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Cyclo(Dehydrohistidyl-L-Tryptophyl), an Inhibitor of Nitric Oxide Production from a Fungal Strain, Fb956

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Abstract In the course of screening for nitric oxide inhibitors in activated microglial BV-2 cells, cyclo(dehydrohistidyl-L-tryptophyl) was isolated from solid-state fermentation cultures of an unidentified fungal strain, Fb956. Its structure was determined by spectroscopic methods including 2D NMR and chiral TLC analyses. Cyclo(dehydrohistidyl-L-tryptophyl) was found to have an inhibitory activity on nitric oxide production with an IC_{50} of 6.5 μM in activated BV-2 cells. The structure determination and biological activity of cyclo(dehydrohistidyl-L-tryptophyl) was reported for the first time in this study.

Keywords: Cyclo(dehydrohistidyl-L-tryptophyl), cyclic peptide, nitric oxide, microglia

Nitric oxide (NO) plays an important role in the physiology and pathophysiology of the central nervous, cardiovascular, and immune systems [2, 9, 20]. NO is produced by the oxidation of L-arginine to L-citrulline by one of three isoenzymes of nitric oxide synthase (NOS): neuronal (nNOS), endothelial (eNOS), and inducible (iNOS) [8, 18]. iNOS is a key mediator of inflammation and host defence systems. Expression of iNOS is induced at a transcriptional level in macrophages or microglia by inflammatory stimuli including bacterial lipopolysaccharide (LPS), interferon, interleukin-1, and tumor necrosis factor-α. Continuous expression of iNOS leads to higher levels of NO, which is implicated in the pathogenesis of various inflammatory diseases such as septic shock, rheumatoid arthritis, inflammatory bowel disease, and neurodegenerative diseases [3, 10, 20]. Thus, intervention of iNOS-driven NO is believed to be effective for the prevention of these diseases [1, 4, 13, 22]. In the course of searching for inhibitors of NO production in activated microglia BV-2 cells from microbial metabolites, we found that cyclo(dehydrohistidyl-L-tryptophyl) (**1**), a cyclic dipeptide isolated from solid-state fermentation cultures of an unidentified fungal strain, Fb956, prevented NO production. Compound **1** has been reported as a metabolite of *Penicillium* sp. [16, 27]. Only UV and MS data of **1** have been reported. The NMR spectral data, absolute stereochemistry, and biological activity of **1**, however, have not yet been reported. In the present study, we report the purification, physicochemical properties, structure determination, and NO-production inhibitory activity of **1**.

The fungal strain Fb956 was isolated from a soil sample that was collected around Odae mountain, Kangwon-do, Korea. Fermentation was carried out in a solid state of moistured wheat-bran. A piece of strain Fb956 was inoculated from a mature plate culture into 500-ml baffled Erlenmeyer flasks containing 100 ml of sterile seed liquid medium containing glucose 2%, yeast extract 0.2%, polypeptone 0.5%, $MgSO_4$ 0.05%, and KH_2PO_4 0.1% (pH 5.7 before sterilization) and cultured on a rotary shaker (150 rpm) at 28°C for 3 days. For the production of **1**, 5 ml of the seed culture was transferred into 500-ml Erlenmeyer flasks (50 flasks) containing 100 g of moistured wheat-bran, and cultivated for 6 days using the same conditions. The solid-state fermented whole medium was extracted with 80% acetone and the extract was concentrated *in vacuo* to an aqueous solution, which was then extracted with an equal volume of EtOAc three times. The EtOAc extract was concentrated *in vacuo* to dryness. The crude extract was subjected to SiO_2 (Merck Art No. 7734.9025) column chromatography followed by stepwise elution with $CHCl_3$-MeOH (50:1, 10:1, 5:1). The active fractions eluted with $CHCl_3$-MeOH (50:1) were pooled and concentrated *in vacuo* to give an oily residue. The active fraction dissolved in MeOH was further purified by reverse-phase HPLC column (20×150 mm, YMC C18) chromatography with a photodiode array detector. The column was eluted with ACN: H_2O (35:65) at a flow rate of 8 ml/min to afford **1**, (62.8 mg) at a retention time of 9.4 min, as a white powder. The physicochemical properties of **1** are as follows: white powder; $[\alpha]_D$+155 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε)

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Table 1. ^{1}H and ^{13}C NMR spectral data for **1**.

Position	δ_H (mult., J_{HH})	δ_C	HMBC
1	11.9 (brs)		C-2, C-3, C-8, C-9
2	7.42 (s)	125.8 CH	C-3, C-8, C-9, C-10
3		109.1 CH	
4	8.00 (d, 7.4)	119.4 CH	C-3, C-6, C-9
5	7.16 (dd, 7.4, 7.8)	119.4 CH	C-7, C-8
6	7.17 (dd, 7.4, 7.8)	121.7 CH	C-4, C-9
7	7.40 (d, 7.8)	111.9 CH	C-5, C-8
8		128.6 C	
9		137.6 C	
10	Ha 3.60 (dd, 14.5, 4.0)	31.9 CH_2	C-2, C-3, C-8, C-11, C-16
	Hb 3.75 (dd, 14.5, 5.9)		C-2, C-3, C-8, C-11, C-16
11	4.82 (dd, 5.9, 4.0)	57.4 CH	C-3, C-13, C-16
12	9.99 (brs)		C-14, C-16
13		162.7 C	
14		123.2 C	
15	11.4 (brs)		C-11, C-13, C-14, C-16, C-17
16		167.3 C	
17	6.33 (s)	108.2 CH	C-13, C-14, *C-18, C-22
18		127.4 C	
20	7.86 (s)	136.8 CH	C-18, C-22
22	7.48 (s)	133.6 CH	*C-18, *C-20

