Pergamon

Tetrahedron 54 (1998) 15871–15878

TETRAHEDRON

Adenopeptin, a New Apoptosis Inducer in Transformed Cells from *Chrysosporium* sp.

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Received 29 July 1998; accepted 20 October 1998

Abstract: A new antitumor antibiotic, adenopeptin (**1**), was isolated from the culture broth of *Chrysosporium* sp. PF1201. The planar structure, which includes a tridecapeptide and a hexahydropyrrolo[1,2-a]pyrimidinium, was elucidated by mass spectrometric and NMR experiments. Adenopeptin (**1**) induced apoptotic cell death in cells transformed with the adenovirus oncogenes.

Keywords: Antibiotics; Antitumor compounds; Peptides and polypeptides

Several oncogenes including *myc*, *E2F* and the adenovirus E1A have been demonstrated to sensitize cells to apoptosis.[1-3] Thus, specific apoptosis inducers in cells expressing such oncogenes may be useful as anticancer agents for treating certain types of tumors. In order to evaluate such substances, we established cell lines by transformation of primary rat glia cells with the adenovirus oncogenes.[4] In the course of our screening for apoptosis inducers using these cells, we isolated linear polyketides, leptofuranins A-D,[4] from *Streptomyces tanashiensis* and a novel 20-membered macrolide, apoptolidin,[5,6] from *Nocardiopsis* sp. Further screening has resulted in the isolation of a unique active metabolite, adenopeptin (**1**), from the culture broth of a fungus identified as *Chrysosporium* sp. This compound induced apoptotic cell death in cells transformed with the adenovirus oncogenes including E1A but not normal glia cells or fibroblasts. In this report, we describe the structure elucidation and biological activity of **1**.

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0040-4020/98/$ - see front matter
PII: S0040-4020(98)00996-X

Structure elucidation

The molecular formula of **1** was determined as $C_{71}H_{123}N_{16}O_{14}^{+}$ (ion) by high-resolution FAB-MS. The strong IR absorption peaks at 3320, 1660 and 1540 cm^{-1} due to amide groups indicated that **1** belongs to the peptide antibiotics.

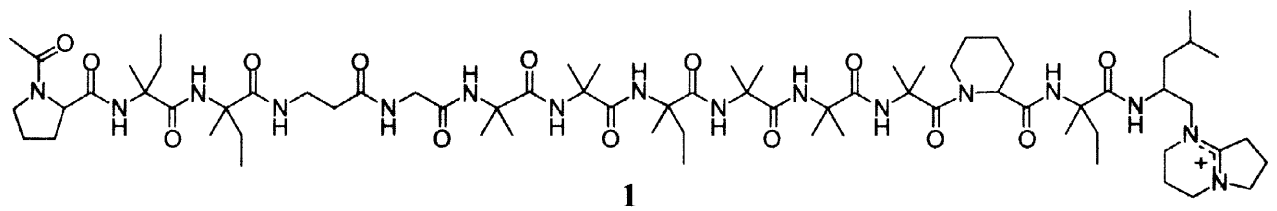

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Figure 1. Structure of adenopeptin (**1**).

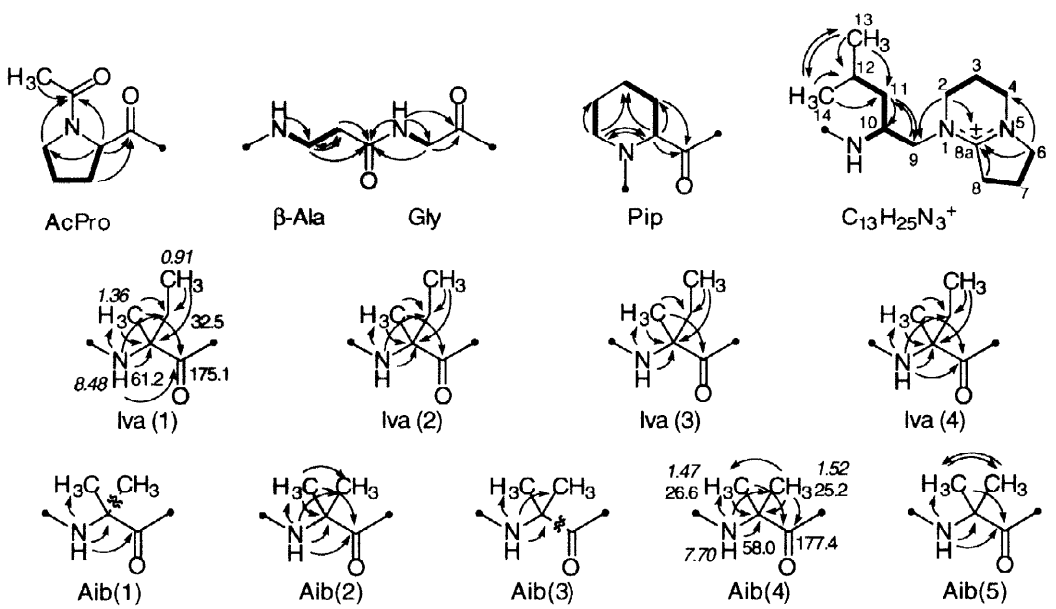

Figure 2. Partial structures of adenopeptin (**1**).
Bold lines show proton spin networks and arrows show ^{1}H-^{13}C long-range correlations.

