Cyclo(Dehydroala-L-Leu), an α -Glucosidase Inhibitor from

Penicillium sp. F70614

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A diketopiperazine (1) has been isolated from the culture broth of *Penicillium* sp. F70614 and its structure has been determined to be *cyclo*(dehydroala-L-Leu) by various spectroscopic analyses. This compound selectively inhibited yeast α -glucosidase and porcine intestinal α glucosidase with IC₅₀ values of 35 and 50 µg/ml, respectively. However, it did not show significant inhibitory effects against almond β -glucosidase, *Aspergillus* α -galactosidase, *Escherichia coli* β -galactosidase and jack bean α -mannosidase.

Glucosidase catalyzes the hydrolysis of alkyl/or aryl glycosides. This hydrolytic enzyme plays physiologically important roles in the digestive process for carbohydrates and processing steps for antigenic sugar chains expressed on the cell surface¹). α -Glucosidase is among the most important carbohydrate-splitting enzymes and also catalyzes the hydrolysis of the α -glucose linkage in the final step of the digestive process of carbohydrates²).

The most beneficial therapy for noninsulin-dependent diabetes mellitus is to control optimal blood glucose concentration after a meal³⁾. α -Glucosidase inhibitors could retard the use of dietary carbohydrates to suppress postprandial hyperglycemia. Thus α -glucosidase inhibitors have been tested for their potential treatment of diabetes melitus^{4,5)}.

In the course of our search for specific inhibitors against α -glucosidase from microbial metabolites, we have isolated a diketopiperazine (1) from *Penicillium* sp. F70614 (Fig. 1). In this paper, we describe the identification and fermentation of the producing strain, isolation, structure identification and inhibitory activity of 1 against different glycosidases.

Materials and Methods

General Experimental Procedures

Specific rotation was determined using a Polartronic polarimeter. High resolution EIMS spectrum was measured using a JEOL JMS-SX 102A spectrometer operating at 70 eV. UV and IR spectra were recorded on a Shimadzu UV-260 and a FT-IR Equinox 55 spectrophotometer, respectively. NMR spectra were obtained on a Varian UNITY 500 NMR spectrometer with ¹H NMR at 500 MHz and with ¹³C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as an internal standard. Analytical silica

Fig. 1. Structure of compound 1.



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gel TLC (Merck, Kiesel gel 60 F_{254} , 0.25 mm) plate was used without activation. HPLC was performed on a Senshu pak ODS column (20×250 mm) with flow rate of 3 ml/minute using 50% aqueous MeOH and by monitoring with a photodiode-array detector.

Microorganism and Taxonomic Studies

The compound **1** producing fungal strain F70614 was isolated from a soil sample collected at Yusong, Chungnam province, Korea. This strain has been deposited in the Korea Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Korea, under the accession number KCTC 8918P.

Morphological characteristics of the spores and mycelia were observed with a scanning electron microscope (Philips SEM 515) and a light microscope (Nikon Labophot-2). Cultural characteristics were determined using the culture grown at 25°C for 7 days on potato dextrose agar (PDA), MEA agar (malt extract 2.0%, peptone 0.1%, dextrose 2.0%, agar 2.0%) and Czapek-Dox agar (K₂HPO₄ 0.1%, NaNO₃ 3.0%, KC1 0.5%, MgSO₄·7H₂O 0.5%, FeSO₄· 7H₂O 0.01%, sucrose 3.0%, agar 1.5%).

Fermentation

A loopful of the slant culture of strain F70614 grown on PDA agar was inoculated into a 500 ml Erlenmeyer flask containing of 100 ml of the seed medium composed of glucose 2.0%, yeast extract 0.2%, polypeptone 0.5%, MgSO₄ 0.05% and KH₂PO₄ 0.1% at pH 6.0. The seed culture was fermentated at 25°C for 3 days on a rotary shaker at 150 rpm. The seed culture was transferred into a 5-liter jar fermentor containing 3 liters of the same medium as the above. The fermentation was carried out at 25°C for 7 days employing aeration at 0.1 v/v and agitation at 300 rpm.

Synthesis of Compound 1

The synthesis of **1** was performed following two procedures. *Cyclo*(L-Ser-L-Leu) was prepared by the method of NITECKI, *et al.*⁶, which is known to proceed without racemization. The dehydroalanine (Deala) moiety was generated by dehydration from the side chain of serine according to a procedure of LEE, *et al.*⁷.

