Isolation of Inhibitors of Adenylate Cyclase from Dan-shen, the Root of *Salvia miltiorrhiza*

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The effect of dan-shen extract, the root of *Salvia miltiorrhiza*, on adenylate cyclase was investigated in both rat brain and rat erythrocytes. The EtOAc fraction of the MeOH extract was proved to have significant inhibitory activity. Potent inhibitory principles in the EtOAc fraction were isolated and identified as 4 polyphenolic acids, rosemarinic acid, lithospermic acid, and their methyl ester derivatives.

Keywords dan-shen; *Salvia miltiorrhiza*; adenylate cyclase; rosemarinic acid; lithospermic acid

Introduction

Folk medicines and crude drugs contain many potentially useful substances and medicinal materials. We have looked for activators and inhibitors of adenylate cyclase in medicinal plants and crude drugs. Adenylate cyclase produces cyclic adenosine monophosphate (cyclic-AMP), which is known to mediate the actions of hormones and neurotransmitters on their target cells.[2] The results of preliminary screening have already been reported.[1] Forskolin, a diterpene extracted from the roots of *Coleus forskohlii* Briq. has been demonstrated to be a unique activator of adenylate cyclase in the brain and various tissues.[3] To date, only the *C. forskohlii* species growing in India is known to produce forskolin.[4]

In a series of investigations, the MeOH extract of dan-shen was found to inhibit this enzyme system. Dan-shen, the dried roots of *Salvia miltiorrhiza* BUNGE, is an important Chinese drug in the treatment of heart disease.[5] Many chemists have studied the physiologically active constituents of this crude drug and have isolated many pigments, abietanoids.[6]

This paper describes the isolation of active components of dan-shen by monitoring its inhibitory activity against adenylate cyclase.

Materials and Methods

Preparation of Membrane Fraction from Rat Erythrocyte All procedures were carried out at 4 °C. Male rats of the Wistar strain (150—200 g) were killed under ether anesthesia and whole blood was collected into a tube containing saline with 10 mM ethylenediaminetetraacetic acid (EDTA). The erythrocytes were sedimented by centrifugation at $500 \times g$ for 10 min and washed 3 times with saline. Then the erythrocytes were lysed hypotonically by addition of 10 volumes of 5 mM Tris–HCl/1 mM EDTA (pH 7.4). The lysate was centrifuged at $20000 \times g$, and the pellet was suspended and washed 3 times with the same buffer as above. The final pellet was suspended in 50 mM Tris–HCl buffer (pH 7.4), and was used as the membrane preparation in the following experiments.

Preparation of Membrane Fraction from Rat Brain Synaptosomal membranes were prepared from brains of male Wistar rats (150—200 g). The brains were homogenized in 10 volumes of 0.3 M sucrose solution containing 5 mM Tris–HCl and 1 mM EDTA (pH 7.4) with a Potter-Elvehjem homogenizer for 3 min. The homogenate was centrifuged at $500 \times g$ for 10 min, and the supernatant was centrifuged at $20000 \times g$ for 30 min. The pellet was resuspended in 20 volumes of 5 mM Tris–HCl buffer (pH 7.4). After standing for 30 min, the suspension was centrifuged at $20000 \times g$ for 30 min. The final pellet was suspended in 0.25 M sucrose–50 mM Tris–HCl buffer (pH 7.4). The membrane suspension was stocked at −20 °C.

Measurements of Adenylate Cyclase Activity and Protein Adenylate cyclase activity was measured according to the method of Salomon *et al.*[7] with some modifications.[8] The assay system consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 8 mM theophylline, 12.0 mM phosphocreatine, 35 units/ml creatine phosphokinase, 0.01 mM guanosine triphosphate (GTP), 0.1 mM adenosine triphosphate (ATP), 1 μCi of [^{3}H]ATP (4×10^5 cpm) as a substrate and sample solution in a final volume of 200 μl. After incubation at 30 °C for 30 min (brain preparation; for 15 min), the reaction was terminated by the addition of 200 μl of 10% sodium dodecyl sulfate/10 mM EDTA solution. The cyclic AMP produced was separated by successive chromatographies on Dowex 50W × 4 and neutral alumina and the radioactivity was measured in a liquid scintillation spectrometer. Protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as the standard. The presence of 5% methanol in the final assay mixture did not effect the basal adenylate cyclase activity.

