4 μm thick.

Colonies on Potato dextrose agar (Difco) grew restrictedly, attaining 2.0 cm in diameter after two weeks at 25°C, and the surface was plane, thin, powdery and pale yellow. The reverse of the colony was the same. This strain could grow at a temperature range from 4 to 35°C with optimum growth at 26°C.

Fig. 1. Scanning electron micrograph of anamorph of the strain F-10129.



By comparison of the above-mentioned characteristics with the description of UDAGAWA and HORIE[13], the strain F-10129 was identified as one strain of *Doratomyces putredinis* (Corda) Morton et G. Smith, and named *Doratomyces putredinis* F-10129. This strain was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM P-7302.

Production of WF-10129 by *Doratomyces putredinis*

The growth of *Doratomyces putredinis* was monitored by packed mycelium volume measured

by centrifugation of cultured broth at 3,000 rpm for 10 minutes.

The production of WF-10129 was monitored by the inhibition of angiotensin converting enzyme and assessed from the standard curve of the purified WF-10129. A time course of WF-10129 production is shown in Fig. 2. The production of WF-10129 was increased by addition of 2% of sterilized glucose (400 g/20 liter medium) at 3 days of cultivation. Maximum production of WF-10129 at 6 days was approximately 10 μg/ml.

Isolation and Characterization

The procedures of the isolation are summarized in Fig. 3. The cultured broth was filtered with the aid of diatomaceous earth. The filtrate (40 liters) was applied to a column of Dowex 1-X2 (OH^-). The active principle was eluted with 0.1 N HCl solution after washing the column with 0.3 N NaOH solution. The eluate was passed through a column of Dowex 50W-X2 (H^+). The column was washed with 0.05 M NaCl and the activity was eluted with 0.1 M NaCl. The eluate was adsorbed to activated carbon and the active principle was eluted with 80% aqueous acetone. The desalted eluate was charged on a column of DEAE-Sephadex A-25 (PO_4^{---}) and eluted with 0.03 M NaCl after washing the column with 0.01 M NaCl. The active fraction was adjusted to pH 2.0 with 6 N HCl

Fig. 2. Time course of WF-10129 production.
● Potency, ○ pH, △ packed mycelium volume (PMV).

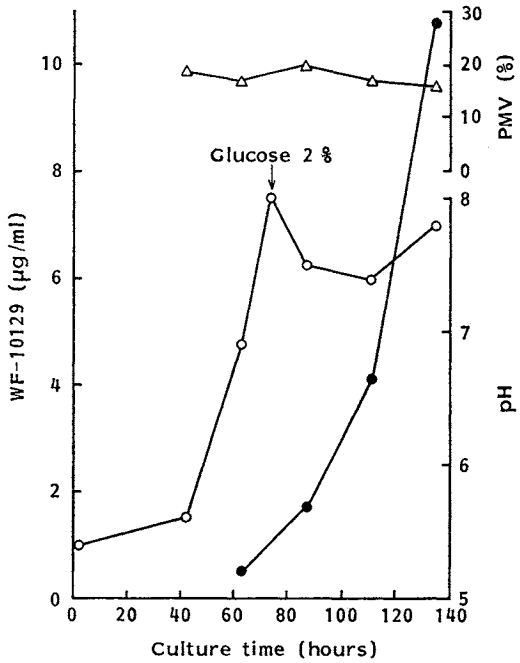


Fig. 3. Isolation procedure of WF-10129.

Filtrate
↓
Dowex 1-X2 (OH^-)
↓ eluted with 0.1 N HCl
Dowex 50W-X2 (H^+)
↓ eluted with 0.1 M NaCl
Active carbon
↓ eluted with 80% Me_2CO
DEAE-Sephadex A-25 (PO_4^{---})
↓ eluted with 0.03 M NaCl, pH was adjusted to 2
Diaion HP-20
↓ eluted with 70% aq MeOH
CM-Sephadex C-25 (H^+)
↓ eluted with H_2O
Sephadex G-15
↓ eluted with 1% AcOH
HPLC

Table 1. Isolation of WF-10129 by HPLC.

Stationary phase	Cosmosil C_{18} (10×250 mm, Nakarai Chemicals Co., Ltd.)
Mobile phase	8% Acetonitrile in 0.05% TFA
Flow rate	5 ml/minute
Detector	UV at 254 nm
Retention time	20 minutes

and adsorbed to Diaion HP-20 for desalting and the activity was eluated with 70% aqueous MeOH. The eluate was concentrated *in vacuo* to a small volume and was chromatographed on a column of CM-Sephadex C-25 (H^+) and eluted with H_2O. The active fraction was subjected to a column chromatography of Sephadex G-15 and developed with 1% acetic acid solution. The active fractions were combined and purified by preparative HPLC. HPLC was carried out on the condition summarized in Table 1. The active fractions obtained by HPLC was lyophilized to give a white powder. The yield from 40 liters filtrate was 17 mg.

