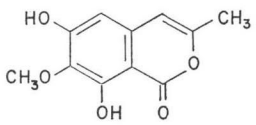
RETICULOL, AN INHIBITOR OF CYCLIC ADENOSINE 3′, 5′-MONOPHOSPHATE PHOSPHODIESTERASE

Sir:

As reported by H. UMEZAWA,[1] microoganisms produce enzyme inhibitors with interesting structures which seem to be unrelated to the substrate structures. We screened microbial culture filtrates for inhibition of cyclic adenosine 3′, 5′-monophosphate phosphodiesterase (PDE) [E.C.3.1.4.c] and found that about 1 % of soil actinomycetes produce inhibitors of this enzyme. One of the active agents was isolated and identified as reticulol (**I**, 6, 8-Dihydroxy-7-methoxy-3-methylisocoumarine) which has no structural relationship with cyclic adenosine 3′, 5′-monophosphate (cAMP). In this paper, the assay method for PDE, the isolation procedures for the active agent, and the type of inhibition are reported.



(I)

A crude PDE was prepared from rabbit brain by a modification of the procedure of DRUMMOND *et al.*[2] The brains from 3 rabbits were homogenized with 3 times their weight of 0.25 M sucrose by a Teflon homogenizer and centrifuged at 100,000×*g* for 20 minutes. The supernatant was brought to 40 % saturation with ammonium sulfate and centrifuged at 10,000×*g* for 20 minutes. The precipitate was dissolved in 0.02 M Tris-HCl, pH 7.5 (1 ml per g brain). This solution was dialyzed overnight at 4°C against 3 liters of the same buffer. The dialyzed solution was stored at −20°C and used as the crude enzyme. Storage of this solution at −20°C did not decrease the activity, and the enzyme could be employed at least for 3 months. The protein content was determined by the method of LOWRY[3] with bovine serum albumin as the standard. Generally the enzyme solution contained 10~15 mg protein/ml. Before use this solution was diluted 30~40 times with 0.02 M Tris-HCl (pH 7.5).

The reaction mixture (total volume, 0.1 ml) consisted of 0.01 ml of 80 mM Tris-HCl (pH 7.5), 0.01 ml of 70 mM $MgSO_4$, 0.01 ml of 20 mM adenosine 5′-monophosphate (5′-AMP), 0.01 ml of 0.5~2 mM cAMP containing 2.2×10^{-3} μCi ^{14}C-cAMP, 0.01 ml of an aqueous solution of a test compound, 0.01 ml of the enzyme solution and 0.04 ml of H_2O. In this reaction mixture incubated at 37°C, 30 % of added cAMP was hydrolyzed in 20 minutes.

In order to avoid subsequent degradation of the reaction product (^{14}C-5′-AMP) by 5′-nucleotidase, a possible contaminant in cultured broths and the crude enzyme, 5′-AMP was added to the reaction mixture. At the concentration added 5′-AMP did not influence the reaction velocity of PDE and did not interfere with the separation process of ^{14}C-cAMP.

The enzyme reaction was started by addition of the enzyme or substrate, continued for 20 minutes at 37°C and stopped by heating in a boiling water bath for 3 minutes. The reaction mixture was diluted with 0.4 ml of distilled water and passed through a dry alumina (neutral, Merck) column (0.5×2.7 cm, 0.5 g). Unhydrolyzed ^{14}C-cAMP was eluted from the column with 2 ml of 10 mM Tris-HCl (pH 7.5), while the reaction product (^{14}C-5′-AMP) was retained on the column.[4] The effluent and eluate were collected directly into counting vials and 8 ml of BRAY's scintillation fluid was added. The radioactivity of the solution was determined with an Aloka LSC-653 liquid scintillation counter. PDE activity was obtained by subtracting d.p.m. of a test run from d.p.m. of an unincubated control run.

Before addition to the reaction mixture, culture filtrate of *streptomyces* was heated in a boiling water bath for 10 minutes, and 10 μl was added to the reaction mixture. In this screening study, the strain MD611-C6 (the strain number in the Institute of Microbial Chemistry) was found to produce an inhibitor of PDE. The properties of this strain suggested that it was most closely related to *Streptomyces mobaraensis*. This strain was cultured in 110 ml of a medium placed in Erlenmeyer flasks (500-ml volume) for 72 hours at 27°C on a rotary shaker. The medium contained starch 2.0 %, glucose 2.0 %, soybean meal 2.0 %, yeast extract 0.5 %, NaCl 0.25 %, $CuSO_4\cdot5H_2O$ 5 mg/ml,