Materials Forskolin was purchased from Calbiochem. ATP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co., [^{3}H]ATP from Amersham International, Ltd., and GTP from Yamasa Co. All other drugs and chemicals were of reagent grade from commercial sources.

Extraction and Separation of Active Components from Dan-shen Dan-shen was purchased from Kojima Shouten Co. for Oriental Medicine (Osaka, Japan). The radix (2 kg) was finely powdered and extracted with MeOH (1.5 l) four times at 70 °C. The concentrated extract (478 g) was dissolved in water and extracted with $CHCl_3$, EtOAc, and 1-BuOH saturated with H_2O, successively. Each fraction was examined for inhibitory activity against adenylate cyclase. The EtOAc soluble fraction (13.4 g) was chromatographed on silica gel (art. 7734, Merck) using stepwise elution with benzene–EtOAc (1 : 1), (1 : 2), (1 : 4), and EtOAc and $CHCl_3$–MeOH (1 : 1) successively, to afford 17 fractions. The activity was distributed in fractions 12 and 13. The active 12 th fraction was separated by HPLC to give 1,2,3, and 4. The following condition were used for the separation: solvents; 40% MeOH (3% CH_3COOH), column, TSK gel ODS-120T (7.6 × 300, 10 μm); flow rate, 2.5 ml/min; room temperature; detection at 254 nm.

Results and Discussion

Figure 1 shows the effect of each fraction separated according to Chart 1 on forskolin-activated and basal adenylate cyclase activities in both rat erythrocyte ghost and rat brain preparation. As shown in Fig. 1, the EtOAc fraction proved to have significant inhibitory activity. Inhibitory activities of the EtOAc fraction against basal and 5 μM forskolin-activated adenylate cyclase activities in rat brain preparation were stronger than those in the case of rat erythrocyte ghosts.

Figure 2 shows the concentration–response curve of EtOAc fraction against basal and 5 μM forskolin-activated adenylate cyclase activities in both rat erythrocyte ghost and rat brain preparation. Figure 3 shows the concentration–response curve for forskolin in the presence or absence of the active EtOAc fraction. The EtOAc fraction has no effect on EC_{50} of forskolin in both rat erythrocyte and rat brain adenylate cyclase systems. These