Physico-chemical Properties and Structural Elucidation

The physico-chemical properties of WF-10129 (**1**) are summarized in Table 2. The amphoteric substance, WF-10129, which is soluble in H_2O and MeOH, while insoluble in hexane, benzene and

Table 2. Physico-chemical properties of WF-10129 (**1**).

Appearance	Colorless powder
MP	90~95°C
Molecular formula	$C_{20}H_{28}N_2O_8$
SI-MS (*m/z*)	425 $(M+H)^+$, 447 $(M+Na)^+$
Specific rotation	$[\alpha]_D^{23}$ +12.9° (*c* 0.375, H_2O)
UV $\lambda_{max}^{H_2O}$ nm (ε)	275 (1,060), 280 (930)
$\lambda_{max}^{H_2O+HCl}$ nm	275, 280
$\lambda_{max}^{H_2O+NaOH}$ nm	277, 283, 292
IR ν_{max}^{KBr} cm^{-1}	3350, 2950, 3000~2300 (br), 1720, 1680, 1620, 1540, 1520, 1445, 1380, 1350, 1260, 1230, 1110, 910, 880, 830
TLC* (Rf value)	
BuOH - AcOH - H_2O (4 : 1 : 2)	0.4
2-PrOH - H_2O (8 : 2)	0.6

* Stationary phase: Silica gel sheet.

Table 3. ^{13}C NMR chemical shifts (ppm) and multiplicities in D_2O for WF-10129 (**1**).

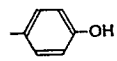
Chemical shift	Multiplicity	Assignment
211.0	s	-CO-
174.7	s	-COO(N)-
171.2	s	
169.4	s	
155.1	s	
131.2 (×2)	d	
129.1	s	
116.0 (×2)	d	
67.5	d	-CHOH
58.2	d	-NHCHCO-
56.7	d	
54.7	d	
42.2	t	$-CH_2-$
38.5	t	
36.9	t	
32.1	t	
22.3	q	$-CH_3$
16.7	q	

Fig. 4. The partial structures A~D and the ^1H-^1H relationships (Hz) in **1**.

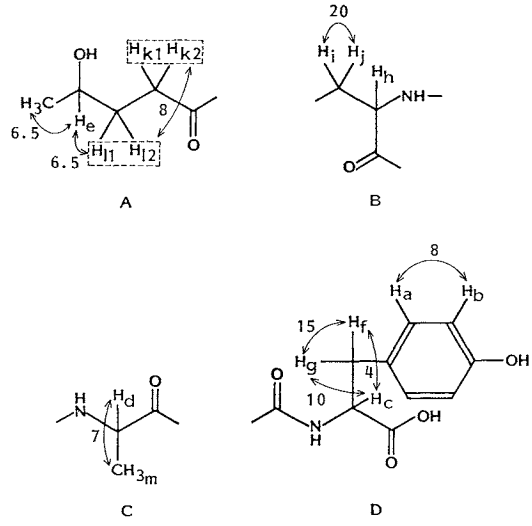
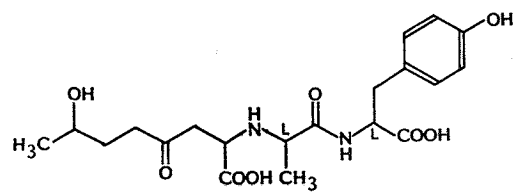


Table 4. ^{1}H NMR chemical shifts, multiplicities, and coupling constants (J, Hz) for **1** and **3**.

Proton	**1**[a]			**3**[b]		
a	7.17	d	(2H, J=8)	7.16	d	(2H, J=8)
b	6.83	d	(2H, J=8)	7.00	d	(2H, J=8)
c	4.75	dd	(J=10, 4)	4.80	m	
d	4.08	q	(J=7)	3.20	m	
e	3.79	m		4.89	m	
f	3.32	dd	(J=15, 4)	3.20	m	(2H)
g	2.78	dd	(J=15, 10)			
h	2.97	m	(2H)	3.53	t	(J=6)
i						
j	3.17	dd	(J=20, 6.5)	2.79	d	(2H, J=6)
k1	2.55	t	(2H, J=8)	2.43	m	(2H)
k2						
l1	1.67	m	(2H)	1.82	m	(2H)
l2						
m	1.52	d	(3H, J=7)	1.21[c]	d	(3H, J=7)
n	1.16	d	(3H, J=6.5)	1.22[c]	d	(3H, J=7)
o				7.90	d	(J=8)
$COOCH_3$				3.74	s	(3H)
				3.70	s	(3H)
$OCOCH_3$				2.29	s	(3H)
				2.02	s	(3H)