Since **1** was resistant to acid hydrolysis for amino-acid analysis, the structure was analyzed based on NMR and MS experiments. The NMR data summary of **1** is presented in Table 1. Twelve amide protons including nine sharp singlets were clearly observed in the ^{1}H NMR spectrum of **1** in CD_3OH, although several ^{1}H and ^{13}C signals exhibited severe overlap and extensive line broadening especially for tertiary methyls. COSY and HMBC experiments

Table 1. ^{13}C and ^{1}H NMR assignments for adenopeptin (**1**) in CD_3OH.

	δ_C	δ_H
AcPro		
1	175.0	
2	61.4	4.33
3	30.6	2.23, 1.92
4	25.8	2.09, 1.97
5	49.5	3.62
MeCO	172.0	
MeCO	22.3	2.06
Iva (1)		
1	175.1	
2	61.2	
3	32.5	1.7~1.9
4	8.2~8.4	0.91
5	21.3	1.36
NH		8.48
Iva (2)		
1	176.8	
2	61.4	
3	32.6	1.7~1.9
4	8.2~8.4	0.86
5	20.7	1.43
NH		7.34
β-Ala		
1	174.9	
2	37.1	2.49, 2.40
3	37.3	3.59, 3.31
NH		7.54
Gly		
1	171.7	
2	45.0	3.76
NH		8.24
Aib (1)		
1	176.6	
2	57.7	
3	25.4	1.45~1.46
4	25~26	1.45~1.46
NH		8.36
Aib (2)		
1	177.1	
2	57.7	
3	25.6	1.41
4	25.0	1.45~1.46
NH		7.76
Iva (3)		
1	177.2	
2	60.8	
3	30.6	
4	8.2~8.4	0.87
5	21.7	1.40
NH		7.72
Aib (3)		
1	176.8	
2	57.8	
3	25.6	1.45~1.46
4	25.0	1.45~1.46
NH		7.92
Aib (4)		
1	177.4	
2	58.0	
3	26.6	1.47
4	25.2	1.52
NH		7.70
Aib (5)		
1	176.6	
2	57.8	
3	25.0	1.58
4	27.6	1.45
NH		8.03
Pip		
1	172.8	
2	58.3	4.54
3	26.3	2.14, 1.74
4	20.2	2.03, 1.63
5	24.8	1.72, 1.61
6	44.0	4.22, 3.37
Iva (4)		
1	176.9	
2	61.8	
3	33.1	2.08, 1.82
4	8.2~8.4	0.91
5	20.7	1.47
NH		7.53
C-terminus		
2	45.0	3.72, 3.35
3	19.7	2.15, 2.03
4	43.3	3.40
6	55.4	3.80, 3.72
7	18.7	2.20, 2.15
8	31.6	3.25, 2.95
8a	166.3	
9	57.6	3.40
10	45.5	4.41
11	42.0	1.53, 1.22
12	25.4	1.71
13	23.7	0.91
14	21.5	0.89
NH		7.33

revealed proton spin networks including three amide protons and ^{1}H-^{13}C long-range correlations as shown in Figure 2. These data identified *N*-acetylproline (AcPro), β-alanylglycine (β-Ala-Gly) and pipecolic acid (Pip) moieties.

In the HMBC spectrum, the nine singlet amide protons were coupled with nine quaternary α-carbons. Among them, long-range correlations were observed from both amide (δ 8.48) and tertiary methyl (δ 1.36) protons to a carbonyl carbon (δ 175.1), an α-carbon (δ 61.2) and a methylene carbon (δ 32.5), the latter two of which were also coupled with an additional methyl (δ_H 0.91), indicating the presence of an isovaline residue, Iva (1). The similar correlations revealed the presence of other three Iva residues, although all the expected couplings were not observed (Figure 2).

One of the remaining five amide protons (δ 7.70) displayed long-range couplings to an α-carbon (δ 58.0) and two methyl carbons (δ 25.2 and 26.6). Additional correlations from both geminal methyl protons (δ 1.47 and 1.52) to the α-carbon and a carbonyl carbon (δ 177.4) identified an α-aminoisovaleric acid residue, Aib (4). The similar correlations for the remaining four amide protons suggested the presence of four other Aib residues, but were insufficient for their complete assignments (Figure 2).

