DEHYDRODICAFFEIC ACID DILACTONE, AN INHIBITOR OF CATECHOL-O-METHYL TRANSFERASE

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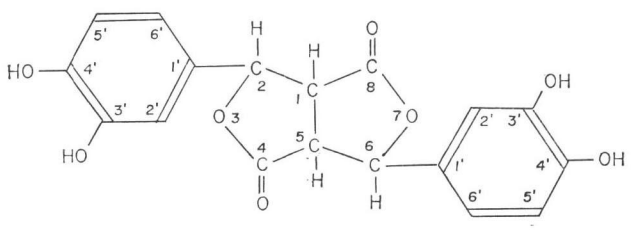
In the screening of catechol-O-methyltransferase inhibitors, three compounds were isolated from the culture filtrate of a mushroom, *Inonotus* sp. One was 3,4-dihydroxycinnamic acid (caffeic acid) which had been reported as an inhibitor of this enzyme. The others were the dextrorotatory 2,6-bis-(3′,4′-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane 4,8-dione (dehydrodicaffeic acid dilactone) and its antipode. These new compounds inhibited both dopamine β-hydroxylase and dopa decarboxylase and showed hypotensive activity in the SH rat.

Catechol-O-methyltransferase was discovered by AXELROD and TOMCHICK[1] and partially purified and characterized[2~4]. Inhibition of this enzyme (COMT) has been studied extensively and its inhibitors have been reviewed in detail by GULDBERG and MARSDEN[5]. Several inhibitors of COMT from cultured broths of microorganisms have been reported by our laboratory[6] and CHIMURA *et al.*[7~9] These include methylspinazarin, dihydromethylspinazarin, 7-O-methylspinochrome B, 6-(3-hydroxy-*n*-butyl)-7-O-methylspinochrome B, 3′,5′,7-trihydroxy-4′,6-dimethoxyisoflavone, 3′,5,7-trihydroxy4′,8-dimethoxyisoflavone and 3′,8-dihydroxy-4′,6,7-trimethoxyisoflavone. In further screening studies, we found caffeic acid (3,4-dihydroxycinnamic acid) and two new products which were elucidated to be (+)- and (−)-dehydrodicaffeic acid dilactone (Fig. 1), {2,6-bis-(3′,4′-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane 4,8-dione}. They were produced by a mushroom culture. In this paper, we describe the isolation, structures and activities of caffeic acid and dehydrodicaffeic acid dilactone.

The method described by NIKODEJEVIC *et al.*[10] was employed to determine the activity of mushroom culture filtrate in inhibiting COMT. Then, addition of 0.05 ml of a twice diluted filtrate of *Inonotus* sp. K–1410 to the reaction mixture for the enzyme assay showed 50% inhibition. The strain K–1410 was shake-cultured at 27°C on a reciprocal shaker in a medium containing glucose, peptone, yeast extract, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$, pH 5.6~5.8. When the pH of the fermentation broth decreased to 3.5~4.0, the activity to inhibit COMT appeared and reached a maximum after 7~11 days of the shaking culture.

Fig. 1. The structure of dehydrodicaffeic acid dilactone.



The activity in the culture filtrate was transferred into butyl acetate at pH 2.0 and the solvent extract was evaporated under reduced pressure, yielding a dark brown powder. The powder thus obtained was subjected to silica gel column chromatography using chloroform - meth-

anol (40: 1) as developing solvent the active fractions were collected, combined and concentrated to dryness. This material was further purified by Sephadex LH-20 column chromatography using methanol as the developing solvent. By this procedure, the activity was separated into two fractions. The active fraction in the early eluate was concentrated and addition of butyl acetate yielded pale yellowish needles (compound A), m.p. 214~220°C (dec). The other active fraction in the late eluate was dried *in vacuo* and subjected to silica gel column chromatography using benzene - acetone (10: 1). Evaporation of the active fraction gave a powder which was a racemic mixture of two optical isomers. To separate these isomers, the mixture was chromatographed on Sephadex LH-20 with methanol, and the compound B-1, (+)-isomer, and the compound B-2, (−)-isomer, were obtained as white powders. Microcrystals of compounds B-1 and B-2 were obtained by crystallization from benzene - acetone.

Compound A has a molecular formula of $C_9H_8O_4$ (MW 180.16) and two acidic functions of pKa' 5.5 and 9.9. The UV spectrum of compound A shows a strong absorption at 329 nm (ε 17,100), suggesting the presence of a conjugated olefin. The IR spectrum of compound A has characteristic absorption for an OH group (3410 cm^{-1}), strongly bonded OH of carboxyl group (3000~2500 cm^{-1}), conjugated carboxylic acid carbonyl group (1660 cm^{-1}) and *trans* –CH=CH– group (970 cm^{-1}). A phenolic OH group was suggested by a brownish color with GIBBS reagent. The pmr spectrum of compound A in DMSO-d_6 shows *trans* olefinic protons (δ 6.19, 7.44, 2H, d, J=16 Hz). From the above fact, compound A was suggested to be 3,4-dihydroxycinnamic acid (caffeic acid) and the identity was confirmed by direct comparison with an authentic sample.

