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# Inhibitors of Cyclic Adenosine Monophosphate Phosphodiesterase in *Polygala tenuifolia*[1]

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Cyclic adenosine monophosphate (AMP) phosphodiesterase inhibitors contained in *Polygala tenuifolia* Willd. were identified as saponins and oleic acid. The concentrations of onjisaponins E, F and G required to give 50% inhibition (IC 50) were of the sameor der as that of papaverine. A kinetic study revealed that onjisaponin F acts non-competitively against cyclic AMP phosphodiesterase, like papaverine. Onjisaponin F exhibited a prolongation effect on hexobarbital sleeping time in mice.

**Keywords**——*Polygala tenuifolia*; cAMP phosphodiesterase; inhibitor; saponin: onjisaponin B: onjisaponin E; onjisaponin F; onjisaponin G; oleic acid

## Introduction

Screening studies aimed at finding inhibitors of cyclic adenosine monophosphate (AMP) phosphodiesterase have shown that a variety of synthetic compounds and natural products have inhibitory effects on this enzyme.[2–8] Weinryb *et al.* reported that various drugs used clinically could inhibit cyclic AMP phosphodiesterase and pointed out that classes of drugs active in the central nervous system appeared to be particularly active inhibitors of cyclic AMP phosphodiesterase *in vitro*, though such findings do not necessarily mean that the pharmacological activity is due to some alteration of cylic AMP metabolism.[8] Chasin *et al.* and Smith reported that a potent inhibitor of cyclic AMP phosphodiesterase was found to have significant anxiolytic activity.[3,4] Vigdahl *et al.* reported that vasodilators inhibited phosphodiesterase[9] and Davies *et al.* showed that phosphodiesterase inhibitors exhibited antibroncho constriction.[10] These observations indicate that phosphodiesterase inhibitors found in the screening of natural products might have a variety of pharmacological activities. It is generally recognized that the detection of biological activity of traditional medicinal drugs by *in vivo* tests, such as blind screening methods, is sometimes very difficult owing to their mild actions. We have demonstrated that measurement of cyclic AMP phosphodiesterase inhibition can be used as a screening method to detect biologically active compounds contatined in medicinal plants used in traditional medicines. Inhibitors contained in the root of *Anemarrhena asphodeloides* and in the fruit of *Forsythia suspensa* were identified as norlignan and lignans, respectively. Several polymethoxy flavonoids were isolated as inhibitors of cyclic AMP phosphodiesterase from the peel of *Citrus reticulata* and from the rhizome of *Iris florentina*. The present paper deals with the identification of phosphodiesterase inhibitors contained in the root of *Polygala tenuifolia*, which has been used as sedative, expectorant and tonic agent in Chinese medicine.

## Results and Discussion

Hot aqueous extract of *Polygala tenuifolia* Willd. roots that showed inhibitory activity against beef heart phosphodiesterase in the previous screening study[11] was further fractionated

