NOTES

Structure of Sporostatin (M5032), an Inhibitor of Cyclic Adenosine 3′,5′-Monophosphate Phosphodiesterase

KENJI KINOSHITA, TOSHIYA SASAKI, MASASHI AWATA, MASAKI TAKADA and SATOSHI YAGINUMA*

Institute for Life Science Research,
Asahi Chemical Industry Co., Ltd.,
632-1, Mifuku, Ohito-cho, Shizuoka 410-23, Japan

(Received for publication July 28, 1997)

In the course of our screening program for unique microbial products with pharmacological activity, we have isolated sporostatin (M5032, **1**) as a new inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase (cAMP-PDE) from the fermentation filtrate of *Sporormiella* sp. M5032 (FERM P-9506)[1]. In this paper, we describe the purification, physicochemical properties, structural elucidation and biological properties of sporostatin (**1**).

Materials and Methods

General Procedure

The IR spectrum was taken with a Hitachi 260-50 IR spectrophotometer. The UV spectrum was recorded on a Shimadzu UV-365 spectrometer. The NMR spectra were obtained with a Jeol JNM-GSX400 spectrometer at 400 MHz (^{1}H) and 100 MHz (^{13}C) with TMS as an internal reference. The mass spectrum was taken with a Jeol JMS-SX102 spectrometer.

Materials

Cyclic adenosine 3′,5′-monophosphate phosphodiesterase (cAMP-PDE) from bovine heart and cyclic adenosine 3′,5′-monophosphate (cAMP) were purchased from Boehringer-Mannheim, papaverine and theophylline were from Sigma Chemical Co.

Fermentation

A 500 ml Erlenmeyer flask containing 100 ml of a seed medium composed of glucose 1%, dextrin 1%, yeast extract 0.5%, casein hydrolyzate 0.5%, $CaCO_3$ 0.1%, celite 1% (pH 6.5 before sterilization) was inoculated with the mycelia of the organism grown on potato-glucose agar slant. The inoculated flask each was incubated on a rotary shaker (200 rpm) at 26°C for 96 hours. Three percent (v/v) of this seed culture was inoculated into one hundred 500 ml Erlenmeyer flasks containing 100 ml of the production medium consisting of glucose 2%, peptone 1%, corn steep liquor 1%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, celite 1% in tap water (pH 6.5 before sterilization) and incubated on a rotary shaker (200 rpm) at 26°C for 3 days.

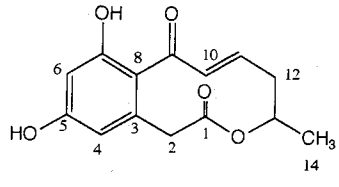
Enzyme Assay

Bovine heart cAMP-PDE activity was measured as follows: The reaction mixture contained in a final volume of 1.0 ml, Tris-HCl buffer (pH7.0) 40 mM, $MgSO_4$ 2 mM, cAMP 0.6 mM as a substrate and the enzyme 7.5 mU/ml. The reaction was started by adding the substrate. Incubation was carried out 30°C for 30 minutes, and the reaction was terminated by adding 0.1 ml of 55% perchloric acid, and the mixture was centrifuged at 3000 rpm for 10 minutes. The reaction product, 5′-AMP in the supernatant, was analyzed with HPLC. The HPLC was performed on a reversed phase silica gel column (Hitachi, #3056, 4 mm i.d. × 150 mm) with 10 mM KH_2PO_4 (pH 2.0)-MeOH (10 : 1) as a solvent system with detection at 260 nm. Flow rate of this mobile phase was 1.5 ml/minute.

X-Ray crystallography

Single crystals of sporostatin (**1**) were obtained in pale yellow prism by recrystallization from MeOH. The crystal was mounted on a Mac Science MXC18 diffractometer with graphite-monochromated Cu Kα radiation ($\lambda = 1.5418$ Å). Crystal structure was solved by direct method with SIR92[2], and refined by full-matrix least-squares method with anisotropic thermal parameters for all non-H atoms and isotropic thermal parameters for all H atoms. Final R values was 0.032.

Fig. 1. Structure of sporostatin.



Results and Discussion

Production, Isolation and Purification

The strain of *Sporormiella* sp. M5032 (FERM P-9506) was isolated from bovine excrement. This strain was cultured in Erlenmeyer flasks at 26°C for 3 days on a rotary shaker to produce the inhibitor, sporostatin (**1**). The inhibitor was presents in the culture filtrate (9 liters) which was separated by centrifugation. The inhibitor was extracted with EtOAc at pH 2, and dried with anhydrous Na_2SO_4. The EtOAc extract was concentrated to a crude powder (*ca.* 700 mg) *in vacuo*. The crude material was chromatographed on a silica gel column eluted with $CHCl_3$-EtOAc (10:1). The active fractions were collected and concentrated, *in vacuo*, to a colorless amorphous powder (230 mg). This powder was dissolved in MeOH and crystallized to obtain pale yellow unaggregated crystals of sporostatin (**1**, 130 mg).