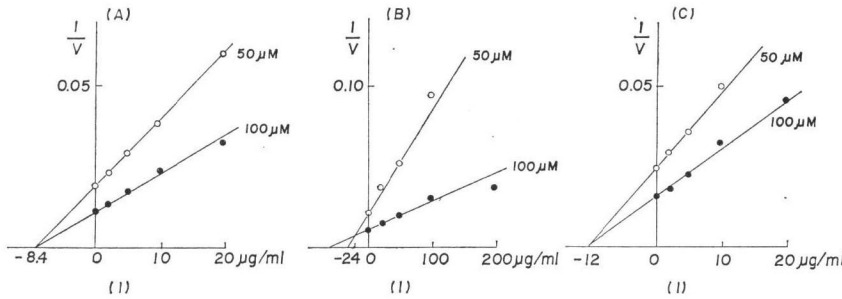
$MnSO_4 \cdot 4H_2O$ 5 mg/ml, $ZnSO_4 \cdot 7H_2O$ 5 mg/ml and water. Addition of 10 μl of culture filtrates after 3 or 4 days sharking culture to the enzyme reaction mixture showed 60~80 % inhibition of PDE. The culture filtrate (8.4 liters) was extracted with 8.4 liters of butyl acetate at pH 2.0, and the extract was concentrated under reduced pressure to a brown syrup. The syrup was submitted to silica gel column chromatography developed with chloroform. The active fractions were evaporated *in vacuo* to 690 mg of colorless crystals which was recrystallized from chloroform-methanol solution by addition of hexane.

Table 1. Ic50 for cyclic AMP PDE

Inhibitor	Ic 50
Reticulol	4.1×10^{-5}M
Theophylline	2.8×10^{-4}M
Papaverine	3.0×10^{-5}M

Ic 50: Inhibitor concentration in the reaction mixture for 50 % inhibition
Substrate: 0.1 mM
Enzyme: 2.7 μg Prot.

Fig. 1. Inhibition of PDE by reticulol, theophylline, and papaverine (DIXON plots)
The preincubation of the reaction mixture (shown in the text) was carried out for 3 minutes at 37°C.
Panel (A): Reticulol, (B): Theophylline, (C): Papaverine.
Substrate concentration (^{14}C-cAMP): 50 μM and 100 μM.
V: n moles of cAMP hydrolyzed min.$^{-1}$ mg prot.$^{-1}$.

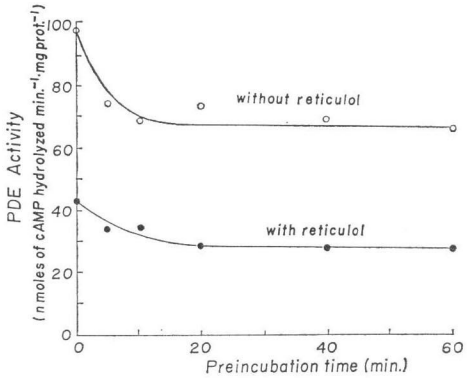


Properties of the purified material are as follows: m.p. 193~195°C, UV λ_{max}^{MeOH} 245 nm (log ε 4.67), 275 nm (log ε 3.84), 337 nm (log ε 3.72), calcd. for $C_{11}H_{10}O_5$: C 59.46, H 4.54, O 36.31; found: C 59.55, H 4.31, O 35.39; M^+ by mass spectroscopy 222. These properties and the nmr spectrum suggested the identity of the active compound with reticulol. The identity was confirmed by direct comparison of the infrared spectrum with an authentic sample and by the mixed melting point.

Reticulol was previously isolated from *Streptomyces rubreticulae* by MITSCHER *et al.*[5] and from *Streptomyces mobaraensis* by Lin *et al.*[6] and EATON *et al.*,[7] but the biological activity was not noted.

The concentrations of reticulol, theophylline or papaverine hydrochloride required for 50 % inhibition (Ic 50) of PDE in our assay system is shown in Table 1. Ic 50 values of reticulol, theophylline and papaverine were 4.1×10^{-5}M, 2.8×10^{-4}M and 3.0×10^{-5}M, respectively.

Fig. 2. Effect of preincubation of PDE with reticulol. The preincubation of the enzyme (2.7 μg as protein) was carried out with or without 1 μg of reticulol at 37°C.
Substrate concentration: 0.1 mM.



As shown by the DIXON plots (1/V to reticulol concentration) of the data in Fig. 1, the inhibition by reticulol was noncompetitive against cAMP and its Ki value was 38×10^{-6}M. Under the same condition theophylline was competitive and papaverine was noncompetitive with cAMP and their Ki values were 133×10^{-6}M and 31×10^{-6}M, respectively.

As shown in Fig. 2, preincubation of the enzyme with or without inhibitor in the absence of the substrate for 10 minutes at 37°C decreased the enzyme activity slightly. The activity decrease curve suggested the presence of not less than two types of PDE, one of which was heat labile, or the presence of an enzyme which inactivated PDE. When PDE solution was preincubated with reticulol for 60 minutes at 37°C, as shown in the same figure, the activity decrease curve with reticulol was almost parallel to that without reticulol. That is, the inhibition was not enhanced by preincubation with the inhibitor. This result and the noncompetitive type of inhibition suggest a reversible binding of reticulol with PDE.

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