ADIPOSTATINS A AND B, NEW INHIBITORS OF GLYCEROL-3-PHOSPHATE DEHYDROGENASE

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As a result of screening for inhibitors of glycerol-3-phosphate dehydrogenase, which may be effective to prevent corpulence, we isolated two inhibitors named adipostatin A and adipostatin B from the culture broth of *Streptomyces cyaneus* 2299-SV1. Their structures have been established to be 5-*n*-pentadecylresorcinol and 5-isopentadecylresorcinol, respectively. Adipostatin A and adipostatin B inhibited glycerol-3-phosphate dehydrogenase at the IC_{50} values of 4.1 μM and 4.5μM, respectively. These compounds prevented triglyceride accumulation in 3T3-L1 cells at a concentration of the μM level.

In some countries, corpulence is getting a serious problem through excessive food intake and lack of exercise. Increases of the number of adipocytes in early life stage and the content of triglyceride in adults are thought to be the cause of this disease. Therefore, an important approach for corpulence prevention may be to control the regulatory system of adipocytes proliferation and accumulation of triglyceride.

The 3T3-L1 cells, a clone of Swiss/3T3 fibroblast, are transformed to adipose cells under certain culture conditions[1~3] being accompanied by an increase of activities of many lipogenic enzymes[4~7]. Therefore, it can be regarded as a good model to study adipocytes differentiation. For example, the activity of glycerol-3-phosphate dehydrogenase (GPDH) increases several hundred fold presumably by playing an important role in the conversion process. This enzyme occupies a central position in the pathway of triglyceride synthesis. Therefore, inhibitors of GPDH are expected to prevent accumulation of triglyceride.

Although some nucleotides such as ATP, ADP, AMP and NAD[8,9], prophosphate[8] and fatty acids[10] were reported to inhibit the activity of GPDH, their activities were too weak to be used as practical inhibitors. Consequently we have screened new inhibitors of GPDH and succeeded in isolating new leads, named adipostatin A and adipostatin B. In this paper, we report the isolation, structure determination and biological activities of these inhibitors.

Materials and Methods

General Experimental Procedures

IR and UV spectra were measured on a Hitachi 270-30 spectrometer and a Hitachi U-3200 spectrophotometer, respectively. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were taken by a Jeol JNM-A500 spectrometer using $CDCl_3$ as the solvent. HR-FAB mass spectra were recorded on a Jeol HX-100 spectrometer. MP's were measured by a Yanaco micro melting point apparatus type MP-S3 and were not corrected.

Chemicals

Chemicals employed were as follows: Silica gel C-300 from Wako Chem. Ind., Osaka, Japan; Toyopearl

HW-40 from Tosoh MFG, Co., Ltd., Tokyo, Japan; a packed column of YMC ODS (20 i.d. × 250 mm, 5 μm) from Yamamura Chemical Laboratories Co., Ltd., Kyoto, Japan; glycerol phosphate dehydrogenase and dihydroxyacetone phosphate from Sigma Chem. Co., St. Louis, U.S.A. All other chemicals were of guaranteed reagent grade.

Glycerophosphate Dehydrogenase Assay

Enzyme assay was carried out by the method of WISE and GREEN[11] with the following modifications. The standard mixture contained 100 mM triethanolamine-HCl buffer (pH 7.5), 2.5 mM EDTA, 0.12 mM NADH, 0.6 mM dihydroxyacetone phosphate and 0.1 mM β-mercaptoethanol in a final volume of 2.84 ml. Methanol solutions (0.01 ml) containing adipostatin A, adipostatin B or other material were added to the standard mixture. The reaction was initiated by adding 0.15 ml of the enzyme solution (GPDH, 2 units/ml) to the reaction mixture at 20°C and the change in absorbance at 340 nm was observed.

Cell Culture and Adipose Conversion

3T3-L1 cells were obtained from the American Type Culture Collection. Cultivation of 3T3-L1 cell and its conversion to adipocytes were carried out according to the method of ARATANI and KITAGAWA[12] with slight modifications. For adipose conversion, cultures and confluence were treated with the DULBECCO's modified EAGLE's medium (DMEM) containing 10% fetal calf serum (FCS), 0.25 μM dexamethasone, 0.5 μM 1-methyl-3-isobutylxanthine, 10 μg/ml insulin (the induction medium) and adipostatins at the concentration indicated in the figure legends. After induction for two days, the medium was replaced with DMEM containing 10% FCS and adipostatins.

