NEW ISOFLAVONES, INHIBITING CATECHOL-O-METHYLTRANSFERASE, PRODUCED BY *STREPTOMYCES*

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In the screening of catechol-O-methyltransferase inhibitors in streptomyces culture filtrates, three new isoflavones were isolated. Their structures were shown to be 3′,5,7-trihydroxy-4′,6-dimethoxyisoflavone (**I**), 3′,5,7-trihydroxy-4′,8-dimethoxyisoflavone (**II**), 3′,8-dihydroxy-4′,6,7-trimethoxyisoflavone (**III**). **I** and **II** inhibited both catechol-O-methyltransferase and dopa decarboxylase, and showed hypotensive action. **III** was a specific inhibitor of catechol-O-methyltransferase, and showed no hypotensive action.

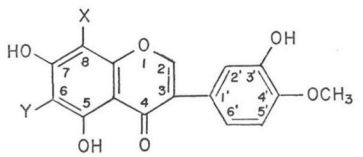
In 1958, AXELROD and TOMCHICK[1] found an enzyme system which catalyses the transfer of the methyl group from S-adenosylmethionine to one of the phenolic hydroxyl groups of epinephrine and other catechols, and the enzyme has been purified from rat and human liver[2~4]. This enzyme reaction is involved in extraneural inactivation of norepinephrine, and the inhibition of this route of catecholamine metabolism has been the subject of considerable research interest. Thus inhibitors found in various known compounds have been studied[5,6]. We were interested in the biological activity of specific inhibitors of this enzyme especially their effect on blood pressure and we screened the activity of culture filtrates of microorganisms in inhibiting this enzyme. In previous papers[7,8], we reported isolation of methylspinazarin and dihydromethylspinazarin from streptomyces and isolation from fungi of 7-O-methylspinochrome B and 6-(3-hydroxy-*n*-butyl)-7-O-methylspinochrome B which inhibit catechol-O-methyltransferase. In this paper, we report the isolation of isoflavone compounds inhibiting this enzyme from streptomyces. The action of one of them was specific to this enzyme and did not show hypotensive effect on spontaneously hypotensive rats.

The method described by NIKODEJEVIC *et al.*[9] was employed to determine the activity of streptomyces culture filtrates in inhibiting catechol-O-methyltransferase. Then, addition of 0.05 ml of a twice diluted culture filtrate of *Streptomyces roseolus* (ISP 5174) to the reaction mixture for the enzyme assay showed a significant inhibition. The activity was produced in media containing one of various carbon sources and plant nitrogen sources such as soybean meal, cotton seed meal or corn steep liquor. The following medium was selected as one of suitable media for the production: glucose 1.0 %, starch 2.0 %, defatted soybean meal 2.0 %, NaCl 0.25 %, $CaCO_3$ 0.35 %, $CuSO_4 \cdot 5H_2O$ 0.0005 %, $MnCl_2 \cdot 4H_2O$ 0.0005 %, $ZnSO_4 \cdot 7H_2O$ 0.005 %, pH 7.4 before sterilization. When cultured in this medium with or without yeast extract 0.5 % or corn steep liquor 0.5 %, addition of 0.05 ml of the twice diluted culture filtrate taken after 5 days on a reciprocating shaking machine at 27°C, showed from 50 to 65 % inhibition of the enzyme which catalysed the transfer of the ^{3}H-methyl group from ^{3}H-methyl-labeled S-adenosyl-

methionine to one of catechol hydroxyl groups of epinephrine.

The activity in the culture filtrate was extracted with butyl acetate at pH 2.0, and that in the mycelial cake was extracted with methanol. The methanol was evaporated under reduced pressure, and the activity in the concentrate was extracted with butyl acetate at pH 2.0. Butyl acetate extracts thus obtained were combined and evaporated under reduced pressure, yielding a brownish oily material. After washing with petroleum ether, the residue was dissolved in acetone and the acetone solution was evaporated and dried to a brownish powder. It was dissolved in benzene - acetone (10 : 1) and subjected to silica gel column chromatography developed with the same solvent. Then, the presence of three active materials was clarified. The compound **I** which appeared in the earliest active peak was crystallized from acetone - *n*-hexane to give pale yellowish needles, m.p. 176°C. The compound **II** in the second active peak was crystallized from hot benzene to give pale yellowish needles, m.p. 197~198°C. The compound **III** which was most slowly eluted was crystallized from acetone - *n*-hexane to give colorless needles, m.p. 215°C.

