VOL. 53 NO. 8, AUG. 2000 THE JOURNAL OF ANTIBIOTICS pp. 793 — 798

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

II. Physico-chemical Properties and Structure Elucidation

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(Received for publication March 21, 2000)

The structure of diheteropeptin (**1**), a TGF-β-like active substance from *Diheterospora chlamydosporia* Q58044, was determined to be a new cyclotetrapeptide, *cyclo*[2-aminoisobutyryl-(*S*)-phenylalanyl-(*R*)-prolyl-(2*S*,8*R*,9*R*)-2-amino-8,9-dihydroxydecanoyl-] by NMR, mass spectrometric and chemical studies.

In the course of our screening for microbial metabolites with TGF-β-like activity by using the luciferase reporter assay for PAI-1 promoter activation, a new active substance, diheteropeptin (**1**, Fig. 1), was isolated from the fermentation broth of *Diheterospora chlamydosporia* Q58044. We have described the fermentation, isolation and biological activity of this metabolite in the preceding paper[1]. We report herein the physico-chemical properties and structure determination of **1**.

Results and Discussion

Physico-chemical Properties

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. IR absorptions at 3300~3400, 1660, 1670 and 1680 cm^{-1} implied the presence of hydroxyl and amide functions. Together with the IR absorptions, four carbonyl carbons observed in the ^{13}C NMR (171.9, 172.8, 174.4 and 175.6 ppm) were assigned to amide carbons in a peptide derivative. The ^{13}C and ^1H NMR spectral data in $CDCl_3$ are shown in Table 2.

Structure Elucidation

The COSY spectrum of **1** revealed the presence of a monosubstituted benzene residue and a sequence from an amide proton at 7.50 ppm to benzylic methylene protons (3′-H) through an α-methine proton (2′-H) as shown in Fig. 2. In the heteronuclear multiple-bond correlation (HMBC)[2] spectrum of **1**, an amide carbonyl carbon (C-1′) exhibited long-range couplings to 2′-H and 3′-H, which were in turn coupled to aromatic carbons (C-4′ and C-5′), respectively. These data established the presence of a phenylalanine residue.

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

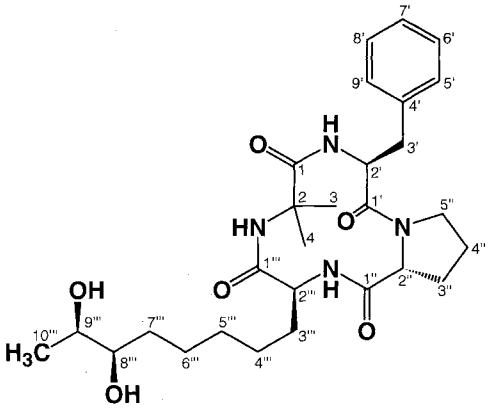


Table 1. Physico-chemical properties of **1**.

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. ^{13}C and ^{1}H NMR data of **1** in $CDCl_3$.

	No.	δ_C	δ_H (multiplicity, *J* = Hz)
Aib*	1	175.6	
	2	58.8	
	3	23.6	1.76 (3H, s)
	4	26.5	1.33 (3H, 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 (2H, d, *J* = 7.0)
	6', 8'	128.6	7.26 (2H, 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 (2H, 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** (2H, 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 (3H, d, *J* = 6.0)
	NH		7.11 (d, *J* = 10.0)

^{13}C and ^{1}H NMR spectra were recorded at 150 MHz and 600 MHz, respectively.

*Aib : α-aminoisobutyric acid, Add : 2-amino-8,9-dihydroxydecanoic acid

**These two assignments are exchangeable.

A proton spin system from 2″-H to 5″-H through 3″-H and 4″-H was detected in the COSY spectrum. C-2″ and C-5″ showed long-range correlations to their appended protons 5″-H and 2″-H, respectively. Moreover, 2″-H and 3″-H were coupled to an amide carbonyl carbon (C-1″). These correlations and the chemical shifts of C-2″ (57.8 ppm) and C-5″ (47.0 ppm) proved the presence of a proline residue (Fig. 2).

Two singlet methyl protons (3-H and 4-H) displayed long-range couplings to a quaternary carbon (C-2) and an amide carbonyl carbon (C-1). In addition to these correlations, long-range couplings from an amide proton at 5.99 ppm to C-2, C-3 and C-4 revealed the presence of an α-aminoisobutyric acid residue (Fig. 2).

