NOTES

A Novel Substance with TGF-β like Activity, Diheteropeptin, Produced by a Fungus, *Diheterospora* sp.

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Transforming growth factor-β (TGF-β) first discovered as a trophic factor to several kinds of cells[1] is now well known to act as a growth inhibitor to various kinds of animal cells[2]. In addition, TGF-β protects neuronal cells from damages caused by stress such as L-glutamate toxicity and β-amyloid toxicity which are considered to be associated with brain ischemia injury and Alzheimer's disease, respectively[3,4]. Thus, substances which mimic TGF-β action are expected to be useful for treatment of diseases such as ischemia injury.

For detecting TGF-β like activities, we constructed a screening system utilizing reporter gene expression. Mink lung epithelial (Mv1Lu) cells, which express excess TGF-β receptors and respond to TGF-β resulting in the expression of plasminogen activator inhibitor-1 (PAI-1)[5], were transfected with the firefly luciferase reporter gene at the downstream of the PAI-1 promoter gene[6]. During the course of our screening using this system, we isolated histone deacetylase inhibitors such as trichostatin A[7]. Further investigation has resulted in the isolation of a novel metabolite with TGF-β like activity, diheteropeptin (**1**, Fig. 1). We report herein the fermentation, isolation and structure determination of **1**.

The diheteropeptin producing organism, identified as *Diheterospora* sp., was cultivated in a medium containing glucose 2.0%, maltose 3.0%, corn steep liquor 1.0%, polypepton 0.3%, soybean meal 1.5% and NaCl 0.3% (pH 7.0) for 5 days at 24°C on a rotary shaker. The active principle was extracted from broth filtrate (2 liters) with EtOAc. The solvent layer was dried over Na_2SO_4, and concentrated to give an oily residue. Then, it was purified successively by silica gel column chromatography ($CHCl_3$ - MeOH = 30 : 1) and Sephadex LH-20 column chromatography ($CHCl_3$ - MeOH = 1 : 1). A pure sample of **1** was finally obtained by HPLC using a PEGASIL ODS column (Senshu-Pak, 20 i.d. × 250 mm) developed with 65% MeOH.

The physico-chemical properties of **1** are summarized in Table 1. The molecular formula of **1** was established as $C_{28}H_{42}N_4O_6$ by high-resolution FAB-MS spectrum. IR absorptions at 3300~3400, and 1660, 1670 and 1680 cm^{-1} implied the presence of –OH, –NH and amide functions. Together with the IR absorptions, amide carbons observed in the ^{13}C NMR (171.9, 172.8, 174.4 and 175.6 ppm) suggested **1** to be a peptide derivative. The ^1H and ^{13}C NMR spectral data are shown in Table 2.

Analysis of the DQF-COSY spectrum of **1** revealed a monosubstituted benzene residue and the sequence from an amide proton at 7.50 ppm to methylene protons 3′-H (2.94, 3.25 ppm) through an α-methine proton 2′-H (5.15 ppm). An amide carbonyl carbon C-1′ (172.8 ppm) was long-range coupled to 2′-H and 3′-H, which were in turn long-range coupled to C-4′ (137.0 ppm) and C-5′

Fig. 1. Structure of diheteropeptin (**1**).

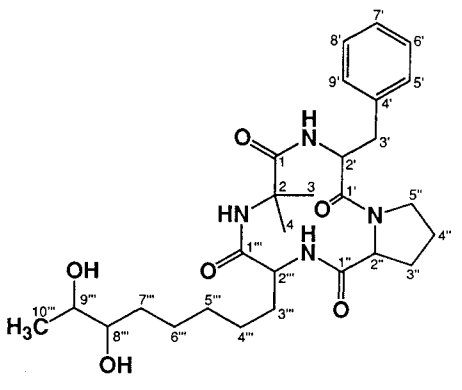


Table 1. Physico-chemical properties of diheteropeptin (**1**).

Appearance	White powder
MP	74 ~ 76°C
$[\alpha]_D^{25}$	-30.3° (*c* 0.19, MeOH)
Molecular formula	$C_{28}H_{42}N_4O_6$
HRFAB-MS (*m/z*)	
Found	531.3188 $(M+H)^+$
Calcd	531.3183
UV λ_{max}^{MeOH} nm (ε)	203 (10,900), 233 (sh, 2,000)
IR ν_{max} (KBr) cm^{-1}	3400, 3300, 1680, 1670, 1660, 1620, 1520

Table 2. ^{1}H and ^{13}C NMR chemical shifts of diheteropeptin (**1**) in $CDCl_3$.