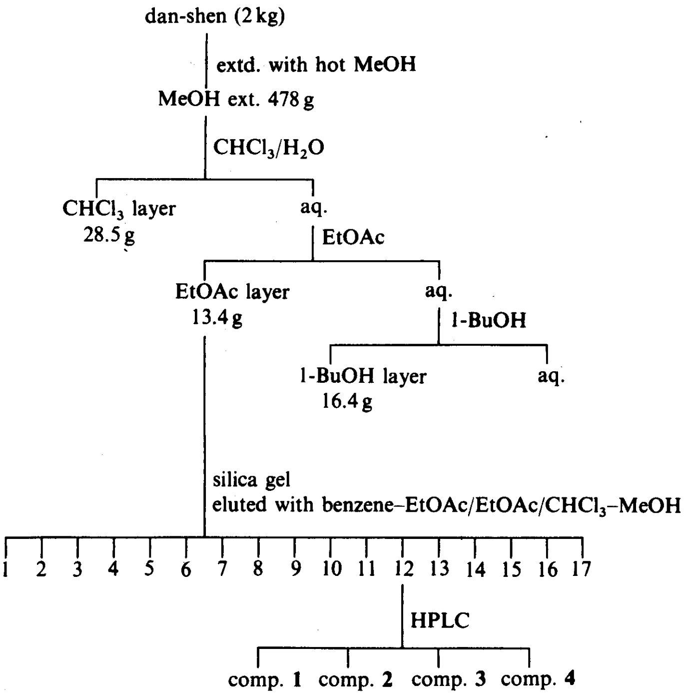


Chart 1. Extraction and Separation of Active Components from Dan-shen

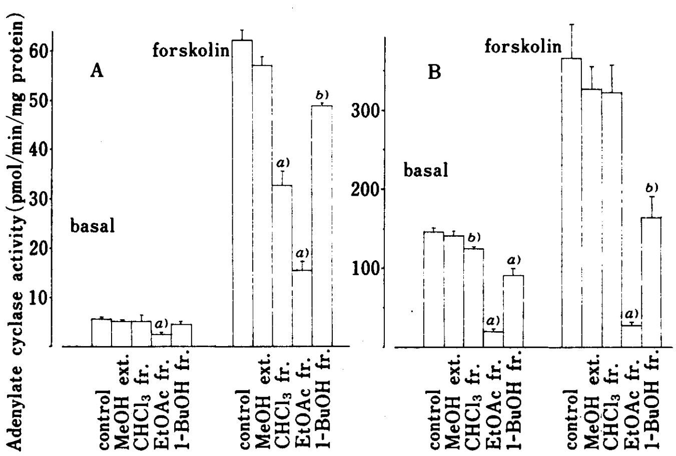


Fig. 1. Effect of Each Fraction on Adenylate Cyclase

A) In rat erythrocyte ghosts. B) In rat brain preparation. Each sample was tested at the concentration of 0.2 mg/ml and in the presence of 5% MeOH and 10^{-5} M forskolin in the final assay mixture. Significant difference from the control value ($n=4$, $\bar{x} \pm$ S.E.): *a*) $p<0.001$, *b*) $p<0.01$.

data demonstrate that the two adenylate cyclase systems are inhibited by EtOAc fraction in a similar manner. Therefore, the difference in the effect of the extract on the erythrocytes and on the brain in Fig. 1 may be due to the lipophilic nature of the latter, since the active principles in the EtOAc extract were expected to be lipophilic. As shown in Fig. 4, inhibitory activity of the EtOAc fraction against basal and 5 μM forskolin-activated adenylate cyclase activities in rat brain preparation was located in the 12th and 13th fractions. Figure 5 shows the inhibitory activity of compounds **1**, **2**, **3** and **4** separated by HPLC. Compounds **1**, **2**, **3**, and **4** all showed strong inhibitory activity against basal and 5 μM forskolin-activated adenylate cyclase activities in rat brain preparation.

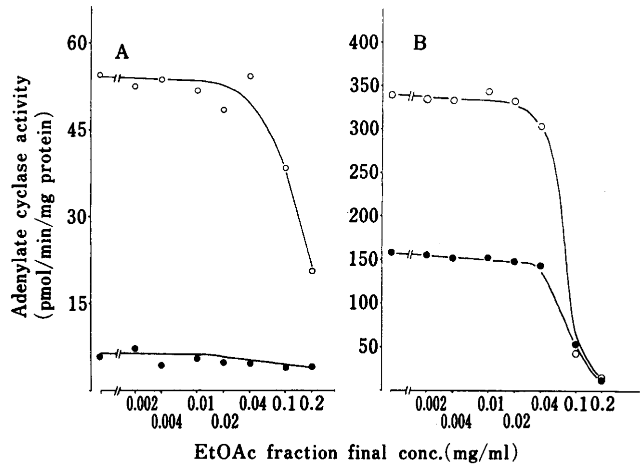


Fig. 2. Concentration–Response Curve of EtOAc Fraction

A) In rat erythrocyte ghosts. B) In rat brain preparation. ●, against basal adenylate cyclase activity; ○, against 10^{-5} M forskolin-activated adenylate cyclase activity.

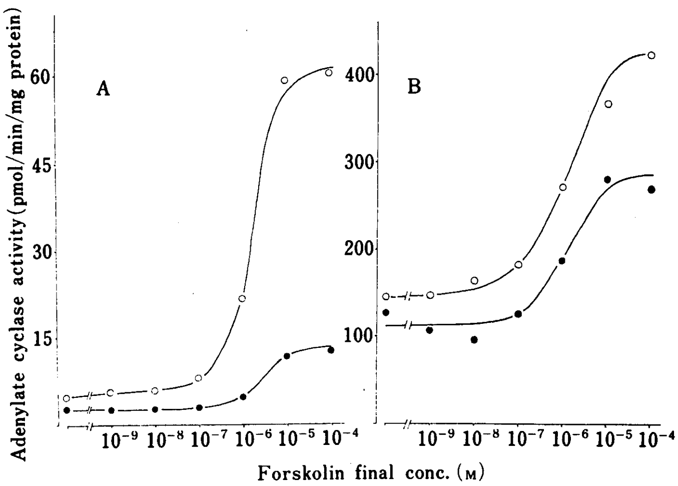


Fig. 3. Concentration–Response Curve of Forskolin

A) In rat erythrocyte ghosts. B) In rat brain preparation. ●, presence of EtOAc fraction; ○, absence of EtOAc fraction.

