ISOLATION OF ISOFLAVONES INHIBITING DOPA DECARBOXYLASE FROM FUNGI AND STREPTOMYCES

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By screening of culture filtrates of fungi and streptomyces for activity to inhibit dopa decarboxylase the following isoflavone compounds were obtained: psi-tectorigenin (**I**), genistein (**II**), orobol (**IV**), 8-hydroxygenistein (**V**) and a new compound (**III**). **III** was elucidated to be 3′,4′,5,7-tetrahydroxy-8-methoxy isoflavone. Among these isoflavones, **IV** and **III** showed the strongest activity in inhibiting dopa decarboxylase. All these isoflavones also inhibited histidine decarboxylase and catechol-O-methyltransferase. Activities of these compounds to inhibit tyrosine hydroxylase and dopamine β-hydroxylase were examined. Orobol which showed no or only slight inhibition of tyrosine hydroxylase and dopamine β-hydroxylase exhibited a significant hypotensive effect on spontaneously hypertensive rats.

As reported in a previous paper[1], we isolated three new isoflavone compounds inhibiting catechol-O-methyltransferase from streptomyces, and two of them, 3′, 5, 7-trihydroxy-4′, 6-dimethoxyisoflavone and 3′,5, 7-trihydroxy-4′, 8-dimethoxyisoflavone, inhibited not only this enzyme but also dopa decarboxylase. These two isoflavone compounds showed hypotensive effect on spontaneously hypertensive rats, but the other one, 3′,8-dihydroxy-4′,6,7-trimethoxyisoflavone, which inhibited only catechol-O-methyltransferase showed no hypotensive effect. We screened activity of culture filtrates of fungi and streptomyces to inhibit dopa decarboxylase and isolated five isoflavone compounds. These compounds exhibited inhibition against dopa decarboxylase, histidine decarboxylase and catechol-O-methyltransferase. In this paper, we report on the isolation of five isoflavones in the screening of dopa decarboxylase inhibitors.

Materials and Methods

Method of Assay for Activity in Inhibiting Dopa Decarboxylase

The method described by AWAPARA *et al.*[2,3] was modified as follows: Rat liver (45 g) taken from 5 rats (160~170 g) was homogenized in 90 ml of 0.003 M mercaptoethanol by Ultra-Turrax (made by Junke and Kungel KG.) and centrifuged for 20 minutes at 13,000 rpm using Tominaga Ultracentrifuge (Model CG-101). To the supernatant (80 ml), 65 ml of saturated ammonium sulfate was added (0.45 saturation) and the precipitate was removed by centrifugation at 8,000 rpm for 10 minutes. To the supernatant (120 ml), 7.8 g of ammonium sulfate was added (0.55 saturation) and the precipitate was collected by centrifugation at 8,000 rpm for 10 minutes. It was dissolved in 10 ml of 0.003 M mercaptoethanol.

The reaction mixture consisted of L-dopa 10^{-3} M, pyridoxal phosphate 7×10^{-5} M, iproniazid phosphate (an inhibitor of monoamine oxidase, purchased from Wako Pure Chemical Industries Ltd.) 10^{-3} M, pH 6.9 phosphate buffer 0.03 M, 50 μl of the enzyme solution, 100 μl of a test material and the total volume was made 1.5 ml with distilled water. The enzyme solution was diluted to give 0.3 optical density of the product (dopamine). The reaction was started by addition of the enzyme solution, and the incubation at 37°C was continued for 25 minutes. The reaction was stopped by heating at 100°C for 3 minutes, and the solution was centrifuged

at 2,000 rpm for 10 minutes. To the supernatant 3.0 ml of distilled water was added and passed through Amberlite CG-50 (NH_4^+ type) column (1.5 ml), and the column was washed with 2.0 ml and 10 ml of distilled water successively. The adsorbed dopamine was eluted with 1.5 ml of acetic acid and optical density of the eluate (1.5 ml) at 279 nm was read. The percent inhibition was calculated by $B-A/B\times 100$, where A was the optical density with a test material and B was the optical density without a test material.

