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

I. Production, Isolation and Biological Activities

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A new metabolite, diheteropeptin, was found in the culture broth of *Diheterospora chlamydosporia* Q58044 by screening for TGF-β-like active substances. Diheteropeptin was extracted from the culture supernatant and purified by a series of chromatographies such as silica gel, gel filtration and HPLC. Diheteropeptin exhibited cytostatic activity in Mv1Lu cells with an IC_{50} value of 20.3 μM and inhibited histone deacetylase.

Transforming growth factor-β (TGF-β) first discovered as a trophic factor for several different cell lines[1] is now well known to act as a growth inhibitor against various kinds of mammalian cells[2]. Transfection with a TGF-β type II receptor gene into SW48 colon cancer cells, which lack functional cell surface TGF-β type II receptors, restores growth inhibition and reverses the *in vitro* and *in vivo* malignant phenotype[3]. In addition, restoration of TGF-β signaling in LNCaP human prostate cancer cells suppresses tumorigenicity by inducing apoptosis[4]. Thus, substances that directly stimulate TGF-β signaling are expected to be useful for treatment of cancer. In order to detect TGF-β-like activities of microbial samples, we constructed an assay system utilizing the expression of a reporter gene. Mv1Lu mink lung epithelial cells express TGF-β receptors abundantly and respond sensitively to TGF-β resulting in the expression of plasminogen activator inhibitor-1 (PAI-1)[5]. The cells were transfected with a plasmid bearing the firefly luciferase reporter gene at the downstream of the PAI-1 promoter[6]. Thus microbial samples with TGF-β-like activity can be detected by production of luciferase which in turn can be measured with a luminometer. During the course of our screening using this system, we isolated diheteropeptin[7] (**1**, Fig. 1), a novel metabolite with TGF-β like activity. We report herein the production, isolation and biological activities of **1**.

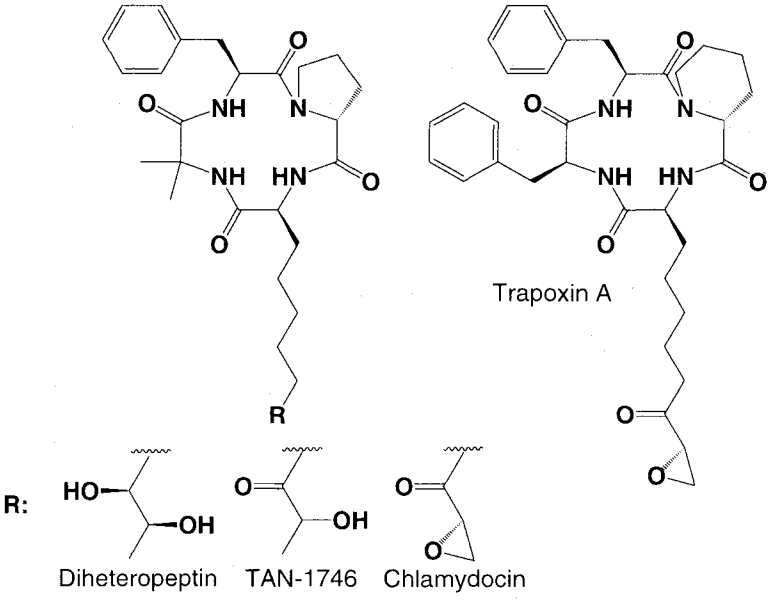
Materials and Methods

Cell Culture and Bioassay for Luciferase Activity

Mv1Lu mink lung epithelial cells bearing the PAI-1 reporter gene were maintained in Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12) supplemented with 5% fetal bovine serum.

For screening, 2 μl each of microbial broth was added into 96 multi-well plates containing 5×10^3 cells and 100 μl of the medium in each well. After incubation for 18 hours,

Fig. 1. Structure of diheteropeptin (**1**) and its related compounds.



the medium was removed and the cells were treated with a lysis buffer (Tris 25 mM, *trans*-1,2-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid 2 mM, dithiothreitol 2 mM, glycerol 10%, Triton X-100 1%, pH 7.8). The lysates were transferred into white 96 multi-well plates for measurement of the luciferase activity. The productivity of luciferase in each well was measured with a luminometer (EG & G Berthold Autolumat LB96P).