The remaining amide proton at 7.11 ppm exhibited a sequence to 4‴-H through 2‴-H and 3‴-H in the COSY spectrum. A long-range coupling between 2‴-H and an amide carbon (C-1‴) extended this substructure to an α-amino acid. In the COSY spectrum of **1**, a proton spin system from methylene protons (7‴-H) to a methyl proton (10‴-H) was recognized through a diol protons at 3.32 ppm (8‴-H) and 3.59 ppm (9‴-H). The remaining two methylenes (C-5‴ and C-6‴) thus could be located between C-4‴ and C-7‴, and this amino acid residue was elucidated as 2-amino-8,9-dihydroxydecanoic acid.

The amino acid sequence was established as shown in Fig. 2 by long-range couplings from 2′-NH to C-1, from 2″-H to C-1′, from 2‴-NH to C-1″, and from 2-NH to C-1‴. Thus, the planar structure of **1** was determined to be a cyclic tetrapeptide as shown in Fig. 2. Diheteropeptin (**1**) is structurally similar to chlamydocin[3], which has the same tetrapeptidyl ring system with an epoxy ketone group instead of the methyl-1,2-glycol function in **1**.

Fig. 2. NMR analyses of **1**.

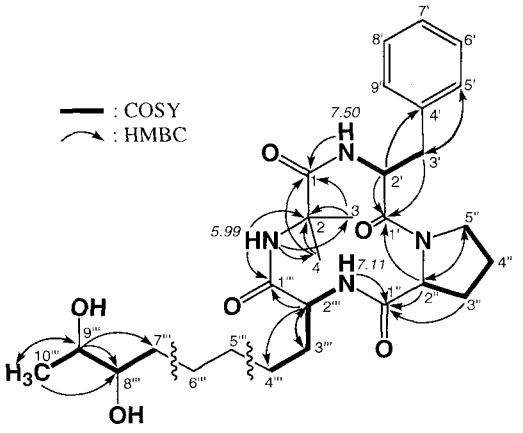


Fig. 3. Derivatization of **1** for determination of the relaive and absolute configrations of a glycol moiety in **1**.

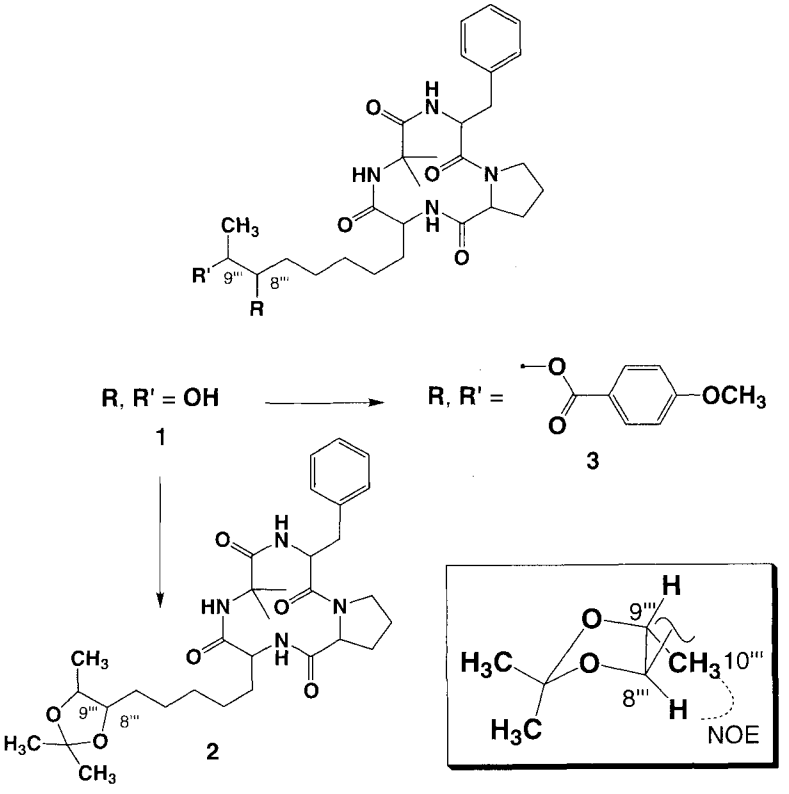
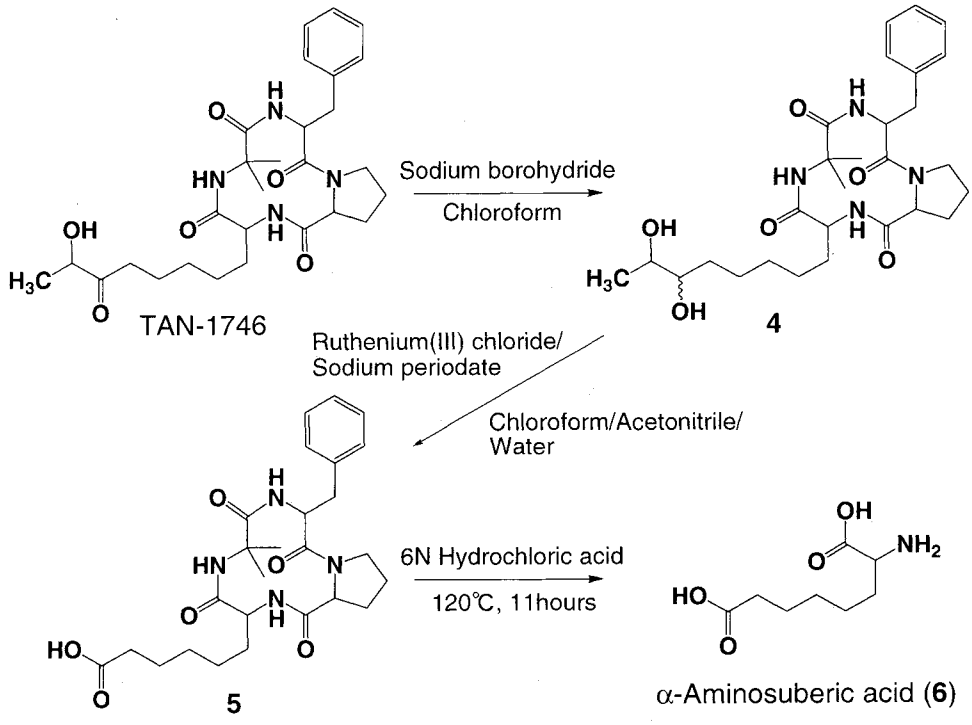