Triglyceride Assay

Cultured cells were washed once with phosphate-buffered saline, scraped off into 0.3 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, and then sonicated. The triglyceride content of the cells was determined enzymatically using a triglyceride G-Test Wako Kit (Wako Chem. Ind., Osaka, Japan).

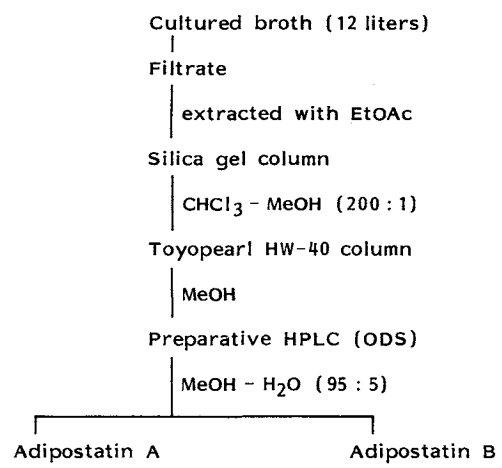
Results and Discussion

Production of Adipostatins

The producing organism, *Streptomyces cyaneus* 2299-SV1, was isolated from a soil sample collected in Nagano Prefecture, Japan. It was inoculated into test tubes containing 15 ml of the seed medium consisting of soluble starch 1%, Polypepton 1%, molasses 1% and beef extract 1% (pH 7.2). After incubation at 27°C for 3 days on a reciprocal shaker, one ml of this suspension was transferred to 500-ml Erlenmeyer flasks each containing 100 ml of the medium consisting of glycerol 2%, molasses 1%, casein 0.5%, Polypepton 0.1% and $CaCO_3$ 0.4%. The medium was adjusted to pH 7.0 before sterilization and the fermentation was carried out at 27°C for 4 days.

The culture filtrate of the strain showed inhibitory activity against GPDH. The isolation scheme for active principles is shown in Fig. 1. The broth filtrate (12 liters) was extracted with ethyl acetate (10 liters) and the extract was concentrated to dryness. The residue (830 mg) was applied to a

Fig. 1. Isolation scheme for adipostatin A and adipostatin B.



silica gel column (150 g) and developed with a mixture of chloroform-methanol (200:1). The crude active material (35 mg) thus obtained was subjected to Toyopearl HW-40 column chromatography (100 g) eluting with methanol. The active fraction was finally subjected to preparative HPLC using a YMC ODS with 95% aqueous methanol to give two active components which were separately concentrated to dryness giving white powders of adipostatin A (9.8 mg) and adipostatin B (4.8 mg).

Structural Determination

The physico-chemical properties of adipostatin A and adipostatin B are shown in Table 1. They are soluble in methanol, ethyl acetate, chloroform, ether and insoluble in water. Their molecular formulae were determined both to be $C_{21}H_{36}O_2$ by HRFAB-MS suggesting structural similarity between them.

The ^1H and ^{13}C NMR data of adipostatin A and adipostatin B are summarized in Table 2. Detailed analysis of ^1H NMR spectral data of adipostatin A (see Fig. 2) revealed the presence of an *n*-alkyl side chain (CH_3, t, δ 0.87; $11\times CH_2$, m, envelope at δ 1.25; CH_2, m, δ 1.29; CH_2, m, δ 1.58; CH_2, t, δ 2.48) connected to a quaternary carbon, a 1,3,5-trisubstituted benzene ring (δ 6.24 (2H, s) and 6.17 (1H, s)) and two exchangeable protons (δ 4.76 (2H, b)). The carbons in adipostatin A as revealed by ^{13}C NMR spectra were classified as follows; $CH_3\times 1$, $CH_2\times 14$, $-CH=\times 3$ and $-C=\times 3$. These results proved that adipostatin A was 5-pentadecylresorcinol.

The structure of adipostatin A was also corroborated by analysis of the HMBC spectrum. Thus, the terminal methylene proton (7-H, δ 2.48)

Table 1. Physico-chemical properties of adipostatin A and adipostatin B.

	Adipostatin A	Adipostatin B
Appearance	White powder	White powder
MP	62~63°C	90~91°C
Molecular formula	$C_{21}H_{36}O_2$	$C_{21}H_{36}O_2$
HRFAB-MS (*m/z*)		
Found:	321.2870 $(M+H)^+$	321.2849 $(M+H)^+$
Calcd:	321.2794	321.2794
UV λ_{max}^{MeOH} nm (ε)	272 (1,590), 278 (1,570), 221 (sh, 7,000)	272 (1,650), 278 (1,620), 221 (sh, 7,430)
IR (film) ν cm^{-1}	3315, 2924, 2856, 1596, 1468	3290, 2916, 2852, 1604, 1470
Rf value*	0.30	0.30

* $CHCl_3$-MeOH=25:1, Silica gel 60.