Physico-chemical Properties

Physico-chemical properties of **1** are summarized in Table 1. Rf values of **1** on a Silica gel f plate (Tokyo Kasei Co., Ltd.) developed in various solvent systems are: $CHCl_3$-MeOH (20:1, Rf 0.55), $CHCl_3$-EtOAc (10:1, Rf 0.24), benzene-EtOAc (10:1, Rf 0.08). Sporostatin (**1**) was visualized with I_2 vapor, $FeCl_3$ or by heating after the plate was sprayed with 50% H_2SO_4-MeOH. The compound was soluble in various organic solvents such as MeOH, $CHCl_3$, EtOAc, and dimethyl sulfoxide, but insoluble in water and hexane.

Table 1. Physico-chemical properties of sporostatin.

Appearance	Pale yellow crystal
MP (°C)	197~200
$[\alpha]_D^{25}$ (c=0.73, MeOH)	−18.8°
Molecular formula	$C_{14}H_{14}O_5$
FAB-MS (m/z)	263 $(M+H)^+$
HRFAB-MS (m/z)	
Obs.	263.0916 $(M+H)^+$
Calcd.	263.0919 for $C_{14}H_{15}O_5$
UV λ_{max} nm (log ε) in MeOH	
Neutral and Basic	202 (4.32), 220 (sh), 235 (sh), 292 (4.12), 330 (sh)
Acidic	202 (4.34), 345 (4.46)
IR (KBr) ν_{max} cm^{-1}	3370, 1730, 1630, 1590, 1490, 1430, 1390
Elemental analysis	
Found (Calcd.) C%:	63.51 (64.12)
H%:	5.40 (5.34)
Color reaction: Positive	$FeCl_3$, I_2, H_2SO_4
Negative	Ninhydrin
Solubility: Soluble	MeOH, $CHCl_3$, EtOAc, DMSO
Insoluble	Hexane, H_2O

Structure Elucidation

The molecular formula of sporostatin (**1**) was determined as $C_{14}H_{14}O_5$ based on HRFAB mass spectrum [$(M+H)^+$, m/z 263. 0916; Calcd for $C_{14}H_{15}O_5$: 263.0919] and elemental analysis [Found (Calcd.) : C% 63.51 (64.12), H% 5.40 (5.34)].

^1H and ^{13}C NMR spectral data in DMSO-d_6 are described in Table 2. The assignments were made on the basis of ^1H-^1H COSY, ^{13}C-^1H COSY, HMBC and NOESY with the 2D NMR experiments. The ^1H NMR spectrum of **1** exhibited one secondary methyl group, two methylenes and one methine protons attached to saturated carbons, and four olefinic protons and two phenolic hydroxyls. In the low field region of the ^1H NMR spectrum, the signals of two phenolic hydroxyl protons (7-OH: δ 13.61 (hydrogen bonded) and 5-OH: δ 10.74) and four aromatic protons (δ 6.91, 6.32, 6.24 and 5.93) were observed. These four methines are assigned to 10-H, 4-H, 6-H and 11-H, respectively. The geometrical configuration of the olefinic bonds were established to be 10E by the vicinal coupling constant (J_{10-11}=16.5 Hz). In the NOE experiments, 12-Ha observed NOE to 14-CH_3 and 10-H, thereby indicated that these protons exist in the same plane. On the other hand, 12-Hb observed NOE to 13-H and 11-H. From these results, the structure of sporostatin (**1**) was determined as shown in Fig. 1. The ^{13}C NMR and other spectroscopic data mentioned above support the conclusion.

Table 2. ^{13}C (100 MHz) and ^1H NMR (400 MHz) assignments in DMSO-d_6 for sporostatin.

Position	^{13}C NMR δ_C (Multi.)		^1H NMR δ_H
1	163.7 (s)		
2	43.9 (t)	Ha	3.82 (1H, d, J=17.4)[a]
		Hb	4.07 (1H, d, J=17.1)
3	111.7 (s)		
4	114.5 (d)		6.32 (1H, s)
5	140.7 (s)		
6	102.1 (d)		6.24 (1H, s)
7	173.1 (s)		
8	167.4 (s)		
9	198.3 (s)		
10	136.3 (d)		6.91 (1H, d, J=16.5)
11	138.0 (d)		5.93 (1H, m)
12	41.6 (t)	Ha	2.12 (1H, dd, J=11.6, 10.7)
		Hb	2.54 (1H, m)
13	74.9 (d)		5.11 (1H, m)
14	19.6 (q)		1.36 (3H, d, J=6.4)
5-OH			10.74 (1H, s)
7-OH			13.61 (1H, s)

[a] Proton number, multiplicity and coupling constants (Hz) are in parentheses.

The structure of **1** was finally determined by X-ray crystallography, but the absolute stereochemistry was not determined. The cell parameters, data collections and refinement details for **1** are summarized in Table 3. Fig. 2 shows the ORTEP view of molecular structure of sporostatin (**1**).

A variety of compounds produced by microorganism have been found to inhibit cyclic adenosine 3′,5′-monophosphate phosphodiesterase (cAMP-PDE), including reticulol[3], PDE-I and II[4], acylpeptides[5], terferol[6], griseolic acid[7], KS-501 and KS-502[8] and TPI compounds[9]. Sporostatin (**1**) was identified as a novel 10-membered macrolide derivatve with inhibitory activities against cAMP-PDE.