These compounds were soluble in alkaline water, methanol, acetone, slightly soluble in butanol, ethyl acetate, butyl acetate. The Rf values on a thin-layer chromatography using silica gel were as follows: **I** 0.30, **II** 0.25 and **III** 0.19 with benzene - acetone (5 : 1); **I** 0.50, **II** 0.30, **III** 0.31 with chloroform - methanol (40 : 1); **I** 0.65, **II** 0.60, **III** 0.50 with benzene - ethyl acetate (1 : 1).



I: X = H, Y = OCH_3
II: X = OCH_3, Y = H

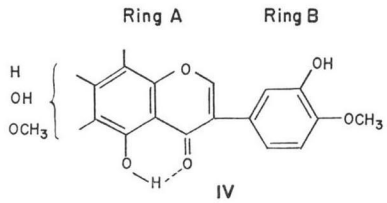
Compound **I** has molecular formula $C_{17}H_{14}O_7$ (MW 330.28). Found: C, 61.92; H, 4.31; O, 33.28. Calcd.: C, 61.82; H, 4.27; O, 33.91. M^+, *m/e* 330. ν_{KBr}: 3500, 1655, 1630, 1580, 1520, 1480, 830 and 815 cm^{-1}. λ_{max}^{EtOH} (log ε): 269 nm (4.31) and 295 nm (shoulder).

Compound **II** has molecular formula $C_{17}H_{14}O_7$ (MW 330.28). Found: C, 61.63; H, 4.50; O, 34.31. Calcd.: C, 61.82; H, 4.27; O, 33.91. M^+, *m/e* 330. ν_{KBr}: 3450, 1655, 1620, 1580, 1515, 825 and 810 cm^{-1}. λ_{max}^{EtOH} (log ε): 269 nm (4.31) and 295 nm (shoulder).

Compound **III** has molecular formula $C_{18}H_{16}O_7$ (MW 344.31). Found: C, 62.86; H, 4.79; O, 32.35. Calcd.: C, 62.79; H, 4.68; O, 32.53. M^+, *m/e* 344. ν_{KBr}: 3500, 1650 (shoulder), 1615, 1580, 1515, 865 and 854 cm^{-1}. λ_{max}^{EtOH} (log ε): 268 nm (4.31) and 295 nm (shoulder).

The preliminary chemical studies suggested that they were structurally related compounds. By oxidative degradation with alkaline hydrogen peroxide they gave 3-hydroxy-4-methoxybenzoic acid which was identified by direct comparison of IR spectra with an authentic sample. The UV spectra suggested that they would have isoflavone structures rather than flavone, and the NMR spectra indicated a singlet proton signal at δ 8.3~8.4 in hexadeuterodimethylsulfoxide (DMSO-d_6), which was characteristic to C_2-proton in isoflavone compounds.

Compound **I** gave positive ferric chloride (dark blue) and GIBBS (violet) tests for the phenolic hydroxyl group. The NMR spectrum in DMSO-d_6 showed the presence of two methoxyl groups at δ 3.78 and 3.82, three hydroxyl protons at δ 13.04 (singlet, hydrogen bonded), *ca.* 10.8 and 9.1, the C_2-proton at δ 8.33, a singlet proton on A-ring at δ 6.52 and three aromatic



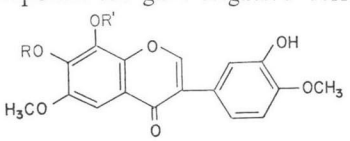
IV

protons on B-ring at δ 7.05 (1H) and 6.97 (2H). These results, isolation of 3-hydroxy-4-methoxybenzoic acid after the oxidative degradation, and the molecular formula indicated the partial structure **IV** for compound **I**.