Method of Assay for Inhibition of Histidine Decarboxylase.

As described in a previous paper[4], we developed a method of assay of histidine decarboxylase activity, where ^{14}C-histamine produced was separated from ^{14}C-histidine by a resin process and determined by the radioactivity. This method was employed.

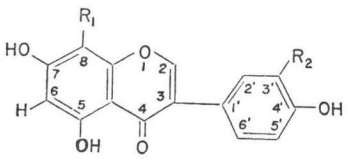
Thin-layer Chromatography of Isoflavone Compounds.

Silica gel (Merck F-254) thin-layer chromatography developed with chloroform - methanol (10 : 1 in volume) or with ethyl acetate - methanol (20 : 1) was employed. The spot was shown by spraying GIBB's reagent.

Methods of shaking culture, deep aerated fermentation, extraction and purification are described in the following section.

Results

Each strain of fungi and streptomyces was inoculated into 125 ml of a medium placed in a 500-ml-volume flask, and shake-cultured at 27°C on a reciprocating shaking machine with 130 strokes, and 100 μl of the culture filtrate after 4, 5 or 6 days was taken and heated at 100°C for 3 minutes and added to the assay medium for dopa decarboxylase inhibitor. Then, a culture filtrate of *Aspergillus niger* NRRL-3122 showed a significant inhibition of dopa decarboxylase. A medium containing glucose 1.0 %, potato starch 2.0 %, soybean meal 2.0 %, KH_2PO_4 0.1 %, $MgSO_4\cdot 7H_2O$ 0.05 % was a suitable one for production of an active agent which was stable to heating at 100°C for 3 minutes or at 60°C for 30 minutes at pH 2.0, or pH 8.0. It was extracted into *n*-butanol, ethyl acetate or butyl acetate. This active agent thus extracted was subjected to silica gel chromatography developed with chloroform - methanol (50 : 1), and the following isoflavone compounds appeared in the following series: psi-tectorigenin (**I**, 4′, 5, 7-trihydroxy-8-methoxyisoflavone), genistein (**II**, 4′, 5, 7-trihydroxyisoflavone), a new isoflavone (**III**, 3′, 4′, 5, 7-tetrahydroxy-8-methoxyisoflavone), orobol (**IV**, 3′, 4′, 5, 7-tetrahydroxyisoflavone), and 8-hydroxygenistein (**V**, 4′, 5, 7, 8-tetrahydroxyisoflavone).



I: $R_1 = OCH_3$, $R_2 = H$
II: $R_1 = H$, $R_2 = H$
III: $R_1 = OCH_3$, $R_2 = OH$
IV: $R_1 = H$, $R_2 = OH$
V: $R_1 = OH$, $R_2 = H$

In an example, a culture filtrate was collected from 100 flasks and filtered. The filtrate (9,000 ml) was extracted 3 times with 4,500 ml of butyl acetate at pH 2.0. The wet mycelial cake (1.2 kg) was extracted with methanol (5,000 ml) and after evaporation of the methanol, it was dissolved in water (1,000 ml) at pH 8.0 with 2 N sodium hydroxide and extracted 3 times with 500 ml of butyl acetate at pH 2.0. The butyl acetate extracts were combined and evaporated under reduced pressure to a blackish brown syrup (12.3 g). Addition of 37 μg to the assay medium showed 50 % inhibition of dopa decarboxylase. This syrup was dissolved in 100 ml of methanol and 20 g of silica gel (Mallinckrodt Silic AR CC-7 special) was added, mixed and dried under reduced pressure. The solid mixture thus prepared was placed on the top of a silica gel (80 g) column (5.6×15 cm) equilibrated with chloroform - methanol (50 : 1 in volume), and the chromatography was de-

veloped with the same solvent. The eluate was cut into each 20-g fraction. Then, 5 active isoflavone compounds appeared in the following series: **I** in 20~30th fractions; **II** in 25~55th fractions; **III** in 55~75th fractions; **IV** in 70~80th fractions; **V** in 100~120th fractions.