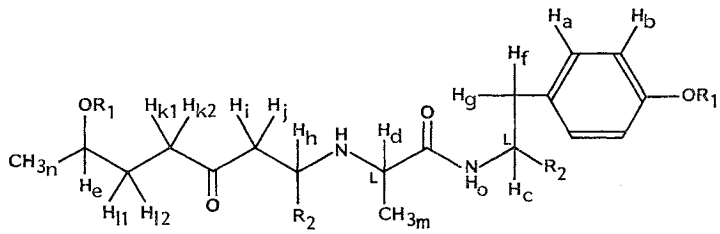
[a] 400 MHz, CD_3OD - D_2O.
[b] 200 MHz, $CDCl_3$.
[c] Assignments may be interchanged.

$CHCl_3$, gave a positive reaction to ninhydrin, Molish, cerium sulfate and iodine, though negative to Dragendorff reagents. The ^{13}C NMR spectrum (D_2O, Table 3) of **1** showed in the sp^2-carbon region 8 signals including four carbonyl carbon signals, one (211.0 (s) ppm) of which is attributable to a ketone function. The remaining four signals at 155.1 (s), 131.2 (d×2), 129.1 (s), 116.0 (d×2) ppm indicated the presence of 1,4-disubstituted phenyl ring in **1**. In the sp^3-carbon region ten signals were assignable to one secondary alcohol carbon (67.5 (d) ppm), three α-amino acid carbons (58.2 (d), 56.7 (d), 54.7 (d) ppm), four methylene carbons (42.2 (t), 38.5 (t), 36.9 (t), 32.1 (t) ppm) and two methyl carbons (22.3 (q), 16.7 (q) ppm). These data together with secondary ion mass spectrum (SI-MS) data (Table 2) disclosed the molecular formula ($C_{20}H_{28}N_2O_8$) of **1**.



1

Extensive spin decoupling of the 400 MHz ^{1}H NMR spectra (Table 4) of **1** revealed ^{1}H-^{1}H relationships as shown in Fig. 4, leading to partial structures A, B, C and D, which are quite consistent with the ^{13}C NMR data described above. The units C and D were confirmed by the fact that hydrolysis of **1** with 6 N HCl (110°C, 22 hours) gave after chromatography on ion-exchange column of Dowex 50W-X8 (0.2 M pyridine - AcOH, pH 3.07) L-alanine and L-tyrosine (*ca.* 1 : 1), which were identified by comparison with authentic samples in amino acid analysis and CD spectra. **1** was subjected to hydrazinolysis (100°C, 6 hours) to provide tyrosine as a sole amino acid product, indicating that L-tyrosine contained in peptide **1** is located at the *C*-terminus.



1 R_1 = H R_2 = COOH

2 R_1 = H R_2 = $COOCH_3$

3 R_1 = $COCH_3$ R_2 = $COOCH_3$

Fig. 5. Fragmentations in the FAB- and SI-MS of **1**.

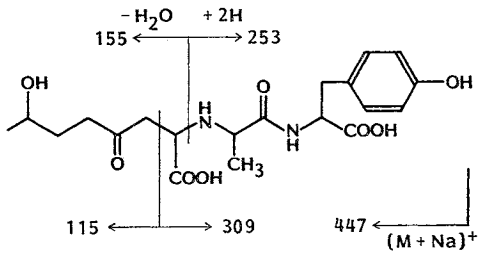


Fig. 7. Lineweaver-Burk plot of inhibition of ACE by WF-10129.

WF-10129 concentration: ● No inhibitor, ○ WF-10129 (1×10^{-8} M), ▲ WF-10129 (2×10^{-8} M).