Compound **I** afforded the triacetyl derivative, m.p. 159°C, by acetylation with acetic anhydride and sulfuric acid, and the trimethyl ether, m.p. 185°C, by treatment with dimethyl sulfate. Five methoxyl peaks in the NMR spectrum of the latter in deuterochloroform ($CDCl_3$) appeared at δ 3.96 (6H), 3.92 (6H) and 3.89 (3H) and shifted to δ 4.02, 3.74, 3.52, 3.44 and 3.17 when hexadeuterobenzene (benzene-d_6) was used as the solvent. This solvent shift is consistent with a 5, 6, 7-oxygenation pattern in ring A as reported by WILSON *et al.*[10] and SIDWELL *et al.*[11]. Nuclear OVERHAUSER effect was observed between the aromatic proton (δ 6.28, benzene-d_6) of A-ring and one of the methoxyl signals (δ 3.17), +30.3 % CH{OCH_3}, in the latter spectrum. The result suggested the presence of the aromatic proton at the 8 position. Addition of aluminium chloride and anhydrous sodium acetate caused a red shift of the main UV absorption band by 15 and 9 nm respectively. These properties are characteristic of hydroxyl groups at 5 and 7 positions[12]. From the data described above, the structure of **I** can deduced to be 3′, 5, 7-trihydroxy-4′, 6-dimethoxyisoflavone.

Compound **II** gave positive ferric chloride (dark blue) and GIBBS (violet) tests. The NMR spectrum in DMSO-d_6 showed the presence of two methoxyl groups at δ 3.78 and 3.81, three hydroxyl protons at δ 12.60 (singlet, hydrogen bonded), *ca.* 10.8 and 9.0, the C_2-proton at δ 8.40 and a singlet proton on A-ring at δ 6.33 and three aromatic protons on B-ring at δ 7.06 (1H) and 6.97 (2H). These results and isolation of 3-hydroxy-4-methoxybenzoic acid after oxidative degradation suggested the partial structure **IV** for compound **II**. Compound **II** gave the triacetyl derivative, m.p. 144°C, and the trimethyl ether, m.p. 163°C. Five methoxyl peaks in the NMR spectrum of the latter in $CDCl_3$ appeared at δ 4.01, 3.98, 3.93 (6H) and 3.91 and shifted to δ 3.78, 3.50, 3.47, 3.43 and 3.26 when benzene-d_6 was used as the solvent. This solvent shift is consistent with a 5, 7, 8-oxygenation pattern in ring A[10,11]. Nuclear OVERHAUSER effect was observed between the aromatic proton (δ 6.00, benzene-d_6) of A-ring and the methoxyl signal at δ 3.26, +14.3 % CH{OCH_3}, and another methoxyl signal at δ 3.50, +13.6 % CH{OCH_3}, in the latter spectrum. The results suggested the presence of the aromatic proton at the 6 (or 7) position. Addition of aluminium chloride and anhydrous sodium acetate caused a red shift of the main UV absorption band by 16 and 10 nm respectively. These properties are characteristic to hydroxyl groups at 5 and 7 positions[12]. Then the structure of **II** was deduced to be 3′, 5, 7-trihydroxy-4′, 8-dimethoxyisoflavone.

Compound **III** gave negative ferric chloride and positive GIBBS (blue) tests. The NMR spectrum in DMSO-d_6 showed the presence of three methoxyl groups at δ 3.90, 3.83 and 3.81, two hydroxyl protons at *ca.* δ 10.0 and 9.0, the C_2-proton at δ 8.39, a singlet proton on A-ring at δ 7.07 and three aromatic protons on B-ring