MTT Reduction Assay

MTT assay was used to determine the growth inhibitory activity of **1** against Mv1Lu cells. Mv1Lu cells were put into each well of 96-well plates at the density of 5×10^3 cells/well in 100 μl of DMEM/F12 (5% FBS). After incubation with various concentration of **1** at 37°C for 18 hours, 50 μg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added into the cell culture, and the plates were incubated at 37°C for 4 hours. The supernatant was removed and 100 μl of dimethyl sulfoxide was added into each well. The cell growth was measured with formazan formation at 570 nm using a multi-well plate reader (ORGANON TEKNIKA Microwell System, Reader 510).

LDH Release Assay

Lactate dehydrogenase (LDH) release assay[8] was used to determine the cytotoxicity of **1**. Mv1Lu cells were treated with **1** as describe above. The culture supernatants were transferred into another 96-well plate, and the remaining cells were lysed with a lysing buffer (0.1 M KH_2PO_4, 0.5% Triton X-100). The supernatants and the cell extracts were separately assayed for LDH release using an LDH-Cytotoxic Test Wako (Wako) according to the manufacture's instruction. The percent cell death was defined as the amount of LDH released into the culture medium divided by the total LDH activity in the culture (supernatants+cell extracts).

Assay of *In Vivo* Histone Deacetylase Activity

Histones in cultured cells were extracted according to the previous report[9]. Briefly, HeLa cells (2×10^6 cells) treated with or without drugs for 24 hours were collected with a cell scraper and washed with phosphate buffered saline. The cells were lysed in 1 ml of an ice-cold lysis buffer (10 mM Tris-HCl, 50 mM sodium bisulfite, 1% Triton X-100, 10 mM $MgCl_2$ and 8.6% sucrose, pH 6.5) by Dounce homogenization. The nuclei were collected by centrifugation and washed three times with the lysis buffer and once with 10 mM Tris-HCl buffer (pH 7.4) supplemented with 13 mM EDTA, successively. After centrifugation, the nuclear pellet was suspended in 0.1 ml of ice-cold water and then concentrated sulfuric acid was added to the suspension to give the final sulfate concentration of 0.2 M. After incubation at 4°C for 1 hour,

the suspension was centrifuged, and the supernatant was taken and mixed with 1 ml of acetone. The coagulated material obtained after overnight incubation at −20°C was collected and air-dried. The acid-soluble histone fraction was dissolved in 50 μl of water. The level of histone acetylation was analyzed by slab gel electrophoresis using an acid/urea/Triton (AUT) gel (1 M acetic acid, 8 M urea, 0.5% Triton X-100, 45 mM ammonia and 16% acrylamide) with an upper gel (1 M acetic acid, 6.3 M urea and 4.4% acrylamide). After the extracted histones were mixed with a loading buffer (7.4 M urea, 1.4 M ammonia, 10 mM dithiothreitol), electrophoresis was performed in a mixture of 0.2 M glycine and 1 M acetic acid, and then the gels were stained with Coomassie Brilliant Blue R-250 (Nacalai Tesque).

