TERFEROL, AN INHIBITOR OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE

I. ISOLATION AND CHARACTERIZATION

FUMIO NAKAGAWA, RYUZOU ENOKITA and ATSUSHI NAITO

Fermentation Research Laboratories

YASUTERU IIJIMA and MITSUO YAMAZAKI

Biological Research Laboratories, Sankyo Co., Ltd.,
1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan

(Received for publication September 10, 1983)

Terferol, a new inhibitor of cyclic adenosine 3′,5′-monophosphate phosphodiesterase (EC 3.1.4.17, cAMP-PDE), was isolated from the cultured broth of *Streptomyces showdoensis* SANK 65080. It was found to have the molecular formula $C_{19}H_{16}O_3$ and to possess inhibitory activity not only against cAMP-PDE but also against cyclic guanosine 3′,5′-monophosphate phosphodiesterase (cGMP-PDE) from various rat tissues. The terferol concentration required for 50% inhibition of cAMP-PDE was 0.82 μM.

Cyclic adenosine 3′,5′-monophosphate (cAMP) plays an important role in the metabolism of mammalian cells as a second messenger mediating the actions of various hormones and is thought to be concerned with the control of many cellular functions[1,2]. Abnormal levels of cAMP are associated with, and appear to contribute to, a number of disease states[3,4]. In living cells, cAMP is formed from adenosine triphosphate by adenylate cyclase and hydrolyzed into 5′-adenosine monophosphate by cAMP-PDE. The concentration of cAMP is controlled by these two enzymes.

Some inhibitors of cAMP-PDE produced by microorganisms have been isolated by FURUTANI,[5] EMOTO[6] and HOSONO[7]. In the course of our screening program for inhibitors of cAMP-PDE in microbial culture filtrates, one of the active substances produced by strain No. 43924 was isolated, characterized as a new compound and named terferol.

This paper deals with the assay method for cAMP-PDE, taxonomy of the producing organism, fermentation, isolation and characterization of terferol.

Determination of cAMP-PDE Activity

Crude cAMP-PDE was prepared from rat tissues by the modified procedure of PICHARD *et al.*[8]. Various rat tissues were homogenized with 3 times their weight of 0.17 M Tris-HCl buffer, pH 7.4, containing 5 mM $MgSO_4$, with a teflon homogenizer at 0°C and centrifuged at 100,000 *g* for 20 minutes. The supernatant enzyme solutions were stored at −20°C. The enzyme preparations thus obtained did not lose activity during 3 months of storage at −20°C. The reaction mixture (total volume 0.1 ml) containing 40 mM Tris-HCl buffer (pH 7.4), 5 mM $MgSO_4$, 50 μM $CaCl_2$, 200 μg/ml snake venom (Sigma, containing 5′-nucleotidase), 0.14 μM [^{14}C]cAMP and an appropriate amount of the enzyme preparation which was diluted 100~200 times with the same buffer containing 5 mM $MgSO_4$, was incubated at 30°C for 20 minutes. Then, the reaction was stopped by adding 1 ml of an IRP-58 resin slurry (Organon, 200~400 mesh resin/water, 1/2 (v/v)). The reaction product, [^{14}C]adenosine, was determined

in a liquid scintillation counter. The protein content was determined by the method of LOWRY *et al.*[9] with bovine serum albumin as the standard.

Assay Method of cAMP-PDE Inhibitors

The enzyme preparation from rat brain was used for the screening of cAMP-PDE inhibitors. As the source of inhibitors, the culture filtrates of microorganisms were heated in a boiling water bath for 10 minutes and 5 μl of the treated filtrate was added to 0.1 ml of the reaction mixture.

The activity of inhibitors was expressed as percent inhibition.

Taxonomy

An actinomycete, strain No. 43924, was isolated from a soil sample collected in Kyoto-fu, Japan. Strain No. 43924 was found to be a *Streptomyces* and further taxonomic characterization was carried out according to the method used in the International Streptomyces Project (ISP). The mature spore chains were generally long with 10~50 spores per chain. The spores were oval in shape with smooth surfaces. The aerial mycelium was brownish gray in color on most of the agar media tested. Melanin was produced both in peptone - yeast extract - iron agar and Tryptone - yeast extract broth.

From the characteristics mentioned above as well as the result of carbohydrate utilization test and other physiological properties, strain No. 43924 was identified as *Streptomyces showdoensis* and designated *Streptomyces showdoensis* SANK 65080.

Fermentation

A loopful of the spore suspension from growth on Bennett agar medium was inoculated

Fig. 1. Time course of terferol production by *S. showdoensis* SANK 65080.

Fermentation was carried out in a 15-liter jar fermentor using the medium described in the text at 28°C with agitation of 200 rpm and aeration of 15 liters/minute. The amount of mycelium is expressed as packed cell volume (ml) per 10 ml of the cultured broth after centrifugation at 3,000 rpm for 15 minutes. Inhibitory activity is expressed as percent inhibition of the enzyme activity.

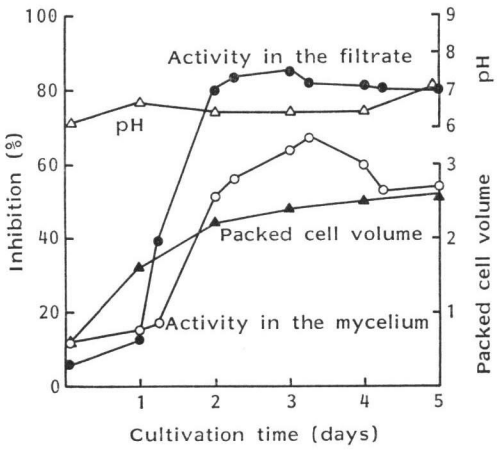

Fig. 2. Isolation procedure for terferol.