III R = CH_3, R' = H
III' R = H, R' = CH_3

at δ 7.07 (1H) and 6.98 (2H). There was no hydrogen-bonded hydroxyl proton. Compound **III** gave the diacetyl derivative, m.p. 165°C. The NMR spectrum in $CDCl_3$ showed the aromatic proton of A-ring at δ 7.58 (singlet) which was isolated from three aromatic protons of B-ring at δ 7.40 (double doublets, J=2.0 and 8.5 Hz), 7.30 (doublet, J=2.0 Hz) and 7.02 (doublet, J=8.5 Hz). Nuclear OVERHAUSER effect was observed between the δ 7.58 proton and one of the methoxyl signals (3.98), +33 % CH{OCH_3}. The result suggested that one of the methoxyl groups is present at *ortho* position of the δ 7.58 proton. Addition of aluminium chloride did not cause UV shift as expected from negative ferric chloride reaction. On these bases, structure of compound **III** should be either 3′, 8-hihydroxy-4′, 6, 7-trimethoxyisoflavone (III, R=CH_3, R′=H) or 3′, 7-hihydroxy-4′, 6, 8-trimethoxyisoflavone (III′, R=H, R′=CH_3). Addition of anhydrous sodium acetate to ethanolic solution of compound **III** caused a red shift of the main UV absorption band by 17 nm which was claimed to be characteristic of 7-hydroxyisoflavones.[12] Therefore, we initially preferred 3′, 7-dihydroxy-4′, 6, 8-trimethoxyisoflavone structure for the compound **III**. Alternative structure, 3′, 8-dihydroxy-4′, 6, 7-trimethoxyisoflavone, however, has not been excluded. The 3′, 7-dihydroxy-4′, 6, 8-trimethoxyisoflavone (**III′**, R=H, R′=CH_3) has now been synthesized by H. SUGINOME *et al.*, the details of which will be reported in a forthcoming paper,[13] and it was found that the synthetic compound was not identical with the natural compound **III**. Therefore compound **III** should be correctly formulated as 3′, 8-dihydroxy-4′, 6, 7-trimethoxyisoflavone (**III**, R=CH_3, R′=H). This was confirmed by a direct comparison of dimethyl ether of the compound (**III**) and dimethyl ether of the synthetic 3′ 7-dihydroxy-4′, 6, 8-trimethoxyisoflavone, which proved that two dimethyl derivatives were identical.

Compounds **I**, **II** and **III** are new species of isoflavones which were isolated from streptomyces for the first time. A structure of the compound **I** was once reported by MORITA *et al.*[14], but these authors' product was not identical with **I**. Hence, it should have a different structure.

Compounds **I**, **II** and **III** inhibited catechol-O-methyltransferase strongly *in vitro*. The ID_{50} was 0.70 μg/ml (2.11×10^{-6} M), 2.0 μg/ml (6.00×10^{-6} M) and 0.2 μg/ml (5.81×10^{-7} M), respectively. Their methylated drivatives did not show any inhibition at 100 μg/ml, but their acetylated derivatives showed 50 % inhibition at 70 μg/ml (1.55×10^{-4} M), 1.6 μg/ml (3.55×10^{-6} M) and 1.0 μg/ml (2.15×10^{-6} M) respectively.

The kinetics were studied using the enzyme which was partially purified by a modified method described by BALL and his associates.[4] The results are shown in Table 1. Compound **I** showed non-competitive type of inhibition, but compounds **II** and **III** showed mixed type of inhibition against epinephrine as substrate. Non-competitive type of inhibition was obtained against S-adenosylmethionine with these three compounds. Very little change of inhibition was observed under various concentrations of magnesium ions, from 3.1×10^{-4} M to 2.0×10^{-2} M.

The effects of compounds **I**, **II** and **III** on tyrosine hydroxylase, dopa decarboxylase and dopamine β-hydroxylase were also studied. In the reaction mixture described by LOVENBERG[15] preparing the enzyme solution as described by DAVIS and AWAPARA[16], dopa decarboxylase was inhibited by 5.0 μg/ml (1.52×10^{-5} M) (ID_{50}) of compound **II** and by 12.5 μg/ml (3.79×10^{-5} M) of compound **I** but not by compound **III** (100 μg/ml). The compounds **I** and **II** inhibited histidine decarboxylase prepared from rat embryo in the reaction mixture as described previously[17]:

50 % inhibition concentration of **I** was 1.8×10^{-5} M and that of **II** was 4.5×10^{-6} M. They did not inhibit the other enzymes at 100 μg/ml. The intravenous administration of 200 mg/kg of each substance to mice did not show any toxic sign. Antimicrobial activity of these compounds was not observed at 100 μg/ml.