Cyclo(L-Ser-L-Leu). To a chilled solution of *t*-Boc-serine (2 mmol) with equimolar concentrations of L-leucine methyl ester, and triethylamine in methylene chloride (10 ml) was added *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (2 mmol). After overnight at -5° C, the reaction mixture was washed with water, citric acid (1 N) and sodium bicarbonate, and evaporated to

dryness. The *t*-Boc-dipeptide methyl ester was kept in formic acid at room temperature for 2 hours. After removal of formic acid *in vacuo*, the residue containing the crude dipeptide ester formate was dissolved in *sec*-butyl alcohol and toluene, and then cyclization of the dipeptide was achieved by boiling for 3 hours, yielding *cyclo*(L-Leu-L-Ser).

Cyclo(Deala-L-Leu). To a solution of cyclo(L-Leu-L-Ser) in pyridine added Tos-chloride on ice bath. After being allowed to stand at 0°C for 12 hours, the solvent was evaporated. The resulting solid was washed with a mixture of ether: petroleum ether (1:1) and dried. The crude cyclo(L-Leu-L-Ser (Tos)) was dissolved in dimethylformamide and to the solution added diethylformamide. After being allowed to stand at 25°C for 5 hours, cyclo-(Deala-L-Leu) was purified by preparative HPLC.

Glycosidase Activity

para-Nitrophenyl (PNP) α -, and β -glucopyranoside, and commercial glycosidases were purchased from Sigma. Porcine intestinal glucosidases were prepared by the method of PAN and ELBEIN⁸⁾.

The enzymatic activities of glycosidases were determined colorimetically by monitoring the release of *p*-nitrophenol from the appropriate PNP-glycoside substrate. The reaction mixture contained 5 mM of PNP-glycoside, and the enzyme in a final volume of 0.5 ml containing 50 mM phosphate buffer, pH 7.0, or 50 mM acetate buffer, pH 5.0 specified in enzyme source. Incubations were for 30 minute at 37°C, and the reactions were terminated by the addition of 2 ml of 0.4 M glycine buffer, pH 10.0. The released *p*-nitrophenol in the reaction was measured at 410 nm. When maltose was used as substrate for α -glucosidase activity, the liberated glucose was determined using a Glzyme kit (Tanabe Seiyaku Co.). The concentration showing 50% inhibition (IC₅₀) was determined from a plot of percent inhibition *vs* the concentration.

Results

Taxonomy of Strain F70614

The compound 1 producing strain F70614 was identified by its cultural and morphological characteristics. The cultural characteristics were observed on potato-dextrose agar, malt extract agar and Czapek-Dox agar. The strain grew rapidly to form pale yellowish green to gray colonies with a diameter of $30 \sim 40$ mm after incubation for 7 days at 25° C. The aerial mycelia were abundantly produced on various agar media. The reverse color was dark green

Media	Diameter of colony (mm)	Color		Quinhia minut
		Surface	Reverse side	Soluble pigment
PDA agar	40	Green	Dark green	None
Czapek-dox a	gar 32	Pale green	Yellowish green	None
Malt extract a	gar 35	Green	Green	None

Table 1. Cultural characteristics of the strain F70614.

(Table 1).

When the strain F70614 was grown on potato - dextrose agar medium, the conidiophores were borne from substrate hyphae and the penicillia were monoverticillate. The size of phialides was about $7.5 \sim 10 \times 2 \sim 3 \,\mu$ m. The conidia were globose to subglobose, $2.0 \sim 2.5 \,\mu$ m in diameter and with a rough. From the above characteristics, the strain F70614 was identified as a member of *Penicillium* and thus named *Penicillium* sp. F70614.

Fermentation, Isolation and Structure

The time course production was investigated by incubating in 5-liter jar fermentor containing 3 liters of production medium. Cell mass was reached to a maximum level after 6 days of fermentation and glucosidase inhibitory activity also exhibited maximum level with about 25 % inhibition after 6 days of fermentation (Fig. 2).

The fermentation broth (3 liters) was centrifuged and the resulting mycelial cake was extracted with 70 % aqueous acetone. After removal of acetone, the aqueous solution was extracted three times with ethyl acetate. The ethyl acetate-soluble portion was concentrated under reduced pressure. The concentrate was applied to a column of silica gel and was eluted with a mixture of chloroform - methanol (10:1) to give a crude powder. The combined active fraction was further purified by Sephadex LH-20 column chromatography developed with methanol to give a white powder. The crude 1 was finally purified by preparative HPLC to afford 5.5 mg of pure 1. 1: white powder; UV λ_{max} nm (ϵ) in MeOH: 219 (2130), 305 (160); IR (KBr): 3447, 2928, 1678, 1637, 1458, 1337, 1021 cm^{-1} ; $[\alpha]_{\rm D} = -23^{\circ}$ (c 0.2, MeOH); ¹H-NMR (CD₃OD, 500 MHz) 0.94 (3H, d, J=6.5 Hz, Leu δ), 0.95 (3H, d, J=6.5 Hz, Leu δ), 1.70 (2H, br. t, J=6.4 Hz, Leu β), 1.83 (1H, m, Leu γ), 4.09 (1H, t, J=6.1 Hz, Leu α), 4.92 (1H, s, Deala β), 5.42 (1H, s, Deala β); ¹³C-NMR (CD₃OD, 125 MHz) 55.8 (CH,

Fig. 2. Time course production of 1.