Each active fraction was evaporated under reduced pressure to dryness, and dissolved in methanol (5 ml). The methanol solution was subjected to Sephadex LH-20 column (2×100 cm) chromatography developed with methanol, and the active eluate was evaporated to dryness. The dried material was dissolved in 10 ml of methanol and 10 g of silica gel (Mallinckrodt Silic AR CC-7, 200~325 mesh) was added and dried. It was placed on a silica gel (40 g) column equilibrated with chloroform - methanol (100 : 1), and the chromatography was developed with the same solvent. Evaporation of the active eluate yielded crude crystals of active agents. They were recrystallized from a mixture of methanol and benzene. The yield of crystals of each active agent from 9,000 ml of the fermented broth was as follows: **I** 4.8 mg, **II** 80.3 mg, **III** 15.8 mg, **IV** 0.1 mg, **V** 4.0 mg.

From a cultured broth which was obtained by aerated fermentation of *Aspergillus niger* NRRL-3122 in 150 liters of medium placed in a 200-liter fermentor, the following amounts of crystals were obtained by the same processes as described above; **I** 10.2 mg, **II** 100 mg, **III** 180 mg, **IV** 20.8 mg, **V** 43.5 mg.

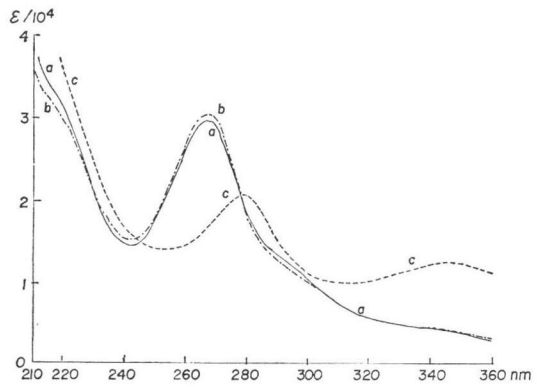
The compound **II**, (m.p. 298°C, $C_{15}H_{10}O_5$, λ_{max} at 263 nm) was identified to be 4′, 5, 7-trihydroxyisoflavone (genistein)[5], and the compound **IV** (m.p. 270°C, $C_{15}H_{10}O_6$, λ_{max} at 263 nm with $E^{1\%}_{1cm}$ 960 and 290 nm with $E^{1\%}_{1cm}$ 400 in methanol) was identified to be 3′, 4′, 5, 7-tetrahydroxyisoflavone (orobol)[6], which had been isolated from *Orobus tuberuosus*.

The compound **I** (yellow needle crystal, m.p. 245°C, $C_{16}H_{12}O_6$ by elemental analysis and mass analysis, λ_{max} at 268 nm with $E^{1\%}_{1cm}$ 957, and a shoulder at 295 nm in methanol, red shift in UV 13 nm with aluminium chloride, red shift by 10 nm with anhydrous sodium acetate) was identified to be 4′, 5, 7-trihydroxy-8-methoxyisoflavone (psi-tectorigenin) which had been synthesized as an isomer of tectorigenin[7].

The compound **V** (a brown powder, m.p. 320°C, $C_{15}H_{10}O_6$ by mass analysis, λ_{max} at 272 nm with $E^{1\%}_{1cm}$ 813 and a shoulder at 310 nm, red shift in UV by 12.0 nm with aluminum chloride and red shift by 8.5 nm with anhydrous sodium acetate) was identified to be 8-hydroxygenistein which had been synthesized[8]. It is the first time to isolate psi-tectorigenin and 8-hydroxygenistein from natural sources. Moreover, it is the first time to isolate psi-tectorigenin, orobol and 8-hydroxygenistein from microorganisms.