Table 1. Inhibition of catechol-O-methyltransferase by compounds **I**, **II** and **III**

	Substrate			
	Epinephrine ($Km=8.3\times10^{-4}$ M)		S-Adenosylmethionine ($Km=1.3\times10^{-4}$ M)	
	Type of inhibition	Ki	Type of inhibition	Ki
I	non-competitive	1.7×10^{-6} M	non-competitive	1.7×10^{-6} M
II	mixed	3.3×10^{-6} M	non-competitive	9.1×10^{-6} M
III	mixed	1.5×10^{-7} M	non-competitive	3.9×10^{-7} M

Measurement of catechol-O-methyltransferase activity was carried out by the modified method of NIKODEJEVIC *et al.*[9] The concentrations of epinephrine were varied from 1.6×10^{-4} M to 2.5×10^{-3} M under the concentration of 7.5×10^{-4} M of S-adenosylmethionine. Similarly, the concentrations of S-adenosylmethionine were varied from 4.7×10^{-5} M to 7.5×10^{-4} M under the concentration of 2.5×10^{-3} M of epinephrine.

Fig. 1. Effect of intraperitoneal injection of compound **I** on blood pressure of SH-rats
Mean depression percentage (4 rats) of each dose Bars show the standard error of 4 rats.

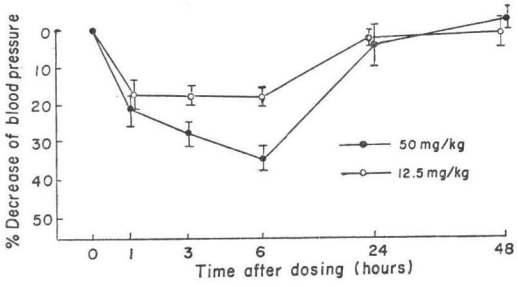
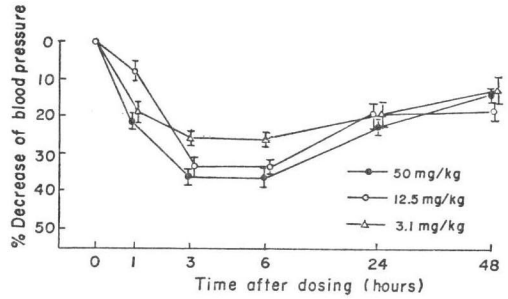


Fig. 2. Effect of intraperitoneal injection of compound **II** on blood pressure of SH-rats
Mean depression persentage (4 rats) of each dose Bars show the standard error of 4 rats.



Hypotensive effect was shown by the compounds **I** and **II**, and the latter showed a strong effect. When 50 mg/kg of **I** was intraperitoneally injected to spontaneously hypotensive rats (4 rats were injected in each dose), the percent blood pressure decrease after various hours of the injection compared with the pressure before the injection is shown in Figs. 1 and 2. The marked decrease in blood pressure was observed 3~6 hours after the injection of both compounds. The compound **II** showed the stronger effect than the compound **I** and the significant decrease was shown even by 3.1 mg/kg during 3~24 hours after the injection. The intraperitoneal injection of 50 mg/kg of **III** did not show a significant decrease of blood pressure. As described above, among compounds **I**, **II**, and **III**, the activity inhibiting catechol-O-methyltransferase was the strongest in **III** and **II** showed the strongest activity in inhibiting dopa decarboxylase. It suggests that inhibition of catechol-O-methyltransferase would not be related to the hypotensive action, but inhibition of dopa decarboxylase might be the reason for the hypotensive effect.

As described in this paper, we found three new isoflavones in streptomyces culture filtrate. Such compounds could not be found in media before inoculation of the strain ISP 5174. However, it is not certain whether streptomyces can synthesize the isoflavone skeleton *de novo*. It is possible that isoflavone compounds in medium ingredients such as soybean meal might be converted to the compounds **I**, **II** and **III** by streptomyces.

Experimental

Equipments for physical determination:

UV was measured by Hitachi UV spectrometer EPS-3T, IR by Hitachi Infrared spectrometer EPI-S2, NMR by Varian HA-100D, mass analysis by Hitachi RMU-6M.