Compounds **1**, **2**, **3** and **4** all showed a positive reaction to iron chloride. The carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra of the four compounds (**1**—**4**) isolated from dan-shen suggested similar chemical structures (Chart 2). The proton magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra of **1** are strikingly similar to those of **3**. The ^{13}C-NMR spectra of **1** and **3** in CD_3OD showed a unit of caffeic acid.[10] The ^{1}H-NMR spectra of **1** and **3** in CD_3OD showed *trans*-olefin proton signals at δ 6.27 and δ 7.55 (each d, $J=15.8$ Hz). The ^{13}C-NMR spectrum of **1** showed 2 carboxyl groups (δ 168.4 ppm, δ 173.4 ppm), 1 methylene (δ 37.9 ppm), and 1 methine linked to oxygen (δ 74.6 ppm). By the ^{13}C- and ^{1}H-NMR analyses, **1** was identified as rosemarinic acid.[10]

The ^{13}C- and ^{1}H-NMR spectra of **3** showed one more methyl ester group (δ 52.7, δ 3.70 ppm) than **1**. Eventually, **3**

The ^{1}H- and ^{13}C-NMR spectra of **2** closely resembled those of **4**. In the ^{13}C-NMR spectrum of **2**, ten more carbon signals compared with **1** were observed, and among them one carbon signal was found to be due to a carbomethoxyl group (δ 53.2 ppm). Moreover, the other 27 carbon signals

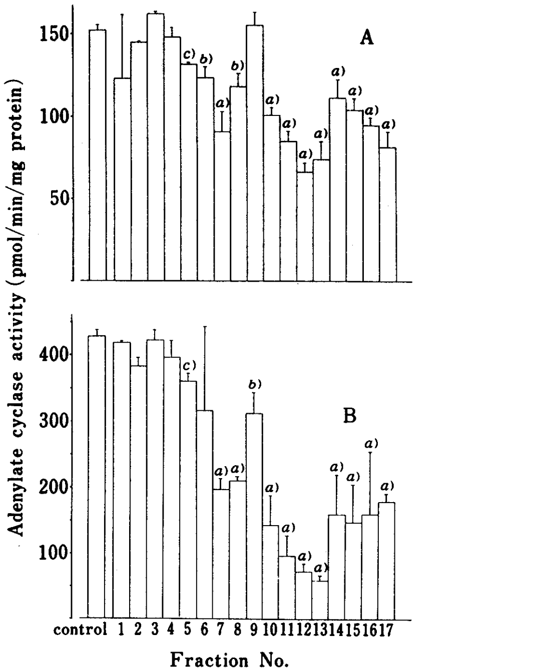


Fig. 4. Effect of Each Fraction of EtOAc Fraction on Rat Brain Adenylate Cyclase

A) Against basal activity. B) Against 10^{-5} M forskolin-activated activity. Each sample was tested at the concentration of 0.1 mg/ml. Significant difference from the control value ($n=6$, $\bar{x}\pm$S.E.): *a*) $p<0.001$, *b*) $p<0.01$, *c*) $p<0.05$.