Fig. 4. Preparation of α-aminosuberic acid from TAN-1746.



Stereochemistry of Diheteropeptin

The relative stereochemistry of the glycol moiety was first determined as follows. Treatment of **1** with 2,2-dimethoxypropane and pyridinium *p*-toluenesulfonate gave an acetonide derivative bridged between C-8‴ and C-9‴. A strong NOE between H-8‴ and H-10‴ required an *anti* orientation for H-8‴ and H-9‴ (Fig. 3). The stereochemistry of the diol moiety (C-8‴ and C-9‴) in **1** was then confirmed by the CD spectrum of its dibenzoate[4].

A di-*p*-methoxybenzoate derivative (**3**) was prepared by treatment of **1** with *p*-methoxybenzoyl chloride. The CD spectrum of **3** in MeOH showed well-split intense positive Cotton effects ($\Delta\varepsilon_{265}$ −37.1 and $\Delta\varepsilon_{247}$ +25.0), indicating that the two *p*-methoxybenzoyl groups at C-8‴ and C-9‴ are in an anticlockwise relationship. Thus the diol moiety was elucidated to have 8‴*R* and 9‴*R* configurations.

A closely related compound to **1**, TAN-1746[5] (Fig. 4), contains a keto function instead of a hydroxyl at C-8‴ in **1**. Reduction of TAN-1746 with sodium borohydride gave a diastereomeric mixture of two diol compounds (**4**), one of which was identical with diheteropeptin. Because of the shortage of diheteropeptin, TAN-1746 was used to determine the absolute configurations of the α-methines in **1**. The diol mixture (**4**) prepared from TAN-1746 was oxidized with ruthenium(III) chloride/sodium periodate to provide a carboxylic acid (**5**, Fig. 4). Acid hydrolysis of **5** afforded phenylalanine, proline, α-aminoisobutyric acid and α-aminosuberic acid (α-aminooctanedioic acid).

The absolute configurations of the phenylalanine and proline moieties were determined as L-phenylalanine and D-proline, by chiral GC-MS analysis of their isopropyl *N*-pentafluoropropionyl derivatives. The optical rotation of α-aminosuberic acid purified by HPLC ($[\alpha]_D^{22}$ +36.4° (*c* 0.12, 5 N hydrochloric acid)) gave an agreement with the literature value of L-α-aminosuberic acid ($[\alpha]_D^{23}$ +20.0° (*c* 1.0, 5 N hydrochloric acid))[6]. The α-methine configurations thus obtained are same as those of chlamydocin[3], a structurally related compound with an epoxide function.