Assay of *In Vitro* Histone Deacetylase Activity

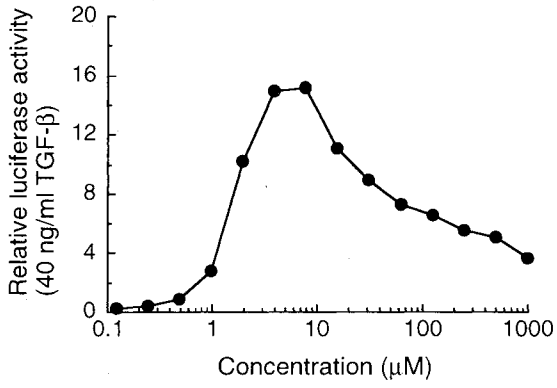
An assay for *in vitro* deacetylation of histones was basically designed according to that described by INOUE and FUJIMOTO[10]. The partially purified enzyme fraction was preincubated with various inhibitors for 5 minutes prior to the enzyme reaction. For the standard assay, [^{3}H]acetyl-labeled histones (6 μl, 2500 cpm/5 μg) were added to 94 μl of the enzyme fraction, and the mixture was incubated at 37°C for 30 minutes. The enzyme reaction was linear for at least 30 minutes under these conditions. The reaction was stopped by the addition of 10 ml of concentrated hydrochloric acid. The released [^{3}H]acetic acid was extracted with 1 ml of ethyl acetate, and the solvent layer (0.9 ml) was transferred into 5 ml of ACS (aqueous counting scintillant) II solution (Amersham Life Science) for determination of the radioactivity.

Results and Discussion

Producing Organisms and Fermentation

The fungal strain Q58044 was isolated from a soil sample collected at Yaku Island, Kagoshima Prefecture, Japan. Its characterization and identification were carried out mainly according to the paper by BARRON and ONIONS[11] and the strain Q58044 was identified as *Diheterospora chlamydosporia*. For the production of diheteropeptin, the fungus was inoculated into a seed medium consisting of glucose 1.0%, potato starch 2.0%, polypepton 0.5%, yeast extract 0.5% and $CaCO_3$ 0.4% (pH 7.0), and cultured on a rotary shaker (200 rpm) at 24°C for 3 days. Two milliliters of the seed culture were transferred into 500-ml Erlenmeyer flasks containing 100 ml of a production medium composed of glucose 2.0%, maltose 3.0%, corn steep liquor 1.0%, Polypepton 0.3%, soybean meal 1.5% and NaCl 0.3% (pH 6.0). The fermentation was carried out on a rotary shaker (200 rpm) at 24°C for 5 days.

Fig. 2. Effect of **1** on PAI-1 reporter expression.



Isolation of Diheteropeptin

The active principle was extracted from the broth supernatant (2 liter) with ethyl acetate (2 liter×3). The solvent layer was dried over Na_2SO_4 and concentrated to give an oily residue. This material was subjected to silica gel column chromatography ($CHCl_3$-MeOH=30:1) followed by 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 (20 i.d.×250 mm) with 65% MeOH. The physico-chemical properties of **1** are described in the accompanying paper[12].

Biological Activities

In the evaluation system we employed, the treatment of Mv1Lu cells with 40 ng/ml of TGF-β increased the expression of luciferase three times. Diheteropeptin induced gene expression from the PAI-1 promoter more than three times at the concentrations from 0.98 μM to 1000 μM (Fig. 2). The expression level increased dose-dependently up to 4 μM. Then the activity decreased gradually at higher concentrations due to the cytostatic activity of **1**. We next evaluated the effect of **1** on the cell growth and cytotoxicity by measuring MTT reduction and LDH release, respectively, since the structurally related compound chlamydocin (Fig. 1) had been reported to show cytostatic activity in mastocytoma cells[13]. Diheteropeptin inhibited the growth of Mv1Lu cells with an IC_{50} of 20.3 μM as determined by the MTT assay. No LDH release

Fig. 3. Cytostatic activity of **1** in MvlLu cells.