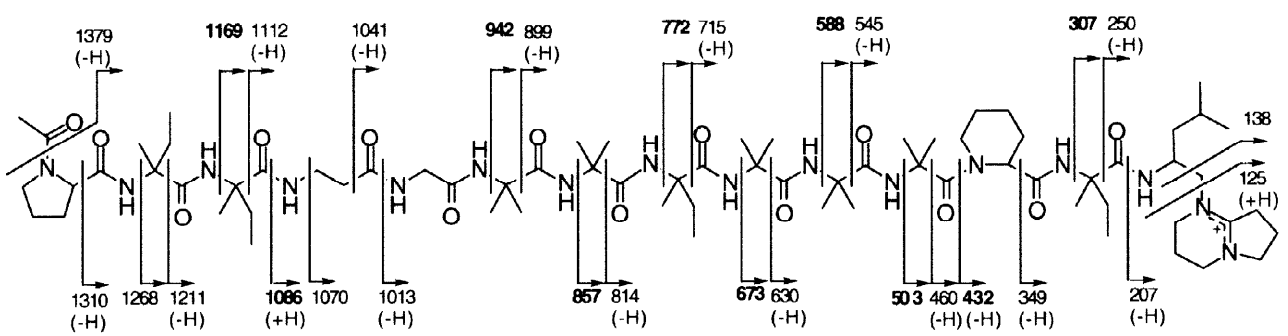

Figure 3. FAB-MS fragmentation in adenopeptin (**1**).
Bold numbers show significant fragment peaks.

In order to establish the structure and sequence of the amino acids, we carried out mass fragmentation analysis for **1**. The FAB mass spectrum revealed distinct fragment peaks with 57 or 43 mass unit differences due to the cleavage at the both sides of quaternary α-carbons (Figure 3), thereby showing that **1** is a linear peptide containing four Iva and five Aib residues. These fragmentation peaks also indicated the amino-acid sequence, which was confirmed by

^{1}H-^{13}C long-range correlations between the amide protons and the carbonyl carbons (Figure 4).

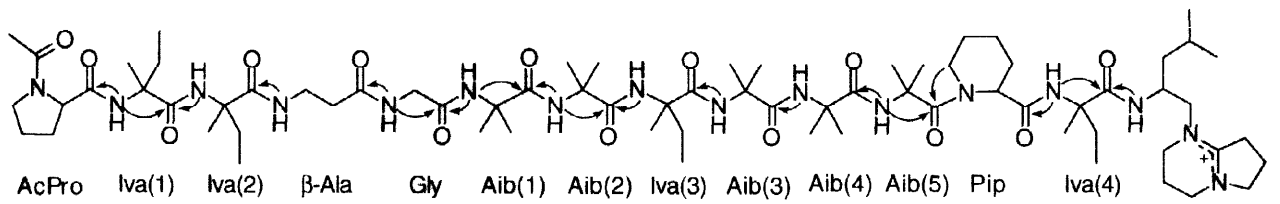

Figure 4. ^{1}H-^{13}C long-range correlations between the amino acid residues in adenopeptin (**1**).

The remaining part, $C_{13}H_{25}N_3^+$, appears to cause sequential fragmentation from the C-terminus of **1**. In this moiety, the COSY spectrum displayed three spin systems (shown by bold lines in Figure 2), one of which was expanded to include geminal methyls attached at C-12 due to long-range couplings from both 13-H_3 and 14-H_3 to C-11 and C-12 (Figure 2). The nitrogen atoms were bound to C-2, C-4, C-6, C-9 and C-10 based on their ^{1}H and ^{13}C chemical shifts. Long-range couplings from 2-H_2 to C-9 and from 6-H_2 to C-4 indicated the connections via nitrogen atoms between C-2 and C-9 and between C-4 and C-6. The remaining carbon (δ 166.3) coupled with the three methylenes (2-H_2, 6-H_2 and 8-H_2) was attributed to an amidinium carbon in a hexahydropyrrolo[1,2-a]pyrimidinium skeleton, and this assignment completed the overall structure of **1**.

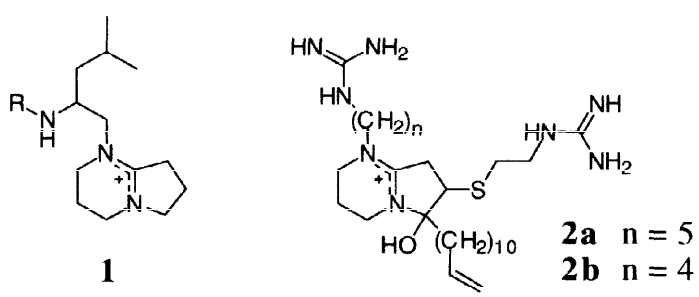

Figure 5. Structures of phloeodictines C1 (**2a**) and C2 (**2b**) and the carboxylic terminus of **1**.