Based upon these experiments, the absolute structure of **1** was determined to be *cyclo*[2-aminoisobutyryl-(*S*)-phenylalanyl-(*R*)-prolyl-(2*S*,8*R*,9*R*)-2-amino-8,9-dihydroxydecanoyl-] (Fig. 1). Diheteropeptin (**1**) is a new cyclotetrapeptide structurally related to chlamydocin, trapoxins[7] and TAN-1746. However, **1** is the first compound possessing a diol moiety at the terminus of the alkyl side chain.

Materials and Methods

General

UV and IR spectra were measured on a HITACHI U-3210 and a JASCO A-102 instruments, respectively. ^1H and ^{13}C NMR spectra were measured on a JEOL JNM A-600 NMR spectrometer. Chemical shifts are given in ppm using TMS as an internal standard. Mass spectra were measured on a JEOL HX-110 instrument. Optical rotations and CD spectra were taken in MeOH on a JASCO DIP-1000 polarimeter and on a JASCO J-20A recording spectropolarimeter, respectively.

Determination of the Absolute Configurations of Phenylalanine and Proline

A sample of TAN-1746 (1.0 mg) was dissolved in 6 N hydrochloric acid (1.0 ml) and heated in a sealed glass tube at 120°C for 11 hours. After cooling, the reaction mixture was evaporated to dryness. To the dried material, 200 μl of a solution (2-propanol : acetyl chloride=4 : 1) was added and heated at 100°C for 45 minutes. After concentration under argon gas at 115°C, the residue was dissolved in 100 μl of methylene chloride and 100 μl of pentafluoropropionic anhydride and heated at 100°C for 15 minutes. The resulting reaction mixture was dried under argon gas and dissolved in methylene chloride and then analyzed with chiral GC-MS (GC: Varian 3400, MS: Finnigan MAT TSQ700 triple stage quadrupole mass spectrometer, column: Chirasil-Val-D 25 m×0.25 mm i.d. (Alltech), column temperature: 50°C (10 minutes hold)~160°C (4°C/minute), injector temperature: 260°C, carrier gas: helium). The retention times for the isopropyl *N*-pentafluoropropionyl derivatives of phenylalanine and proline were 28.42 minutes and 18.29 minutes, respectively. Under the same condition, standard samples displayed retention times at 29.36 minutes for D-phenylalanine, 28.48 minutes for L-phenylalanine, 18.30 minutes for D-proline and 18.15 minutes for L-proline.

8‴,9‴-Isopropylidenediheteropeptin (2)

To a solution of **1** (5 mg) in chloroform (3 ml), 2,2-dimethoxypropane (100 μl) and pyridinium *p*-toluenesulfonate (1 mg) were added, and the mixture was stirred at room temperature for 2 hours. After evaporation to dryness, 1 ml of water was added to the reaction mixture. The material was extracted with ethyl acetate and dried over sodium sulfate. The extract was evaporated *in vacuo* to give 8‴,9‴-isopropylidenediheteropeptin (**2**: 4.5 mg, 84%). FAB-MS *m/z* 571 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 1.22 (3H, s), 1.24 (3H, s), 1.32 (3H, s), 1.35 (1H, m), 1.36 (3H, d J=6.0), 1.38 (5H, m), 1.48 (2H, m), 1.62 (m), 1.71 (3H, m), 1.75 (3H, s), 2.15 (m), 2.30 (m), 2.92 (dd J=13.5, 5.5), 3.21 (dt J=10.0, 7.5), 3.24 (dd J=13.5, 10.0), 3.47 (m), 3.66 (dq J=6.5, 6.0), 3.83 (ddd J=10.0, 8.0, 4.5), 4.16 (dt J=10.0, 7.5), 4.62 (dd J=8.0, 2.0), 5.13 (dt J=10.0, 5.5), 5.90 (s), 7.05 (d J=10.0), 7.19 (3H, m), 7.25 (2H, m), 7.50 (d J=10.0).

8‴,9‴-Di-*p*-methoxybenzoyldiheteropeptin (3)

To a solution of **1** (5 mg) in pyridine (3 ml), *p*-methoxybenzoyl chloride (4 mg) was added, and the mixture was stirred at room temperature for 2 hours. The reaction was stopped with addition of excess MeOH. After concentration, the residue was purified by preparative silica gel TLC ($CHCl_3$: MeOH=50 : 1, Rf=0.32) and HPLC (PEGASIL ODS, 90% MeOH) to give a 8‴,9‴-di-*p*-methoxybenzoyldiheteropeptin (**3**: 2.7 mg, 35%). FAB-MS *m/z* 799 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 1.32 (3H, s), 1.35 (m), 1.36 (3H, d J=6.0), 1.38 (5H, m), 1.48 (2H, m), 1.62 (m), 1.71 (3H, m), 1.75 (3H, s), 2.14 (m), 2.28 (m), 2.92 (dd J=13.5, 5.5), 3.19 (dt J=10.0, 7.5), 3.23 (dd J=13.5, 10.0), 3.82 (m), 3.83 (6H, s), 4.12 (dt J=10.0, 7.5), 4.61 (dd J=8.0, 2.0), 5.13 (dt J=10.0, 5.5), 5.29 (2H, m), 5.90 (s), 6.88 (4H, m), 7.02 (d J=10.0), 7.19 (3H, m), 7.25 (2H, m), 7.49 (d J=10.0), 7.97 (4H, m).