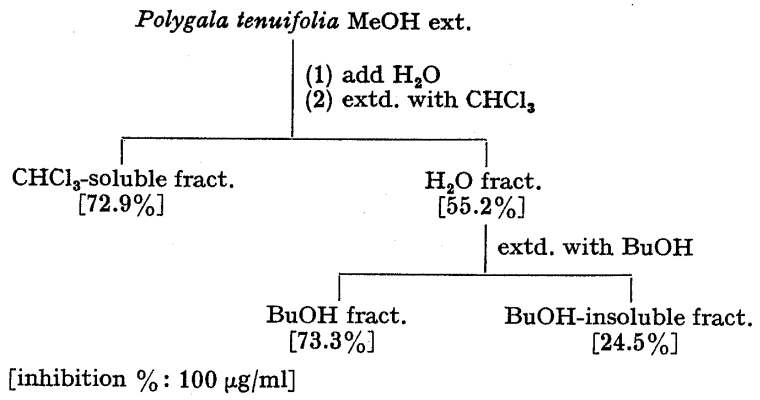


Chart 1

with chloroform. Both the chloroform-soluble and insoluble fractions showed considerable inhibition of cAMP phosphodiesterase. In order to identify the phosphodiesterase inhibitors, *Polygala tenuifolia* WILLD. roots were extracted with hot methanol on a large scale and the extract was fractionated as shown in Chart 1. The chloroform-soluble fraction of the methanol extract was fractionated by column chromatography on silica gel and monitored by thin layer chromatography (TLC) and also by measurement of inhibitory activity against phosphodiesterase. The active fractions were refractionated by gel filtration on LH-20 to give an active compound as a colorless oil. The oily compound was identified as oleic acid by comparing its infrared (IR), $^1$H-nuclear magnetic resonance (NMR) and gas liquid chromatography-mass spectrum with those of an authentic sample.

The butanol-soluble fraction also showed considerable inhibitory activity (73.3% inhibition at 100 μg/ml) against phosphodiesterase. Since the main compounds in the butanol fraction were saponins, onjisaponins (A, B, C, D, E, F and G), which have been isolated from this plant by Shoji *et al.*,[12–16] were tested for inhibition of cAMP phosphodiesterase. Among the saponins tested, onjisaponins B, E, F and G showed higher inhibitory effects than the other onjisaponins (Table I). Onjisaponins E, F and G are as potent as papaverine which was used as a reference inhibitor of this enzyme. Onjisaponin F showed a non-competitive kinetics

TABLE I. IC 50 for cAMP Phosphodiesterase

| Sample | IC 50 ($\times 10^{-5}$ M) |
|---|---|
| Onjisaponin B | 6.0 |
| Onjisaponin E | 3.1 |
| Onjisaponin F | 2.9 |
| Onjisaponin G | 3.7 |
| *cf.* Papaverine | 3.0 |

IC 50: inhibitor concentration in the reaction mixture required to give 50% inhibition.

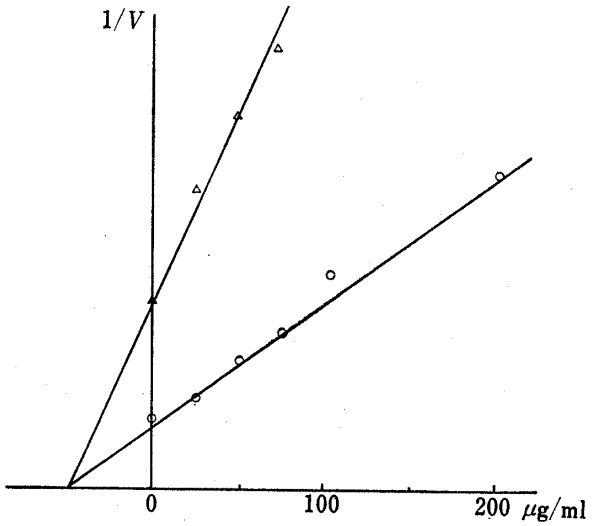


Fig. 1. Inhibition of cAMP Phosphodiesterase by Onjisaponin F (Dixon Plots)

The assay was carried out by the method described in the previous paper.[11]
Substrate concentration ($^3$H-cAMP): 50 μM (△) and 100 μM (○).
Enzyme amount: 4.5 mU (Boehringer).

pattern and the $K_i$ value determined from Dixon plots was $32.8 \times 10^{-6}$ M (Fig. 1, *cf.* papaverine: $K_i = 31 \times 10^{-6}$ M.[5])