Leu α), 45.2 (CH₂, Leu β), 25.1 (CH, Leu γ), 23.2 (CH₃, Leu δ), 22.5 (CH₃, Leu δ), 169.0 (C, Leu CO), 135.4 (C, Deala α), 101.8 (CH₂, Deala β), 161.2 (C, Deala CO); HREI-MS: m/z 182.1056 (M⁺), C₉H₁₄N₂O₂ requires 182.1055. The above physico-chemical properties of compound 1 suggested that this compound was a diketopiperazine, and the chemical structure was unambiguously assigned as cyclo(Deala-L-Leu) by the ¹H-¹H COSY, DEPT and HMBC experiments. The configuration of leucine was established to be L by chiral TLC analysis of the acid hydrolysate (121°C, 24 hours) of compound 1. Although cyclo(Deala-L-Leu) was previously isolated from the culture medium of Vibrio parahaemolyticus by CARMELI's group⁹⁾, the details for 1 were reported in the present paper. 1 was synthesized as described in Materials and Method for its mass production. Its physico-chemical properties and NMR spectral data were compared with those of natural 1. The synthesized compound was identical to the natural 1. The structureactivity relationships of diketopiperazines for glucosidase inhibition are under investigation.

Biological Activity

Compound 1 inhibited yeast and porcine intestinal α glucosidase with IC₅₀ values of 35 and 50 µg/ml, respectively, in a dose-dependent fashion (Fig. 3). Inhibitory activity of 1 against yeast α -gucosidase was more active than those of 1-deoxynojirimycin, castanospermine and acarbose, well known α -gucosidase inhibitors, as shown in Table 2. A reciprocal plot analysis revealed that 1 behaved as a non-competitive inhibitor (Fig. 4). The *Ki* value of 1 was found to be 3.8×10^{-5} mol/liter. To extend this finding, various commercially available glycosidases were tested for their sensitivity to 1.

Fig. 3. Dose dependent inhibition of α -glucosidase by compound 1.

•, Yeast α -glucosidase enzyme; \bigcirc , Porcine intestine α -glucosidase enzyme.



Table 2. Comparison of inhibitory activity of compound 1, 1-deoxynojirimycin, acarbose and castanospermine against Brewer's yeast α -glucosidase.

Compound	IC_{50} value (µg/ml)	
Compound 1	35	
1-Deoxynojirimycin	75	
Castanospermine	45	
Acarbose	61	

Fig. 4. Lineweaver-Burk plot of inhibition of yeast α -glucosidase by 1.





Table 3. Inhibitory activity of compound 1 against the different glycosidases.

Engrand	Sources	% of inhibition (µg/ml)		
Enzyme		50	100	
α-Glucosidase	Brewer's yeast	70.4 (25) ^a	84.0 (44)	
	Bacillus	64.0 (31)	89.0 (54)	
	Rice	34.0 (27)	67.0 (49)	
	Porcine intestine	50.0 (23)	67.0 (42)	
α-Galactosidase	Aspergillus niger	12.7	17.1	
	Porcine intestine	10.2	16.0	
β-Galactosidase	Escherichia coli	10.9	13.4	
	Porcine intestine	2.8	4.3	
α-Mannosidase	Jack Bean	3.8	4.1	
β-Glucosidase	Almond	8.5	10.9	
	Porcine intestine	10.2	14.0	

^a Value in parentheses is the inhibition of enzymatic activity when maltose was used as the natural substrate Compound 1 inhibited α -glucosidases from different organisms, but showed the inhibitory effect at basal level against almond β -glucosidase, porcine intestine β glucosidase, *Aspergillus* α -galactosidase, *Escherichia coli* β -galactosidase, porcine intestine β -galactosidase and jack bean α -mannosidase, as shown in Table 3. Thus, 1 was presumed to be a specific inhibitor to α -glucosidase.

It has been recently reported that nojirimycin inhibits the HIV infection of T-cells¹⁰. We investigated the preventive effect of **1** against HIV infection to T-cells. However, compound **1** did not show preventive activity against HIV infection (data not shown).

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