Table 2. ^{13}C and ^1H NMR spectral data of adipostatin A and adipostatin B.

	Functional group	Adipostatin A		Adipostatin B	
		δ_C	δ_H	δ_C	δ_H
1, 3	>C=	156.53		156.57	
2	-CH=	100.09	6.17 (s)[a]	100.11	6.17 (s)
4, 6	-CH=	108.01	6.24 (s)	108.03	6.24 (s)
5	>C=	146.13		146.15	
7	$-CH_2-$	35.79	2.48 (t)	35.82	2.48 (t)
8	$-CH_2-$	31.02	1.58 (m)	31.05	1.57 (m)
9	$-CH_2-$	31.89		39.06	1.16 (m)
18	$-CH_2-$	29.32		27.42	
19	*	29.25		27.97	1.50 (m)
20	**	22.65		22.65	0.87 (d)
21	$-CH_3$	14.09	0.87 (t)	22.65	0.87 (d)

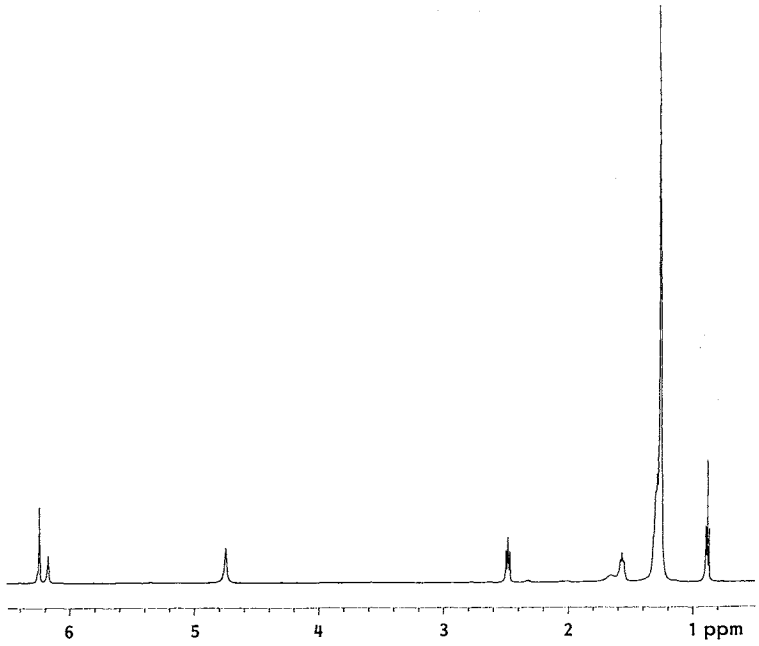
Methylene signals from C-10 to C-17 are as follows; adipostatin A, δ_C 29.64 ($\times 6$), 29.55 and 29.48, δ_H 1.25; adipostatin B, δ_C 29.95, 29.72, 29.67 ($\times 3$), 29.58, 29.51 and 29.28, δ_H 1.25.

[a] Multiplicities in ^1H NMR spectra.

* CH_2 in adipostatin A and CH in adipostatin B.

** CH_2 in adipostatin A and CH_3 in adipostatin B.

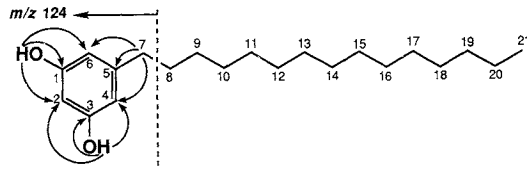
Fig. 2. 500 MHz ^{1}H NMR spectrum of adipostatin A in $CDCl_3$.



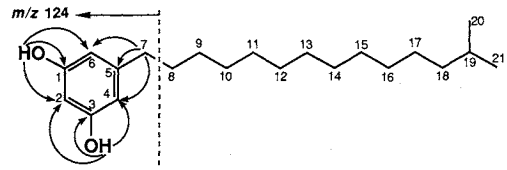
of the *n*-pentadecyl side chain was coupled to C-5 (δ 146.1), C-4 and C-6 (δ 108.0). Based on the ^{13}C chemical shifts, C-1 and C-3 (δ 156.5) were assigned to phenolic carbons. The phenolic hydroxyl protons (1-OH and 3-OH, δ 4.76) were coupled to C-1 and C-3 (δ 156.5), C-2 (δ 100.1), C-4 and C-6 (δ 108.0).