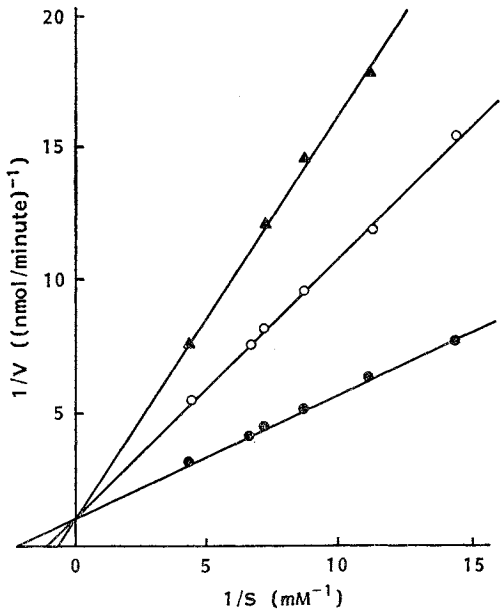
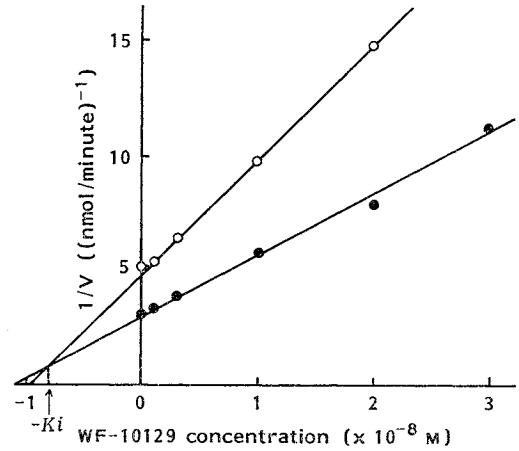


Fig. 6. Dixon plot of inhibition of ACE by WF-10129.

Substrate concentration (ABz-Gly-Phe(NO_2)-Pro): ● 0.23 mM, ○ 0.115 mM.



Treatment of **1** with excess CH_2N_2 in MeOH-ether for 10 minutes at 0°C gave a dimethyl ester derivative **2** (field desorption mass spectra (FD-MS) *m/z* 453 $(M+H)^+$, 475 $(M+Na)^+$; IR ($CHCl_3$) cm^{-1} 1735, 1660; ^1H NMR ($CDCl_3$) 3.68 (3H, s), 3.76 (3H, s)), which was acetylated with Ac_2O - pyridine (room temperature 20 hours) to lead to the diacetyl dimethyl ester derivative **3** (SI-MS *m/z* 537 $(M+H)^+$; ninhydrin positive). The ^1H NMR spectrum ($CDCl_3$) of **3** was analyzed with the aid of spin decoupling experiments and ^1H shift correlation spectrum, resulting in the assignments of proton signals as shown in Table 4.

Table 5. Inhibition of angiotensin I pressor response by WF-10129 in rats.

Dose	Inhibition (%) of angiotensin I* response (mean±SE, n=4)		
	10 minutes	40 minutes	50 minutes
300 μg/kg iv	74.4±5.9	36.9±6.5	25.8±10.3

* 1 μg/kg iv.

In this ^1H shift correlation spectrum, a long range coupling between two methylene protons resonated at 2.43 (H_{k1} and H_{k2}) and 2.79 (H_i and H_j) ppm was observed. These data made it possible to connect the partial structures A, B, C and D in WF-10129 and to lead to the full structure **1**. The deduced structure **1** was assisted by analysis of fragmentation peaks in the fast atom bombardment (FAB)- and SI-MS as shown in Fig. 5.

Biochemical Properties

WF-10129 inhibited ACE in a dose dependent manner and IC_{50} was 1.4×10^{-8} M. IC_{50} for captopril was 1.7×10^{-8} M when assessed in the same experiment. The kinetic study of WF-10129 was carried out and Dixon plot and Lineweaver-Burk plot for ACE inhibition are shown in Figs. 6 and 7. WF-10129 was a competitive inhibitor of ACE with a *Ki* value of 8×10^{-9} M.

Inhibition of the Pressor Response Induced by Angiotensin I

WF-10129 was tested intravenously at a dose of 0.3 mg/kg in anesthetized normotensive rats. As shown in Table 5, WF-10129 inhibited the angiotensin I induced pressor response significantly but did not reveal any effect on angiotensin II (0.3 μg/kg) induced pressor response.

Discussion

Most of the currently reported ACE inhibitors of microbial origin were produced by the strains of actinomycetes[2~11] and only aspergillomarasmines A and B were originated from the culture broth of a fungus[1]. WF-10129 was isolated as a fungal product of *Doratomyces putredinis*. The structure was deduced to be a novel dipeptide shown as **1**. IC_{50} of WF-10129 for ACE is 1.4×10^{-8} M and the potency is almost the same with that of captopril, indicating WF-10129 is one of the most potent ACE inhibitor which has been isolated from microbial product. It is of interest to note that WF-10129 (**1**), an ACE inhibitor found from microbial origin is a substituted *N*-carboxymethyl dipeptide which is similar in structure[14] with the synthetic potent ACE inhibitor MK421, enalapril.

Acknowledgment

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