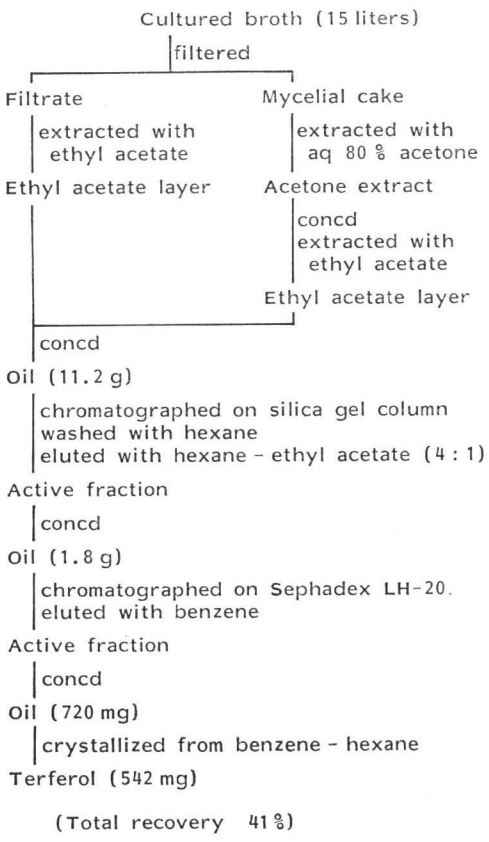

Fig. 3. Double reciprocal plot analysis of the effect of terferol on cAMP-PDE from rat brain.

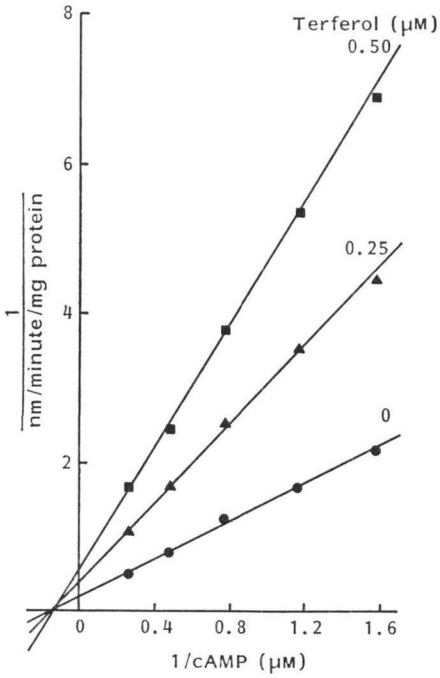

Table 1. Inhibition by terferol of cyclic nucleotide phosphodiesterases from various rat tissues.

Values are expressed as the concentration required for 50% inhibition of the enzyme activity. Substrates at the following concentrations were used; a) 0.14 μM cAMP, b) 0.14 μM cGMP.

Tissue	I_{50} (μM)	
	cAMP	cGMP
Brain	0.82	0.96
Aorta	2.7	0.49
Heart	18	20
Platelet	39	13
Kidney	12	3.0
Liver	58	18

into 100 ml of a seed medium consisting of 5.0% glucose, 1.0% soybean meal, 0.4% Polypepton, 0.4% meat extract, 0.1% yeast extract, 0.25% NaCl and 0.5% $CaCO_3$, in a 500-ml Erlenmyer flask. After incubation at 28°C for 96 hours on a rotary shaker, 300 ml of the seed culture were transferred into 15 liters of the same medium in a 30-liter jar fermentor. The fermentation was carried out at 28°C for 48 hours with aeration (15 liters/minute) and agitation (200 rpm). An example of the time course of the fermentation is shown in Fig. 1. The inhibitory activity against cAMP-PDE was detected after 24 hours and reached a maximum after 48 hours of cultivation.

Isolation and Purification

A flow diagram for the isolation procedure of terferol is shown in Fig. 2.

Biological Activity

Fig. 3 shows the double reciprocal plots of the cAMP concentration *versus* reaction velocity of cAMP-PDE from rat brain in the presence of terferol at various concentrations. The inhibition of terferol was non-competitive in regard to cAMP with a *Ki* value of 0.22 μM.

Terferol inhibited cAMP-PDE obtained from rat brain, aorta, heart, platelet, kidney and liver (Table 1). The concentration for half-maximal inhibition was the lowest for the brain enzyme and highest for the liver enzyme. Terferol also inhibited cGMP-PDE. Mice tolerated the intraperitoneal administration of 200 mg/kg of terferol.

References

1) Greengard, P.; R. Paoletti & G. A. Robison: Advances in Cyclic Nucleotide Research. Vol. 1. Raven Press, New York, 1972
2) Burger, M. M.; B. M. Bombic, B. M. Breckenridge & J. R. Sheppard: Growth control and cyclic alterations of cyclic AMP in the cell cycle. Nature New Biol. 239: 161~163, 1972
3) Amer, M. S.: Cyclic adenosine monophosphate and hypertension in rat. Science 179: 807~809, 1973
4) Parker, C. W. & J. W. Smith: Alterrations in cyclic adenosine monophosphate metabolism in human bronchial asthma. J. Clin. Invest. 52: 48~59, 1973

5) 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
6) 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
7) 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
8) PICHARD, A.-L. & W. Y. CHEUNG: Cyclic 3′,5′-nucleotide phosphodiesterase. J. Biol. Chem. 251: 5726~5737, 1976
9) LOWRY, O. H.; N. J. ROSEBROUGH, A. L. FARR & R. J. RANDALL: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 262~275, 1951