^{1}H and ^{13}C NMR spectral data were measured at 600 and 150 MHz, respectively, in pyridine-d_5. The assignments were aided by ^{1}H-^{1}H COSY, DEPT, HMQC, and HMBC. *Measured in $CDCl_3$+CD_3OD.

217 (4.48), 281 (3.98), 289 (4.02), 323 (4.15) nm; IR (KBr) ν_{max} 3,439 (OH), 2,926, 1,675 (CO), 1,430, 1,100, 743 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS $[M+H]^+$ *m/z* 322.1302 (calcd for $C_{17}H_{15}N_5O_2$+H, 322.1304).

The ^{1}H and ^{13}C NMR data of **1** together with ^{1}H-^{1}H COSY, DEPT, and HMQC data (Table 1) suggested the presence of a 1,2-disubstituted benzene (δ 8.00, d, *J*= 7.4 Hz, δ 119.4; δ 7.16, dd, *J*=7.4, 7.8 Hz, δ 119.4; δ 7.17, dd, *J*=7.4, 7.8 Hz, δ 121.7; δ 7.40, d, *J*=7.8 Hz, δ 111.9), four isolated olefinic methines (δ 7.86, δ 136.8; δ 7.48, δ 133.6; δ 7.42, δ 125.8; δ 6.33, δ 108.2), a methylene linked to an α-methine of an amino acid (δ 3.60, δ 3.75, δ 31.9; δ 4.82, δ 57.4), three quaternary sp^2 carbons (δ 127.4, 123.2, and 109.1), two amide carbonyls (δ 167.3 and 162.7), and three exchangeable protons (δ 11.9, 11.4, and 9.99). The connectivity among these partial structures was determined by the HMBC spectroscopic data (Fig. 2). The isolated olefinic methine proton at δ 7.42 (H-2) was long-range coupled to two quaternary sp^2 carbons at δ 137.6 (C-9) and δ 128.6 (C-8) of the 1,2-disubstituted benzene. In addition, the methylene protons (H_2-10) adjacent to the α-methine showed the HMBC correlations with C-2, C-3, C-8 (δ 128.6), and the amide carbon at δ 167.3 (C-16). These data indicate the presence of a tryptophan moiety. One exchangeable proton at δ 11.4 (NH-15) was long-range coupled to the amide carbon at δ 162.7 (C-13) and the α-methine carbon at δ 57.4 (C-11), whereas the other exchangeable proton at δ 9.99 (NH-12) was long-range coupled to C-16 and the quaternary sp^2 carbon at δ 123.2 (C-14). In addition, the α-methine proton (H-11) showed the HMBC correlation with C-3 and C-13. The isolated olefinic methine at δ 6.33 (H-17) was long-range coupled to C-13, C-14, and C-22 (δ 133.6). These spectral data together with the molecular formula indicated the presence of a cyclodipeptide composed of tryptophan and dehydrohistidine. The presence of the imidazole moiety was confirmed by the HMBC experiment in $CDCl_3$+CD_3OD, as shown in Table 1. The configuration of the double bond at C-14 in the dehydrohistidine moiety was determined by the NOESY spectrum (Fig. 2). H-17 was determined to have a *trans* configuration with NH-15 by the NOE effect between H-17 and NH-15. The absolute configuration of the tryptophan moiety was determined by acidic hydrolysis and chiral TLC analysis [14]. Compound **1** (1 mg) was hydrolyzed in 6 M HCl at 110°C for 8 h. After the resulting hydrolysate was evaporated to dryness and dissolved in 50% aqueous MeOH, it was applied to the chiral plate with authentic samples of D- and L-tryptophans and the plate was developed with MeOH/H_2O/ACH (1:1:4, v/v/v) followed by visualization with ninhydrin. As a result, R_f values of D- and L-tryptophans were 0.48 and 0.60, respectively, and the tryptophan component of the hydrolysis mixture of **1** was assigned to be D. Thus, the absolute structure of **1** was determined as shown in Fig. 1.

The BV-2 cells are a mouse microglial cell line that produces large amounts of nitric oxide on inflammatory stimuli including bacterial LPS, interferon, interleukin-1, and tumor necrosis factor-α [6, 29]. Thus, the mouse microglia BV-2 cells stimulated with LPS were used as an *in vitro* model of activated microglia. The mouse microglia

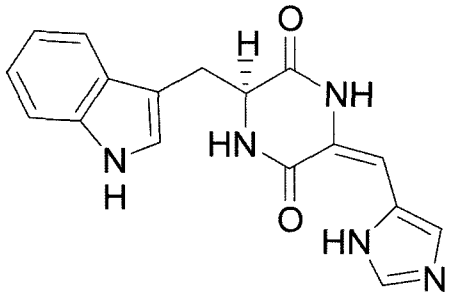

Fig. 1. The structure of cyclo(dehydrohistidyl-L-tryptophyl) (**1**).