Since *Polygala tenuifolia* root has been used as a sedative agent in Chinese medicine, each fraction of methanol extract (Chart 1) was tested for prolongation of hexobarital sleeping time to clarify its pharmacological potential. The butanol fraction was more active than the other fractions, and onjisaponins were suggested to be the active principles in this pharmacological test (Table II). Thus, onjisaponins B, F and G were similarly tested. Onjisaponin F induced a prolongation of hexobarbital sleeping time at a dose of 5 and 20 mg/kg, but pharmacological tests with higher doses were not possible due to the lack of enough material. In the case of the administration of crude extracts, marked excited behavior such as running, jumping and biting, and vocalization was seen just after the administration of methanol extract (100 and 200 mg/kg), chloroform-insoluble fraction (25, 50 and 100 mg/kg) and butanol fraction (50 mg/kg), but this disappeared within 5 min. Weak excited behavior such as spontaneous and exploratory movements were also seen at lower doses of the methanol extract (50 mg/kg), chloroform-insoluble fraction (12.5 mg/kg) and butanol fraction (6.25, 12.5 and 25 mg/kg). Diarrhea was seen 30 min after the administration of methanol extract (50—200 mg/kg) and chloroform-insoluble fraction (25—100 mg/kg). In the case of the administration of saponins, the behavior and symptoms mentioned above did not appear, suggesting that onjisaponin F was one of the active constituents. This result (Table III) provides some support for the use of this drug in oriental medicine.

Table II. Effect of *Polygala* Extracts on Hexobarbital Sleeping Time in Mice

| Fraction | Control | Dose (mg/kg, *i.p.*) 6.25 | 12.5 | 25.0 | 50.0 | 100.0 | 200 | CPZ[a] (3 mg/kg) |
|---|---|---|---|---|---|---|---|---|
| MeOH ext. | 31.3± 2.9 | | | | 45.8± 5.3 | 69.1± 6.3[c] | 90.6± 11.1[c] | 52.5± 5.5[c] |
| $CHCl_3$ insol. fract. | 25.3± 2.3 | | 32.2± 1.5 | 42.3± 6.3[b] | 46.5± 6.6[c] | 50.6± 6.4[c] | | 60.8± 3.6[c] |
| BuOH insol. fract. | 25.2± 2.8 | 29.5± 2.6 | 43.7± 4.8[c] | 41.3± 4.2[c] | 51.6± 5.7[c] | 50.0± 4.2[c] | | 60.9± 3.6[c] |
| BuOH sol. fract. | 26.3± 3.0 | 51.6± 7.4[c] | 60.7± 10.9[c] | 84.5± 10.7 | 62.1± 14.1[c] | | | 61.0± 6.1[c] |
| $CHCl_3$ fract. | 30.3± 4.9 | | 38.8± 4.7 | 37.0± 4.2 | 44.1± 3.3 | | | 55.3± 5.1[c] |

All figures are mean sleeping time (min) ±S.E.
*a*) Chlorpromazine hydrochloride.
*b*) significantly different from control ($p<0.05$).
*c*) significantly different from control ($p<0.01$).

Table III. Effect of Onjisaponins on Hexobarbital Sleeping Time in Mice

| Saponins tested | Dose (mg/kg) Control | 5 | 10 | 20 | CPZ[a] (2 mg/kg) |
|---|---|---|---|---|---|
| Onjisaponin B | 31.4±1.6 | 38.0±2.4 | 28.5±3.0 | 31.4±3.6 | 60.2±2.7[c] |
| Onjisaponin F | 23.6±2.1 | 32.9±1.8[b] | 23.3±2.6 | 35.5±4.6[b] | 42.4±2.4[c] |
| Onjisaponin G | 30.7±2.5 | 30.3±1.8 | 32.9±2.7 | 27.6±4.6 | 46.7±2.0[c] |

All figures are mean sleeping time (min) ±S.E.
See footnotes to Table II.

It has been pointed out that various therapeutic drugs inhibit the cyclic AMP phosphodiesterase reaction *in vitro*.[2-10] Although the pharmacological acctivities of these drugs do not necessarily arise from alterations of cyclic AMP metabolism, the *in vitro* test of phosphodiesterase inhibition has been used as a screening method to find bio-active compounds.[3,4,9,10]

Our results provide additional support for the effectiveness of cyclic AMP phosphodiesterase inhibition measurement as a screening method to detect biologically active compounds contained in medicinal plants.