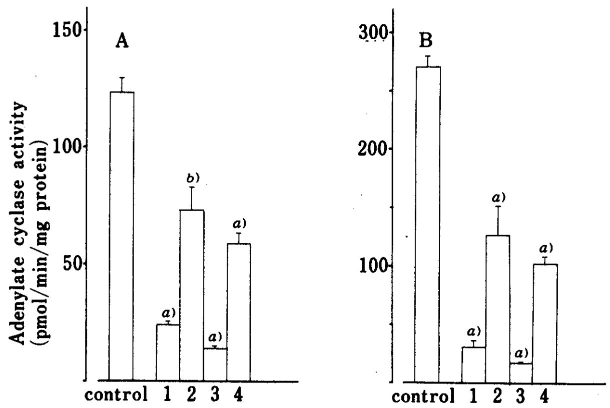
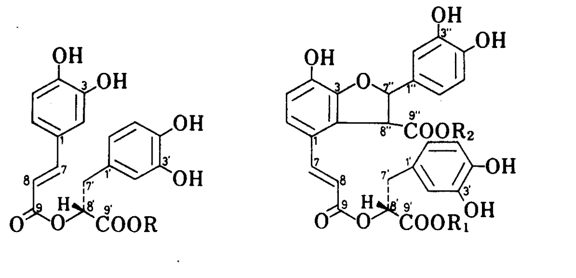


Fig. 5. Effect of Each Compound Separated by HPLC

A) Against basal activity. B) Against 10^{-5} M forskolin-activated activity. Each sample was tested at the concentration of 0.1 mg/ml. Significant difference from the control value ($n=4$, $\bar{x}\pm$S.E.): *a*) $p<0.001$, *b*) $p<0.01$, *c*) $p<0.05$.



R	
1 : H	
3 : CH_3	

	R_1	R_2
2 :	H	CH_3
4 :	CH_3	CH_3

Chart 2. Structure of Active Components

TABLE I. ^{13}C-NMR Spectral Data for Polyphenolic Acids (**1**—**4**)

	1	**2**	**3**	**4**
1	127.7	124.4	127.6	124.4
2	114.5	126.8	114.2	127.0
3	146.8	148.7	146.8	148.8
4	149.8	146.6	149.8	146.7
5	116.5	118.4	116.5	118.4
6	123.2	121.9	123.2	121.8
7	147.7	143.9	148.0	144.0
8	115.3	116.4	115.3	116.3
9	168.4	168.1	168.3	168.0
1′	129.3	129.2	128.8	128.7
2′	117.6	117.5	117.5	117.5
3′	146.2	146.4	146.2	146.5
4′	145.3	145.0	145.4	145.3
5′	116.3	116.4	116.3	116.4
6′	121.9	121.9	121.8	121.8
7′	37.9	37.7	37.9	37.8
8′	74.6	74.6	74.7	74.7
9′	173.4	173.6	172.2	172.1
1″		133.3		133.3
2″		113.4		113.5
3″		146.0		146.1
4″		145.0		145.3
5″		116.4		116.3
6″		118.4		118.4
7″		88.3		88.4
8″		57.1		57.2
9″		173.6		173.6
COO–Me		53.2		53.2
COO–Me			52.7	52.7

NMR spectra were taken at 25 °C using tetramethylsilane (TMS) as an internal standard; ^{13}C-NMR at 25.15 MHz (δ relative to TMS in CD_3OD).

of **2** corresponded closely with those reported for lithospermic acid,[10] and the 3-(3,4-dihydroxyphenyl)lactic acid moiety was essentially the same as that of **1**.[10] Therefore, the carbomethoxyl group of **2** should be located on the dihydrobenzofuran ring. These results indicated that **2** is the monomethylester of lithospermic acid.

In the ^{1}H- and ^{13}C-NMR spectra of **4**, two carbomethoxyl groups were indicated. Therefore, **4** was concluded to be the dimethylester of lithospermic acid.

Rosemarinic acid was first isolated from *Rosemarinus officinalis*, and has been reported to occur in several species of the family Labiatae.[11] This compound has been reported to be an anti-inflammatory constituent of *Symphytum officinale*[12] and *Ehretia microphylla*.[13] Though the isolation of lithospermic acid was first reported from *Lithospermum ruderale*,[14] the structure reported was not correct. In 1975, Carmack *et al.* undertook a study of the structure and antigonadotropic activity in animals.[15] This was the first demonstration that these polyphenolic acids have a strong inhibitory effect against adenylate cyclase. Further studies of the inhibitory effects of polyphenolic acids on adenylate cyclase as well as the relationship between this effect and the reported antigonadotropic effect are in progress.

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