Structurally related pyrrolo[1,2-a]pyrimidines are rarely found in natural products, which include phloeodictins (Figure 5),[7] cytotoxic guanidine alkaloids from the sponge *Phloeodictyon* sp. A series of fungal peptides are known as the peptaibols,[8,9] which contain a high proportion

of α,α-dialkylated amino acids such as Aib and Iva, an acetylated N-terminus and a C-terminal amino alcohol. The structure of **1** appears to be related to those of the peptaibols, but is distinguishable due to the inherent sequence of amino acid moieties and the presence of the novel C-terminus.

Biological activity

The antitumor activity of **1** was investigated using transformed rat glia cells and rat 3Y1 fibroblasts[10-12] (Table 2). In cells transformed with the adenovirus type 12 oncogenes including E1A, **1** induced apoptotic cell death, which was confirmed by observing chromatin condensation, nuclear fragmentation and DNA ladder formation in adenopeptin-treated cells. However, **1** showed no cytotoxicity against other transformed cells or normal cells, although their growth was inhibited at higher concentrations (Table 2). Thus, adenopeptin (**1**) seems to be a good candidate for selective anticancer drugs.

Table 2. IC_{50} values of adenopeptin (**1**) against normal and transformed cells.

Cell line	Oncogene	IC_{50} (ng/ml)
Glia		13,000
RG-E1A-7	E1A	6.4
RG-E1-4	E1A, E1B	49
3Y1		16,000
HR-3Y1	v-H-*ras*	4,600
SR-3Y1	v-*src*	840
SV-3Y1	SV40 large T antigen	8,500
Ad12-3Y1	E1A, E1B	16

Experimental

General. Specific optical rotations were obtained on a Jasco DIP-371 spectropolarimeter at 589.6 nm. Mass spectra were measured on a JEOL HX-110 spectrometer in the FAB mode

using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. NMR spectra were obtained on a JEOL JNM-A500 spectrometer with ^{1}H NMR at 500 MHz and with ^{13}C NMR at 125 MHz. Chemical shifts are given in ppm using TMS as internal standard.

Production and isolation of ***1***. The producing organism, *Chrysosporium* sp. PF1201, was cultivated in 500 ml-Erlenmeyer flasks containing 100 ml of a medium consisting of glucose 5.0%, soybean meal 1.0%, meat extract 0.4%, yeast extract 0.1%, sodium chloride 0.25% and calcium carbonate 0.5% (pH 7.0) on a rotary shaker at 25°C for 4 days. The culture filtrate (3 liters) was applied to a Diaion HP-20 column, which was washed with water and 25% methanol and eluted with acetone - 0.2M HCl (1 : 1). The eluate was evaporated and then partitioned between EtOAc and water. The aqueous layer was extracted with chloroform, and the extract was subjected to Sepabeads SP20SS column chromatography with 0.1% trifluoroacetic acid - 80% methanol. The active fraction was concentrated to dryness to give a colorless powder of **1** (207 mg): MP 106~110°C; $[\alpha]_D^{20}$ -3.53° (*c* 1.03, MeOH); molecular formula (ion) $C_{71}H_{123}N_{16}O_{14}^{+}$; high-resolution FAB-MS *m/z* 1423.9393 (M^+ Δ1.1 mmu); UV λ_{max} 201 nm (ε 30,400) in MeOH; IR ν_{max} 3320, 1660, 1540 cm^{-1} (KBr).

Cell culture. All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose, and grown at 37°C in a humidified atmosphere of 5% CO_2. Normal rat glia cells were obtained from primary cultures of Wistar rat (18-day embryo) cerebral cortex cells. All 3Y1 cell lines were obtained from Japanese Cancer Research Resources Bank. Cells at 50% confluence were plated at one tenth lower cell density and incubated for 3 days with various concentrations of samples. The growth was measured at 570 nm with formazan formation after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C.

Apoptosis assay. Chromatin structure was visualized by fluorescence microscopy after staining fixed cells (1 x 10^6 cells) with Hoechst Dye 33258. For the DNA fragmentation assay, cells (2 x 10^6 cells) were incubated in 100 µl buffer containing 50 mM Tris-HCl, 10 mM EDTA, 0.5% sodium *N*-lauroylsarcosinate and 100 µg/ml RNase (pH 8.0) at 37°C for 30 min. After

treatment with Proteinase K (100 μg/ml) at 55°C for 1 hour, the low-molecular-weight DNA was recovered by ethanol precipitation and analyzed by electrophoresis on 2% agarose gels.

Acknowledgments We thank Dr. Toru Sasaki, Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd. for the identification and fermentation of the producing organism. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, The Ministry of Education, Science, Sports and Culture, Japan, and Research for Future, Japan Society for the Promotion of Science.

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