The compound **III** is a new isoflavone compound which has the following properties: pale brownish color crystals, m.p. 252°C, $C_{16}H_{12}O_7$ shown by elemental analysis and mass analysis; calcd.: C 60.76, H 3.82, O 35.41; found: C 60.43, H 3.86, O 35.39, M^+ 316; soluble in methanol, ethanol, butanol, acetone, dimethylsulfoxide, and slightly soluble in benzene, chloroform, toluene. As shown in Fig. 1, maxima are present at 268 nm ($E^{1\%}_{1cm}$ 960) and 295 nm ($E^{1\%}_{1cm}$ 350) in methanol, at 268 nm ($E^{1\%}_{1cm}$ 980) and 295 nm ($E^{1\%}_{1cm}$ 350) in 0.01 N HCl and at 279 nm ($E^{1\%}_{1cm}$ 660) and 345 nm ($E^{1\%}_{1cm}$ 420) in 0.01 N sodium hydroxide-methanol. In IR, the bands are seen at the following wave numbers: 3400, 1660, 1530, 1450, 1380, 1270, 1240, 1180, 1115, 1065, 1035, 995, 910, 865, 830, 780, 730, 680 cm^{-1}. It gives positive ferric chloride, 2, 6-dichloroquinonechlorimide, 2, 4-dinitrophenylhydrazine and negative EHRLICH. As described in another paper, its structure was elucidated to be 3′, 4′, 5, 7-tetrahydroxy-8-methoxyisoflavone.

Fig. 1. Ultraviolet spectrum of 3′,4′,5,7-tetrahydroxy-8-methoxyisoflavone: a: methanol b: 0.01 N HCl-90 % methanol c: 0.01 N NaOH-90 % methanol



Thin-layer chromatography using silica gel and chloroform - methanol (10 : 1) or ethyl acetate - methanol (20 : 1) was utilized for detection of these compounds in extracted material . Rf values are as follows: the compound **I** 0.40, **II** 0.39, **III** 0.385, **IV** 0.22, **V** 0.37 with chloroform - methanol; **I** 0.85, **II** 0.80, **III** 0.70, **IV** 0.40, **V** 0.60 with ethyl acetate - methanol. Rf of **IV** with butanol - acetic acid - water (4 : 1 : 1) was 0.80.

Orobol (**IV**) was also obtained from a culture filtrate of a fungus classified as *Stemphilium* sp. (the strain number in authors' institute is No. 644). It was shake - cultured for 5 days at 27°C in a medium containing glucose 1.0 %, starch 2.0 %, soybean meal 3.0 %, KH_2PO_4 0.5 %, $MgSO_4 \cdot 7H_2O$ 0.25 %, and 15.4 mg of pale yellowish crystals was obtained from 9,000 ml of the cultured filtrate. Furthermore, this compound was obtained from a strain of streptomyces classified as *Streptomyces neyagawaensis* var. *orobolere*. This strain showed the same properties as *Streptomyces neyagawaensis* ISP 5588 except for utilization of raffinose and D-mannitol and positive milk coagulation by the former.

Activities of compounds **I**, **II**, **III**, **IV** and **V** to inhibit dopa decarboxylase and histidine decarboxylase are shown in Table 1. All of them inhibited both enzyme reactions. Among them, orobol showed the strongest inhibition of both enzymes. All isoflavone compounds also inhibited catechol-O-methyltransferase, and 50 % inhibition concentrations shown by the method described in a previous paper[1] were as follows: **I** 5.8 μg/ml (1.9×10^{-5} M), **II** >20 μg/ml, **III** 6.5 μg/ml (2.0×10^{-5} M), **IV** 70 μg/ml, (2.4×10^{-4} M), **V** 1.0 μg/ml (3.5×10^{-6} M). In this case, activity of orobol was weakest.