BV-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were grown at 37°C in 5% CO_2 in a humidified atmosphere. To determine NO concentration, BV-2 cells were cultured at an initial cell density of 5×10^4 cells/well in 24-well plates. After 48 h, the medium was replaced with DMEM supplemented with 10% FBS containing 1 μg of LPS/ml and various concentrations of **1**. Culture supernatants were collected 24 h after stimulation. NO production was assessed by measuring the concentration of nitrite, a stable degradation product of NO, with the Griess reagent [7].

Compound **1** inhibited the nitrite accumulation in a dose-dependent manner, as shown in Fig. 3. The IC_{50} was 6.5 μM. To determine whether **1** had a cytotoxic effect on BV-2 cells, **1** was treated up to 100 μM for 24 h and cell viability was measured using the crystal violet assay. However, compound **1** had no cytotoxic effect on BV-2 cells at the concentrations used (data not shown). It indicates that the inhibitory activity of **1** on NO production was not due to cytotoxicity. Since most known inhibitors of NO production in activated microglia or macrophages from natural sources have been known to inhibit the signaling pathway and result in the decrease in the expression of iNOS protein, the effect of **1** on iNOS expression was examined. However, compound **1** showed no inhibitory activity against iNOS

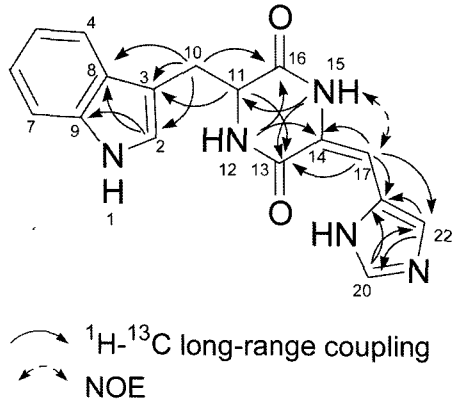

Fig. 2. Key HMBC and NOE data in **1**.

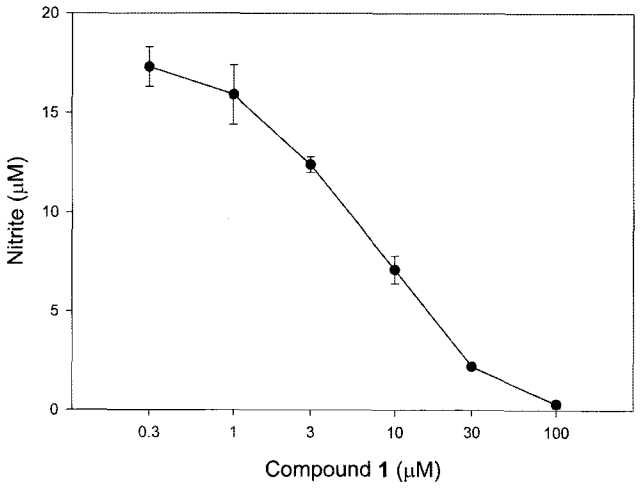

Fig. 3. Effect of **1** on NO production in activated BV-2 cells.
BV-2 cells were treated with 1 μg of LPS/ml and the indicated doses of **1** for 24 h. NO production was assessed by measuring the concentration of nitrite in culture supernatants with the Griess reagent. The values represent the mean±SD obtained from two independent experiments performed in triplicate.

expression, even at 100 μM (data not shown). The precise mechanism of the inhibitory activity of **1** on NO production will be reported elsewhere.

Fungi have proved to be a rich source of secondary metabolites with unusual structures as well as interesting biological activities [11, 12, 17, 24, 28]. Cyclo(dehydrohistidyl-tryptophyl) has been reported as a metabolite of *Penicillium* sp. [16, 27]. The structure determination and biological activity of cyclo(dehydrohistidyl-tryptophyl), however, has not yet been reported. Cyclic dipeptides are among the simplest peptide derivatives commonly found in nature [22]. Most cyclic dipeptides appear to have emerged as by-products of food processing or metabolites of microorganisms. However, a few cyclic dipeptides among them have biological activities, including antibacterial and plant growth regulating activities [5, 14, 15, 23]. In humans, only cyclo(His-Pro) is present as an endogenous neuropeptide. Recently, cyclo(His-Pro) was reported to be related to glucose metabolism and to have antidiabetic activity [25, 26]. The NO inhibitory activity of cyclic peptides, however, has not yet been reported.

In conclusion, we found that cyclo(dehydrohistidyl-L-tryptophyl), a cyclic dipeptide from the fungal strain Fb956, had an inhibitory effect on NO production without cytotoxic activity in microglial BV-2 cells. The structure determination and biological activity of cyclo(dehydrohistidyl-L-tryptophyl) were reported for the first time in this study.

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