The base peaks at *m/z* 124 in the EI-MS spectrum of adipostatin A supported the compound to be a 5-alkyl resorcinol[13]. Therefore, the structure of adipostatin A was established to be 5-*n*-pentadecylresorcinol (Fig. 3).

Fig. 3. Structures of adipostatin A and adipostatin B.



Adipostatin A



Adipostatin B

The ^{1}H NMR spectrum of adipostatin B was quite similar to that of adipostatin A except for the appearance of a doublet signal corresponding to a gem-dimethyl residues (6H, d, δ 0.87) in place of a methyl triplet resonance in adipostatin A. This signal was straightforwardly assigned to an isopentadecyl side chain. Therefore, the structure of adipostatin B was determined to be 5-isopentadecylresorcinol. The fragment ion at *m/z* 124 supported this conclusion. As far as we know, adipostatin B has never been reported as a natural product, while adipostatin A is contained in wheat[14] and rye[15]. In addition, both compounds were obtained by acid hydrolysis of panosialin[16].

Inhibitory Activity against GPDH

In order to study the structure-activity relationship of adipostatin A and adipostatin B, their inhibitory

Fig. 4. Inhibition of glycerol phosphate dehydrogenase by adipostatins and other related compounds.

□ Adipostatin A, ■ adipostatin B, ● 3-pentadecylphenol, ○ stearic acid.

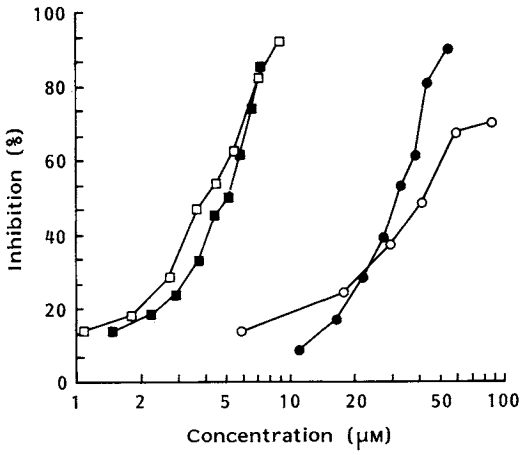
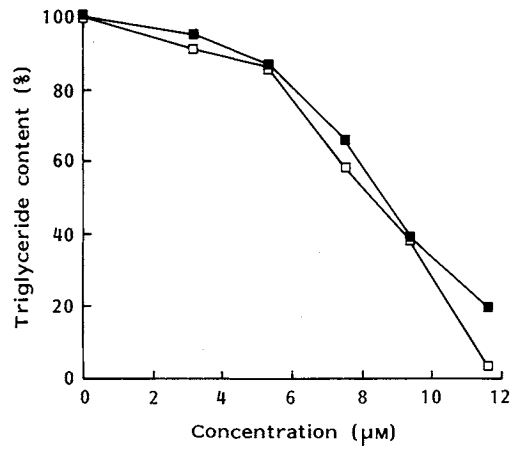


Fig. 5. Dose-dependent effects of adipostatins on the triglyceride content of 3T3-L1 cells.

□ Adipostatin A, ■ adipostatin B.



activities to GPDH were compared with those of 3-pentadecylphenol, 5-methylresorcinol and stearic acid. The IC_{50} values of adipostatin A and adipostatin B, 4.1 μM and 4.5 μM, respectively, showed clearly that adipostatins are approximately ten times as active as the other compounds tested (Fig. 4). 5-Methylresorcinol had no inhibitory activity against GPDH even at 1 mM and 3-pentadecylphenol was obviously less active than adipostatins. The activity of stearic acid, which was reported to inhibit GPDH, was also over ten times lower than that of adipostatins. These data indicated that the length of the alkyl side chain and the number of the hydroxy groups on the aromatic ring were important factors for GPDH inhibitors.

Biological Activity

The inhibitory activities of adipostatin A and adipostatin B on triglyceride accumulation were examined. The effects of adipostatin on triglyceride content of 3T3-L1 cells are shown in Fig. 5. They inhibited markedly the triglyceride accumulation in 3T3-L1 cells at 5~7.5 μM. Adipostatins did not show cytotoxicity to cultured 3T3-L1 cells at 7.5 μM.

Detailed studies on the biological activity of adipostatins are now under way.

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