Table 1. Fifty percent inhibition concentration of isoflavones against dopa decarboxylase (DDC) and histidine decarboxylase (HDC)

	DDC	HDC
I	51.0 μg/ml (1.7×10^{-4} M)	39.0 μg/ml (1.3×10^{-4} M)
II	55.0 μg/ml (2.0×10^{-4} M)	58.0 μg/ml (1.9×10^{-4} M)
III	0.2 μg/ml (6.0×10^{-7} M)	3.6 μg/ml (1.1×10^{-5} M)
IV	0.01 μg/ml(3.5×10^{-8} M)	0.9 μg/ml (3.1×10^{-6} M)
V	2.6 μg/ml (9.1×10^{-6} M)	0.7 μg/ml (2.3×10^{-6} M)

I=psi-tectorigenin. **II**=genistein. **III**=3′, 4′, 5, 7-tetrahydroxy-8-methoxyisoflavone. **IV**=orbol. **V**=8-hydroxygenistein.

Fifty % inhibition concentrations against dopamine β-hydroxylase and tyrosine hydroxylase were as follows: dopamine β-hydroxylase: **I** 40 μg/ml, **II** 40 μg/ml, **III** 100 μg/ml, **IV** >100 μg/ml, **V** >100 μg/ml; tyrosine hydroxylase: **I** >100 μg/ml, **II** >100 μg/ml, **III** >100 μg/ml, **IV** >100 μg/ml, **V** >100 μg/ml.

When orobol was intraperitoneally injected, it showed a hypotensive effect against hypertension of spontaneously hypertensive rats with a strength similar to L-α-methyl dopa (Table 2).

All isoflavone compounds described above have low toxicity, and the intraperitoneal injection of 250 mg/kg (dissolved in 25 % dimethylsulfoxide water) to mice showed no toxic sign.

Plant isoflavones including genistein have been reported to have estrogen-like activity and to promote an increase of body weight of sheep. The isoflavone compounds described above

Table 2. Hypotensive effect of orobol and α-methyldopa on SH-rats

Compounds	Dosis (mg/kg)	Percent decrease of blood pressure after the intraperitoneal injection				
		1 hr	3 hr	6 hr	24 hr	48 hr
Orobol	100	2.7	21.9	20.8	24.6	15.3
	50	2.7	21.5	26.9	28.0	20.4
	12.5	5.5	15.3	18.0	15.3	12.0
	3.1	4.7	21.6	15.8	12.9	7.6
α-Methyldopa	100	15.8	26.6	32.1	21.7	15.2
	50	19.3	22.0	24.7	14.0	8.6
	12.5	11.6	13.8	16.6	9.4	5.5
	3.1	4.3	9.3	13.1	9.8	7.1

examined for their activity to inhibit binding of estradiol with its uterus-binding protein by the method described by STANLEY[9]. As the result will be reported in another paper together with activities of the other compounds, all except **V** showed inhibition, and genistein and orobol showed the strongest inhibitions.

As described above, the activity of isoflavones described in this paper is not specific to dopa decarboxylase and they inhibit histidine decarboxylase and catechol-O-methyltransferase. All these compounds contain the 5-hydroxyl group which chelates with an adjacent carbonyl. As already noticed, many compounds which contain such a chelating group inhibited tyrosine hydroxylase or dopamine β-hydroxylase. However, these activities were weak in isoflavone compounds described in this paper. Among isoflavone compounds described in this and the previous paper[1], only one of them does not contain the 5-hydroxyl group and exhibited specific inhibition of catechol-O-methyltransferase.

As discussed in a previous paper[1], isoflavone compounds were produced by fermentation in media containing plant nitrogen sources such as soybean meal, cotton seed meal or corn steep liquor, and therefore it is not certain whether these isoflavones were produced by their *de novo* biosynthesis by fungi or streptomyces, although MARCHELLI *et al.*[10] reported *de novo* synthesis of a flavonoid antibiotic (chloroflavonin) by *Aspergillus candidus*.

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