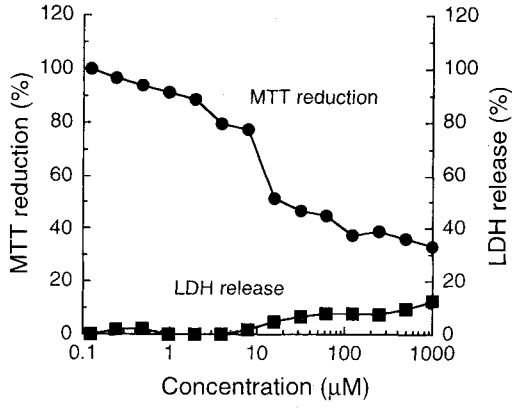
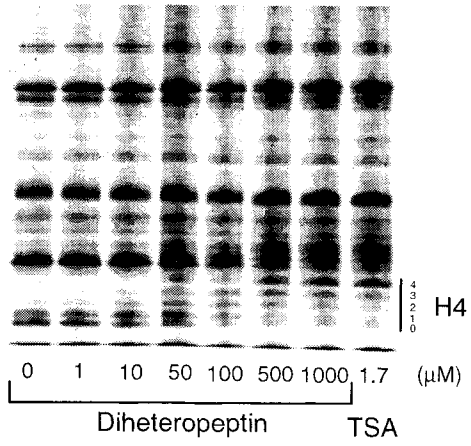


Fig. 4. Effect of **1** on histone acetylation *in vivo*.



H4: histone H4, TSA: trichostatin A

Table 1. Inhibitory activity of **1** on partially purified histone deacetylase.

Compound	IC_{50} (nM)
Diheteropeptin	24,600
TAN-1746	88.10
Trapoxin A	0.47
Trichostatin A	1.44
Sodium *n*-butyrate	150,000

was observed at the growth inhibitory concentration, suggesting that **1** exhibited cytostatic activity without cytotoxicity in Mv1Lu cells (Fig. 3).

Our screening for TGF-β-like substances using the PAI-1 reporter system detected various histone-deacetylase inhibitors including trichostatin A[9] (Table 1). This compound has been reported to inhibit histone deacetylase resulting in hyperacetylation of histones causing increased expression of several genes[14,15]. Therefore, we investigated the inhibitory effect of **1** on histone deacetylase in HeLa cells by adding **1** at concentrations ranging from 1 to 1000 μM. After incubation for 24 hours, histones were isolated from HeLa cells and analyzed by acid/urea/Triton (AUT) gel electrophoresis. Fig. 4 shows the profiles of histones on AUT gel electrophoresis that separated histones to components with different degree of acetylation. The non acetylated histone H4 band was followed by four additional bands corresponding to mono-, di-, tri- and tetra-acetylated forms with the tri- or tetra-acetylated form being predominant at high concentrations of **1** (500~1000 μM). Although the effective inhibitory concentrations of **1** on histone deacetylase were higher than that of trichostatin A (1.7 μM), both AUT gel electrophoresis patterns were almost identical, suggesting that **1** also inhibits histone deacetylase *in vivo*.

The level of histone acetylation is mainly controlled by the equilibrium between acetyltransferase and deacetylase[16]. The most likely reason for the induction of histone hyper acetylation by **1** is the direct inhibition of histone deacetylase. This possibility was checked by analyzing the *in vitro* effect of **1** on the partially purified histone deacetylase. The enzymatic activity was monitored by measuring the amounts of [^{3}H]acetic acid released from the ^{3}H-acetylated histones used as the substrate. As shown in Table 1, **1** inhibited histone deacetylase with an IC_{50} of 24.6 μM.

The structure of **1** is similar to those of TAN-1746[17] and trapoxin A[18] (Fig. 1). TAN-1746, an analog with a ketone moiety in the alkyl side chain, showed histone-deacetylase inhibitory activity at 280 times lower concentration than **1**, and trapoxin A, a known histone-deacetylase inhibitor with ketone and epoxide moieties, showed much stronger activity.

The epoxide group in trapoxin A was reported to be essential for enzyme inhibition, and a reduced derivative of trapoxin A did not cause any inhibitory effect on histone acetylation[19]. Since the effect of trapoxin A but not of trichostatin A was irreversible, it is speculated that trapoxin A binds covalently to the histone deacetylase *via* its epoxide moiety. While **1** and TAN-1746 contain no epoxide group at the terminus of the alkyl side chain, they showed

weak histone-deacetylase inhibitory activity with TAN-1746 being stronger than **1**. Although the epoxide and ketone groups play an important role for the histone-deacetylase inhibitory activity, it appears that the ketone function is not an essential moiety for the inhibition of the enzyme.

Acknowledgments

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