Dihydro Derivatives (4) of TAN-1746

To a solution of TAN-1746 (70 mg) in chloroform (4 ml), sodium borohydride (6 mg) was added, and the mixture was stirred at room temperature for 4 hours. The reaction was stopped by adding hydrogen peroxide at 0°C and the reaction mixture was neutralized with 2 N hydrochloride. The chloroform layer was removed under reduced pressure and the residue was extracted with ethyl acetate (15 ml). The ethyl acetate solution was dried over sodium sulfate and concentrated *in vacuo* to afford a diastereomeric mixture of dihydro derivatives (**4**, 66.5 mg, 95%). FAB-MS *m/z* 531 $(M+H)^+$. One of the diastereomers (**4**) revealed an identical peak with that of **1** on HPLC using a PEGASIL ODS column (90% MeOH).

Cyclo[2-aminoisobutyryl-(*S*)-phenylalanyl-(*R*)-prolyl-(*S*)-2-aminosuberoyl-] (5)

A flask was charged with acetonitrile (1.88 ml), chloroform (1.88 ml), water (2.88 ml), **4** (53 mg, 0.1 mmol) and sodium periodate (86 mg, 0.4 mmol). To this biphasic solution, ruthenium(III) chloride (0.41 mg, 0.02 equiv.) was added, and the reaction mixture was vigorously stirred for 3 hours at room temperature. To the reaction mixture, diethyl ether (10 ml) was added, with stirring for 10 minutes. The

organic phase was separated, and the aqueous phase was extracted with diethyl ether (2×5 ml). The combined organic phase was dried over sodium sulfate and concentrated to dryness. The crude mixture was purified by silica gel column chromatography ($CHCl_3$: MeOH= 20 : 1) to give *Cyclo*[2-aminoisobutyryl-(*S*)-phenylalanyl-(*R*)-prolyl-(*S*)-2-aminosuberoyl-] (**5**: 45 mg, 90%). FAB-MS *m/z* 501 $(M+H)^+$; ^1H NMR ($CDCl_3$) δ 1.32 (3H, s), 1.33 (5H, m), 1.61 (m), 1.72 (4H, m), 1.75 (3H, s), 2.15 (m), 2.30 (m), 2.40 (2H, dt J=2.0, 7.0), 2.92 (dd J=13.5, 5.5), 3.21 (dt J=10.0, 7.5), 3.24 (dd J=13.5, 10.0), 3.83 (ddd J=10.0, 8.0, 4.5), 4.16 (dt J=10.0, 7.5), 4.63 (dd J=8.0, 2.0), 5.13 (dt J=10.0, 5.5), 5.90 (s), 7.08 (d J= 10.0), 7.19 (3H, m), 7.25 (2H, m), 7.48 (d J=10.0).

(*S*)-α-Aminosuberic Acid (**6**)

5 (45 mg) was dissolved in 6 N hydrochloric acid (1.0 ml) and heated in a sealed glass tube at 120°C for 11 hours. After cooling, the reaction mixture was evaporated to dryness. The material was subjected to HPLC using a PEGASIL ODS column (20 mm i. d.×250 mm) with 7% acetonitrile and 0.1% trifluoroacetic acid to give (*S*)-α-aminosuberic acid (**6**: 5 mg). $[\alpha]_D^{22}$ +36.4° (*c* 0.12, 5 N HCl); FAB-MS *m/z* 190 $(M+H)^+$; ^1H NMR (D_2O) δ 1.20 (4H, m), 1.40 (2H, m), 1.72 (2H, m), 2.20 (2H, t), 3.85 (t).

Acknowledgments

This work was supported in part by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists to Y.M., a Grant-in Aid for Scientific Research (C) to K.S., Research for the Future, Japan Society for the Promotion of Sciences to H.S. and a Grant-in-Aid for Scientific Research on Priority Areas (Diagnosis and treatment of cancer), The Ministry of Education, Science and Culture, Japan, to H.S.

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