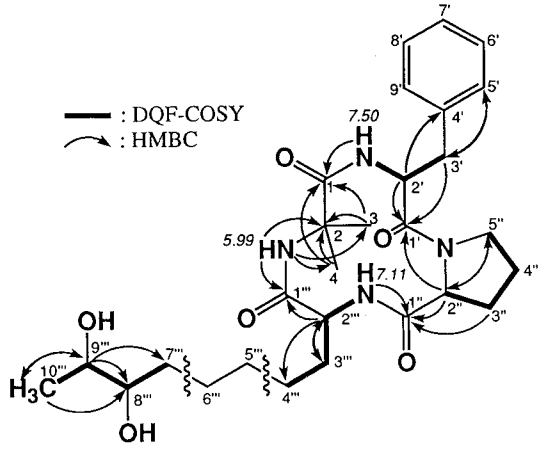
	No.	δ_C	δ_H
+Aib	1	175.6	
	2	58.8	
	3	23.6	1.76 (s)
	4	26.5	1.33 (s)
	NH		5.99 (s)
Phe	1'	172.8	
	2'	53.4	5.15 (dt, J = 5.5, 10.0)
	3'	35.8	2.94 (dd, J = 5.5, 13.5), 3.25 (dd, J = 5.5, 13.5)
	4'	137.0	
	5', 9'	129.0	7.21 (d, J = 7.0)
	6', 8'	128.6	7.26 (t, J = 7.0)
	7'	126.7	7.19 (d, J = 7.0)
	NH		7.50 (d, J = 10.0)
Pro	1"	171.9	
	2"	57.8	4.65 (dd, J = 2.0, 8.0)
	3"	24.7	1.76 (m), 2.32 (m)
	4"	25.0	2.16 (m)
	5"	47.0	3.21 (dt, J = 7.5, 10.0), 3.85 (ddd, J = 4.5, 8.0, 10.0)
+Add	1"'	174.4	
	2"'	54.4	4.18 (dt, J = 7.5, 10.0)
	3"'	28.8	1.63 (m), 1.82 (m)
	4"'	29.1	1.29 (m), 1.39 (m)
	5"'	25.2	1.38++ (m)
	6"'	25.2	1.38 (m), 1.48++ (m)
	7"'	33.1	1.38 (m), 1.48 (m)
	8"'	76.1	3.32 (m)
	9"'	70.9	3.59 (dq, J = 6.0, 6.5)
	10"'	19.5	1.19 (d, J = 3.0)
	NH		7.11 (d, J = 10.0)

^{1}H and ^{13}C NMR spectra were recorded at 600 MHz and 150 MHz, respectively.
+ Aib : α-aminoisobutyric acid, Add : 2-amino-8,9-dihydroxydecanoic acid
++ These two assignments are exchangeable.

(129.0 ppm), respectively, in the HMBC spectrum of **1**. These results established a phenylalanyl moiety in Figure 2.

The proton spin system from 2"-H (4.65 ppm) to 5"-H (3.21, 3.85 ppm) through 3"-H (1.76, 2.32 ppm) and 4"-H (2.16 ppm) was detected in the DQF-COSY spectrum, and C-2" (57.8 ppm) and C-5" (47.0 ppm) were long-range coupled to their appended protons 5"-H and 2"-H, respectively. Moreover, 2"-H and 3"-H were long-range coupled to an amide carbonyl carbon C-1" (171.9 ppm). These long-range couplings and the chemical shifts of C-2" and C-5" proved the presence of a prolyl moiety (Fig. 2). Two singlet methyl protons 3-H (1.76 ppm) and 4-H (1.33 ppm) were long-range coupled to C-2 (58.8 ppm) and an amide carbonyl carbon C-1 (175.6 ppm). In addition to these correlations, long-range couplings from an amide proton at 5.99 ppm to C-2, C-3 (23.6 ppm) and C-4 (26.5 ppm), revealed the presence of an α-aminoisobutyryl skeleton (Fig. 2).

Fig. 2. ^{1}H-^{1}H and ^{1}H-^{13}C correlations revealed by DQF-COSY and HMBC experiments of diheteropeptin (**1**).



The only residual amide function was revealed by long-range couplings from 2‴-H (4.18 ppm) to an amide carbon C-1‴ (174.4 ppm) and methylene carbons C-3‴ (28.8 ppm) and C-4‴ (29.1 ppm); this sequence being also supported by proton spin couplings from an amide proton at 7.11 ppm to 4‴-H (1.29, 1.39 ppm). The alkyl side chain linked to C-4‴ was determined as follows. In the DQF-COSY spectrum of **1**, a proton spin system from methylene protons 7‴-H (1.38, 1.48 ppm) to a methyl proton 10‴-H (1.19 ppm) was recognized through methine protons 8‴-H (3.32 ppm) and 9‴-H (3.59 ppm) which were concluded to be attached to oxygenated carbons from the relevant ^{13}C chemical shifts. Accordingly, the remaining two methylenes were attributed to C-5‴ (25.2 ppm) and C-6‴ (25.2 ppm). Thus, this amino acid residue was elucidated to be 2-amino-8,9-dihydroxydecanoic acid.

The connectivities among these amide functional units were established by long-range couplings from the amide protons to their neighbored carbonyl carbons, *i.e.*, the amide protons at 7.50 ppm, 5.99 ppm and 7.11 ppm to C-1, C-1‴ and C-1″, respectively (Fig. 2). A long-range coupling from 2″-H to C-1′ revealed the relationship between the prolyl and phenylalanyl residues. Thus, the structure of **1** was determined to be a cyclic peptide (Fig. 2). **1** is structurally very similar to chlamydocin[8] that has the same tetrapeptidyl ring system with an epoxy ketone group instead of the 3-methyl-1,2-glycol function in **1**. The determination of the absolute stereochemistry of **1** is now under way.

In the evaluation system we employed, the addition of TGF-β at the concentration of 40 ng/ml to Mv1Lu cells increased the expression of luciferase three times. **1** induced the PAI-1 promoter gene expression more than three times at the concentrations from 0.98 μM to 1.0 mM. In order to compare the biological activities of **1** and chlamydocin that showed cytostatic activities, we evaluated the effect of **1** on the cell growth by measuring MTT reduction and LDH release[9]. **1** exhibited the cytostatic effect to Mv1Lu cells with IC_{50} value of 20.3 μM as determined by the MTT assay. No LDH release, however, suggested that **1** showed only the cytostatic activity to Mv1Lu cells. This biological activity pattern similar to that of TGF-β. Detailed investigations on other biological activities of **1** are now under way.

Acknowledgments

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