Method of assay for activity inhibiting catechol-O-methyltransferase:

The reaction mixture consisted of distilled water 0.125 ml, 0.1 M phosphate buffer at pH 8.0 0.05 ml, 0.1 M $MgCl_2$ 0.1 ml, 0.05 M epinephrine 0.05 ml, 0.05 mM ^{3}H-methyl-labeled S-adenosylmethionine (2.2×10^5 cpm) 0.075 ml, a test sample or distilled water 0.05 ml, the enzyme solution 0.05 ml. The reaction mixture except enzyme was mixed at 0°C and the reaction was started after addition of the enzyme solution and the incubation at 37°C was continued for 20 minutes. The reaction was stopped by addition of 1.0 ml of 0.5 M borate buffer at pH 10.0 and the reacted mixture was extracted with toluene - isoamylalchohol (3 : 2) and the radioactivity in the solvent layer was counted by Beckmann sintillation counter LS-250.

The enzyme solution was prepared from rat liver. It was homogenized with 3 volume of 0.25 M sucrose and centrifuged at 105,000 *g*. This supernatant was divided into each 2 ml and kept in frozen. Before use, it was diluted 10 times with distilled water and employed. Epinephrine solution was prepared as follows: 832.5 mg of epinephrine bitartarate (purchased from Tokyo Kasei Co.) was dissolved in 50 ml of 0.1 N HCl and pH was adjusted to 5.0 and kept in frozen. The percent inhibition was calculated from the radioactivity as follows $\left(1-\frac{A-C}{B-C}\right)\times100$, where B is that without inhibitor, A is that with inhibitor and C is that without the enzyme. The culture filtrate was heated at 100°C for 3 minutes before assay.

For kinetic study, catechol-O-methyltransferase was purified as follows: Rat liver (31.25 g) was homogenized with 2 volumes of 0.25 M sucrose and centrifuged at 105,000 *g* for 1 hour. The supernatant was made pH 5.0 by addition of acetic acid and the precipitate was removed by 10 minutes centrifugation at 10,000 *g*. To this supernatant 0.5 M phosphate buffer at pH 7.8 was added until pH became 6.8 (the total volume became 61 ml). To this solution 10.7 g of ammonium sulfate was added and the precipitate was removed by centrifugation at 10,000 *g* for 10 minutes. To the supernatant 15.3 g of ammonium sulfate (to 65 % saturation) was added and centrifuged at 10,000 *g* for 20 minutes. The precipitate was dissolved in 2 mM phosphate buffer (11.5 ml) at pH 7.4 and sujected to Sephadex G 100 column (3×70 cm) chromatography developed with the same buffer. The effluent was cut into each 5 g, and the active effluent (from 20th to 28th fractions) was passed through CM-Sephadex column (2×6 cm) equilibrated with 2 mM phosphate buffer at pH 6.7. The effluent was collected in 4-ml fractions. The active fractions (3rd~10th fractions) were collected. About 20 fold purification was accomplished by this method. This partially purified enzyme solution was employed for the kinetic study.

Extraction and isolation of compounds I, II, and III:

S. roseolus ISP 5174 was shake-cultured in a medium containing glucose 1.0 %, starch 2.0 %, defatted soybean meal 2.0 %, NaCl 0.25 %, $CaCO_3$ 0.35 %, $CuSO_4\cdot5H_2O$ 0.0005 %, $MnCl_2\cdot4H_2O$ 0.0005 %, $ZnSO_4\cdot7H_2O$ 0.005 % (pH 7.4 before sterilization). After 3 days shaking culture at 27°C, 500 ml of the culture broth was inoculated to 12 liters of the same medium placed in a jar fermentor and 1.2 ml of silicone resin was added to suppress the foaming. The fermentation was continued for 105 hours at 27°C under aeration of 12 liters/minute and 250 rpm stirring. The culture broth of 4 fermentors was combined and centrifuged at 2,500 rpm. The