Biological Activity

Inhibitory activities of sporostatin (**1**) against cAMP-PDE from bovine heart expressed in terms of 50% inhibition (IC_{50}) was 41 μg/ml, and it was noncompetitive againt cAMP. In the same condition, theophylline (IC_{50}: 470 μg/ml) was competitive, and paraverine (IC_{50}: 25 μg/ml) was noncompetitive against cAMP.

Sporostatin (**1**) at 1000 μg/ml exhibited no antimicrobial activity against *Staphylococcus aureus* FDA-209P, *Bacillus subtilis* PCI-219, *Escherichia coli* HIHJ34, *Pseudomonas aeruginosa* ML-4262R$^-$ or *Candida albicans* NI-7491. No acute toxicity of this compound **1** was observed at 50 mg/kg in mice injected intraperitoneally.

Table 3. Single crystal X-ray crystallographic analysis for sporostatin.

Crystal parameters:	
Chemical formula	$C_{14}H_{14}O_5$
Formula weight	262.26
Crystal dimensions	$0.18 \times 0.15 \times 0.10\,mm^3$
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Cell dimensions	a = 8.744 (3) Å
	b = 20.020 (7) Å
	c = 7.104 (3) Å
	V = 1243.7 (8) Å^3
Z	4
D_{calcd} (g/cm^3)	1.402
Refinement parameters:	
Unique reflections	917 ($2\theta_{max}$ = 99.12°)
Reflections with $I > 3\sigma(I)$	863
R factor	0.032

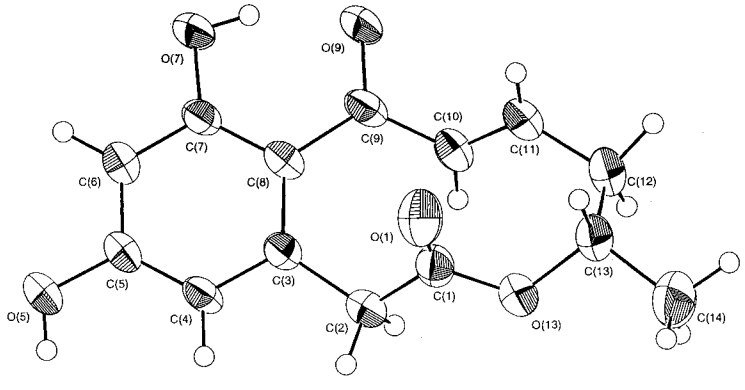
Acknowledgments

The authors wish to thank Mrs. K. YAMASHITA for X-ray crystallography.

References

1) YAGINUMA, S.; T. SASAKI, M. AWATA, M. TAKADA, M. HAYASHI & K. MIZUNO (Toyo Jozo): Biologically active substance M5032 and its preparation. Jpn. Kokai 83078 ('89), March 28, 1989
2) ALTOMARE, A.; G. CASCARANO, C. GIACOVAZZO, A. GUAGLIARDI, M. C. BURLA, G. POLIDORI & M. CAMALLI: SIR92—a program for automatic solution of crystal structures by direct methods. J. Appl. Cryst. 27: 435, 1994
3) FURUTANI, Y.; M. SHIMADA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Reticulol, an inhibitor of adenosine 3′,5′-monophosphate phosphodiesterase. J. Antibiotics 28: 558～560, 1975
4) EMOTO, Y.; Y. FURUTANI, H. NAGANAWA, M. HAMADA, T. TAKEUCHI & H. UMEZAWA: Isolation and characterization of PDE-I and II, the inhibition of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. Agric. Biol. Chem. 42: 1331～1336, 1978
5) HOSONO, K. & H. SUZUKI: Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase I. Purification, physicochemical properties and structures of fatty acid residues. J. Antibiotics 36: 667～673, 1983
6) NAKAGAWA, F.; R. ENOKITA, A. NAITO, Y. IIJIMA & M. YAMAZAKI: Terferol, an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase I. Isolation and characterization. J. Antibiotics 37: 6～9, 1984
7) NAKAGAWA, F.; T. OKAZAKI, A. NAITO, Y. IIJIMA & M.

Fig. 2. A ORTEP drawing of the molecular structure of sporostatin.



YAMAZAKI: Griseolic acid, an inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase I. Taxonomy, Isolation and characterization. J. Antibiotics 38: 823～829, 1985

8) NAKANISHI, S.; K. ANDO, I. KAWAMOTO & H. KASE: KS-501 and KS-502, new inhibitors of Ca^{2+} and calmodulin-dependent cyclic-nucleotide phosphodiesterase from *Sporothrix* sp. J. Antibiotics 42: 1049～1055, 1989

9) YAGINUMA, S.; M. AWATA, M. TAKADA & K. KINOSHITA (Toyo Jozo): cyclic adenosine 3′,5′-monophosphate phosphodiesterase inhibitors. Jpn. Kokai 215551 ('87), Sept. 22, 1987