## Experimental

The following instruments were used to obtain the physical data. An Aloka LSC-903 liquid scintillation counter was used. Silica gel 60 (Merck, precoated plates, 0.25 mm) was used for TLC and detection was achieved by illumination with an ultraviolet (UV) lamp or by spraying 10% $H_2SO_4$ followed by heating. For column chromatography, silica gel C-200 (Wako) was used and for gel filtration, Sephadex LH-20 (Pharmacia Fine Chemicals). IR spectra were recorded on a Hitachi EPI-G3 spectrometer. GC was carried out on a Hitachi 063 gas chromatograph equipped with a hydrogen flame ionization detector using a stainless steel column (3 mm × 1 m) packed with 2% SE-30 on Chromosorb-W (60—80 mesh) with $N_2$ carrier gas at a flow rate of 30 ml/min.

**Assay of Phosphodiesterase**——Phosphodiesterase activity was assayed by the method of Thompson[17] and Brooker[18] as modified in the previous paper.[11]

**Extraction and Separation**——The dried roots of *Polygala tenuifolia* (10 g, purchased from Uchida Pharmacy for Oriental Medicine, Tokyo) were extracted three times with hot water (150 ml each) for 6 h and the extract was concentrated, frozen and dried to give a powder (2.10 g), which was further dissolved in water and partitioned with $CHCl_3$ (100 ml × 3). Removal of the solvent under reduced pressure gave a solid residue ($CHCl_3$ fraction; 0.4 g). The aqueous layer was frozen and dried to give a powder ($CHCl_3$-insoluble fraction; 1.6 g). These extracts were tested for inhibitory effect on phosphodiesterase, and gave inhibitions of 84.4% and 36.9%.[11]

In order to identify biologically active compounds, dried roots of *Polygala tenuifolia* (300 g) were extracted with MeOH (1000 ml × 3) under reflux for 4 h. The MeOH extract (113.8 g) was partitioned between $CHCl_3$ and water. The $CHCl_3$ layer gave a solid residue ($CHCl_3$-soluble fraction; 23 g) on removal of the solvent by evaporation, and the $CHCl_3$-insoluble fraction was extracted three times with BuOH (*ca.* 10 times by volume). On removal of the solvent under reduced pressure, the BuOH extract gave a residue (BuOH fraction; 28 g), and the aqueous layer was frozen and dried to give a powder (BuOH-insoluble fraction; 51 g). These extracts were tested for inhibitory effect against phosphodiesterase and for prolongation of hexobarbital sleeping time in mice (Chart 1, Table III).

**Oleic Acid**——The $CHCl_3$-soluble fractions were fractionated by means of silica gel chromatography using $CHCl_3$–MeOH. Each fraction was monitored by TLC [solvent: $CHCl_3$–MeOH (10: 1)] and also by determination of inhibitory activity against phosphodiesterase. Active fractions eluted with $CHCl_3$–MeOH (1: 1) were refractionated by gel filtration on LH-20 using MeOH as an eluent. The main constituent of this fraction was identified as oleic acid (IC 50, $13.1 \times 10^{-5}$ M) by IR, gas chromatography (GC), $^1$H-NMR, mass-spectrum (MS) and GC-MS in comparison with an authentic sample (P-L Biochemical Inc.).

**Purified Saponins**——Purified onjisaponins A, B, C, D, E, F and G were obtained from *Polygala tenuifolia* root in the course of structural studies.[12–16]

**Prolongation of Hexobarbital Sleeping Time in Mice**——Male mice, weighing 20—24 g, were used in a group of six. Crude extracts and onjisaponins were administered intraperitoneally, and 30 min later 70 mg/kg of sodium hexobarbiturate was injected *via* the same route. The duration of loss of the righting reflex was measured. Chlorpromazine (CPZ) was used as the reference agent.

**Dixon Plots**——The assay was carried out by the method described in the previous paper.[11] Substrate concentration was 50 μM or 100 μM. The amount of cyclic AMP phosphodiesterase (Boehringer) used was 4.5 mU.

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## References and Notes

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