liquid part (40 liters) and the wet solid part (5 kg) were thus separated. The wet solid was extracted with 5 liters of methanol. Addition of 0.05 ml of the 4 times diluted methanol solution (4.8 liters) or of the twice diluted culture filtrate to the reaction mixture for assay of an inhibitor of catechol-O-methyltransferase showed 50 % inhibition. This methanol solution was concentrated to 500 ml under reduced pressure and added to the liquid part of the culture broth and extracted with 40 liters of butyl acetate at pH 2.0. The evaporation of the butyl acetate solution under reduced pressure gave 80 g of oily material. It was washed with petroleum ether yielding 35.0 g of brown powder (ID_{50}=111 μg). The powder was dissolved in 300 ml of acetone and the insoluble part was removed. To the acetone solution, 90 g of silica gel (Mallinckrodt, Silicic Acid AR 100 mesh) was added and dried under reduced pressure. This powder was placed on the top of the silica gel column (6.5×58.0 cm), and the column was developed with the same solvent. A yellow powder (790 mg) containing compound **I** was obtained by evaporation of the first active fraction (800 ml) under reduced pressure. Another active yellow powder (250 mg) containing compound **II** was obtained from the second active eluate (1,500 ml) and the active brown powder (150 mg) containing compound **III** was obtained from the third active fraction (1,500 ml). These powders showed 50 % inhibition of catechol-O-methyltransferase in the following doses: **I** powder 20 μg; **II** powder 50 μg; **III** powder 2.0 μg.

The **I** powder was dissolved in 10 ml of methanol and passed through Sephadex LH-20 (500 ml). The activity appeared in one peak (25 ml) and the eluate was evaporated to dryness under reduced pressure. The powder was dissolved with 5 ml of acetone and 20 ml of *n*-hexane was added. After overnight, **I** crystallized: 18.5 mg of pale yellow needle crystals.

The **II** powder described above was subjected to Sephadex LH-20 chromatography similar to that described above. The powder thus obtained was dissolved in 4 ml of benzene at 60°C. Then, after 1 week at room temperature, 20 mg of **II** crystals was obtained.

The **III** powder described above was mixed with 3 times weight of alumina and placed in a column and the compound **III** was dissolved in methanol which was passed through the column. The brown-colored impurity was removed by this method. The methanol solution was passed through Sephadex LH-20 column (500 ml) and the active eluate was evaporated to dryness. The crystallization by the same method as used for compound **I** gave 11.5 mg of colorless needle crystals of **III**.

Method of measuring the activity in inhibiting dopa decarboxylase[15,16]: The reaction mixture consisted of L-dopa 1×10^{-3} M, pyridoxal phosphate 7.5×10^{-5} M, 0.05 ml of dopa decarboxylase solution, phosphate buffer (pH 6.9) 0.03 M, iproniazid 1×10^{-3} M, 0.1 ml of a test material, and the total volume was made 1.5 ml with distilled water. The reaction was started by addition of the enzyme solution. The incubation time at 37°C was 25 minutes. The reaction was stopped by heating at 100°C for 3 minutes. The reacted solution was passed through Amberlite CG-50 column (0.5×7.5 cm) and dopamine adsorbed was eluted with 1.5 ml of 1 N acetic acid. The optical density at 279 nm of the eluate was read.

Dopa decarboxylase was prepared from rat liver. It was homogenized with 2 volumes of 0.003 M mercaptoethanol. The homogenate was centrifuged at 13,000 rpm for 20 minutes, and the precipitate was obtained by 0.45~0.55 saturation of ammonium sulfate. It was dissolved in 0.003 M mercaptoethanol. The concentration of the enzyme solution was adjusted as its addition of 0.05 ml gave optical density 0.3 of dopamine. Generally, the protein content of this enzyme solution was 1.0 mg/ml.

Testing the hypotensive effect: Four spontaneously hypertensive rats (male) of 15~20 weeks age were employed for each dosis. One, 3, 6, 24 and 48 hours of intraperitoneal injection of 50 mg/kg or 12.5 mg/kg of compound **I**, 50 mg/kg, 12.5 mg/kg or 3.1 mg/kg of compound **II** and 50 mg/kg of compound **III**, the blood pressure was measured by the plethysmographic tail method. Percent decrease from the pressure before injection was calculated (Figs. 1 and 2).

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