The molecular formulae of both B-1 and B-2 were shown to be $C_{18}H_{14}O_8$ (MW 358.29) by elemental analysis. In addition, m.p. (199~201°C, dec.), color reaction by GIBBS test (dark green), UV (Fig. 2), IR (Fig. 3), pmr and mass spectra of compounds B-1 and B-2 were completely identical for both compounds. But compounds B-1 and B-2 were different in the direction of the rotation; B-1, $[\alpha]_D^{20}$ +91°

Fig. 2. UV spectrum of compound B-1

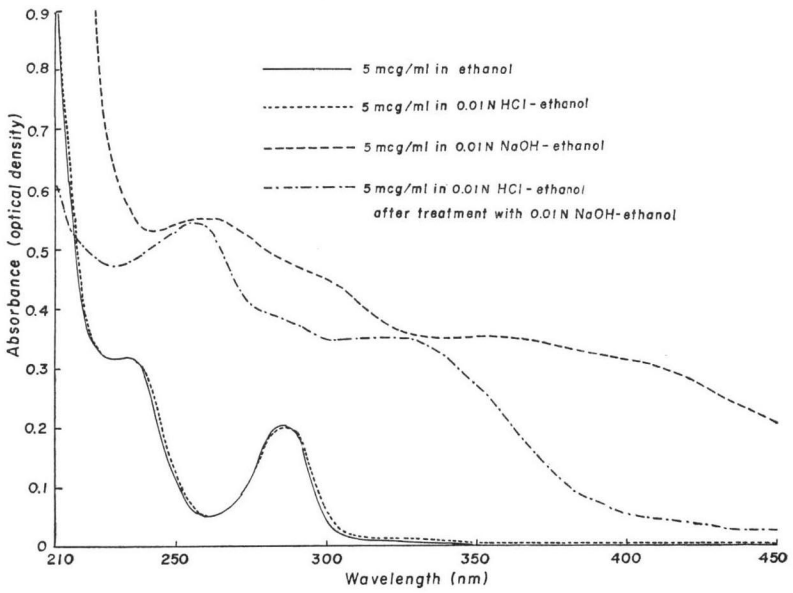
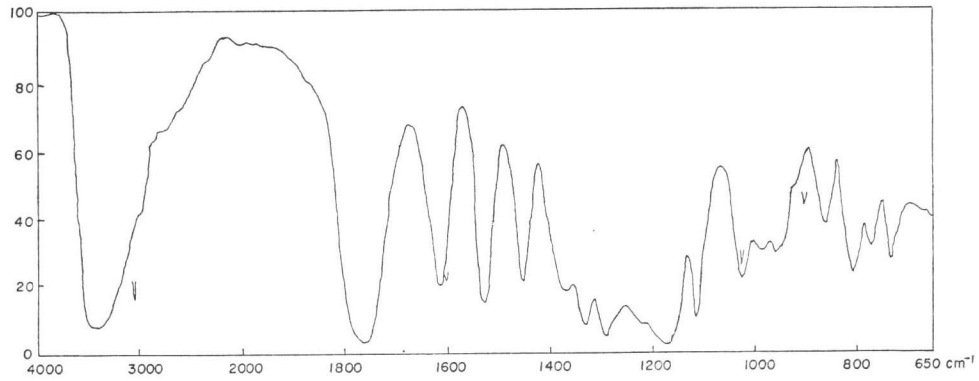
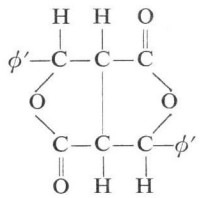


Fig. 3. IR spectrum of compound B-1 (KBr)



(*c* 1, methanol); B-2, $[\alpha]_D^{20}$ −91° (*c* 1, methanol). These facts indicate that compound B-1 is the antipode of compound B-2. As shown by the UV spectrum (Fig. 2), compounds B-1 and B-2 were labile in alkaline solution. The IR spectrum (Fig. 3) shows the presence of OH group (3450, 1170 cm^{-1}), lactone (1765 cm^{-1}) and ring-C=C-(1615, 1530 cm^{-1}). It was suggested that compounds B-1 and B-2 had phenolic OH groups by the dark green color with GIBBS reagent. Compounds B-1 and B-2 showed the same mass spectrum indicating the fragment peak (M^+ −44, *m/e* 314). The pmr spectrum of compounds B-1 and B-2 in DMSO-d_6 is as follows: δ 4.08 (2H), δ 5.72 (2H), δ 6.60~7.00 (6H) and δ 9.14 (4H). Signals at δ 9.14 (4H) and at δ 6.60~7.00 (6H) can be assigned to phenolic hydroxyl protons and aromatic protons, respectively and signals at δ 5.72 (2H) to protons attached to carbon atoms bearing oxygen atoms and linking to aromatic carbon atoms. Signals at δ 4.08 (2H) can be assigned to protons attached to carbon atoms bound to carbonyl carbon atoms. The presence of a lactone is supported by the IR spectrum ($\nu_{c=0}$ 1765 cm^{-1}). The off-resonance spectrum in CMR of compound B-2 (MeOH-d_4) showed five doublets and four singlets. Nine peaks in the CMR spectrum indicate that compound B-2 has a symmetrical unit ($C_9H_7O_4$) in its structure. From these facts, it is deduced that both compounds have the partial structure shown:



A similar compound (ϕ'=3',4'-dimethoxyphenyl) has been synthesized by CARTWRIGHT and HAWARTH[11] in 1944. In order to confirm the structure of compounds B-1 and B-2, this 3',4'-dimethoxyphenyl compound was synthesized according to the procedures of these authors and compounds B-1 and B-2 were methylated with diazomethane. Methylation of compounds B-1 and B-2 gave tetramethyl ether derivatives. The pmr spectra of methylated compounds B-1 and B-2 in $CDCl_3$ showed four methoxy signals at δ 3.87 and 3.89. The IR spectrum of methylated compound B-1 was identical with that of methylated compound B-2, but the values of specific rotation were +48° and −48°, respectively. All the physico-chemical properties of the 3',4'-dimethoxyphenyl compound synthesized, tetra-O-methyl B-1 and tetra-O-methyl B-2 were identical except for optical rotation. Thus, the structures of compounds B-1 and B-2 were elucidated to be (+)-dehydrodicaffeic acid di-

lactone and (−)-dehydrodicaffeic acid dilactone, respectively, that is, 2,6-bis-(3′,4′-dihydroxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane 4,8-dione. X-Ray analysis for determination of the absolute configuration of tetra-O-methyl B-2 will be reported in another paper.

Compounds A, B–1 and B–2 inhibited COMT *in vitro*. The ID_{50} was 15 μg/ml (8.3×10^{-5} M), 14 μg/ml (3.9×10^{-5} M) and 18 μg/ml (5.0×10^{-5} M), respectively. 3,4-Dihydroxycinnamic acid has been reported to be an inhibitor of COMT[12].

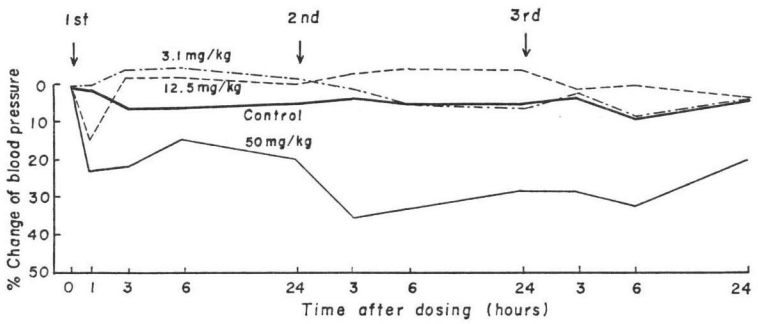
The kinetics were studied using enzyme which was partially purified by a modification of the method described by BALL and his associates[4]. Compounds B–1 and B–2 showed mixed type inhibition with adrenaline and non-competitive type inhibition with S-adenosylmethionine. *Ki* values of compounds B–1 and B–2 against adrenaline were 1.43×10^{-5} M and 2.77×10^{-5} M and those against S-adenosylmethione were 6.26×10^{-5} M and 8.72×10^{-5} M, respectively.

The effects of these compounds on dopamine β-hydroxylase (DβH)[13~14] and dopa decarboxylase[15~17] were studied. At 100 μg/ml, compound A did not inhibit DβH but compounds B–1 and B–2 showed 42.5% and 65.2% inhibition of DβH. ID_{50} of compounds A, B–1 and B–2 on dopa decarboxylase was 6.8 μg/ml (3.8×10^{-5} M), 7.4 μg/ml (2.1×10^{-5} M) and 4.5 μg/ml (1.3×10^{-5} M), respectively.

Compounds A, B–1 and B–2 showed 50% inhibition of growth of L–1210 cells *in vitro* at 34 μg/ml (2.9×10^{-4} M), 23 μg/ml (6.4×10^{-5} M) and 30 μg/ml (8.4×10^{-5} M), but these compounds had no effects against L–1210 and EHRLICH carcinoma *in vivo* at 100 mg/kg/day for 10 days. Antimicrobial activity of these compounds was not found at 100 μg/ml. Compounds B–1 and B–2 have a low toxicity with an LD_{50} greater than 1 g/kg (i.p.).

A hypotensive effect was shown by the racemic mixture of compounds B–1 and B–2. When 50 mg/kg, 12.5 mg/kg and 3.1 mg/kg of the racemic mixture were intraperitoneally injected into spontaneously hypertensive rats (once a day for 3 days), as shown in Fig. 4, 50 mg/kg caused a significant decrease of the blood pressure. This hypotensive effect of compounds B–1 and B–2 may not be due to the inhibition of catechol-O-methyltransferase, because a specific inhibitor of this enzyme has been shown to be not hypotensive.[9]

Fig. 4. Effect of a racemic mixture of B–1 and B–2 on the blood pressure in SH-rats.



Experimental

UV spectra were taken by Hitachi UV spectrometer EPS–3T. IR spectra were taken as KBr disks by Hitachi Infrared spectrometer EPI–S2. NMR spectra were taken by Varian HA–100D and XL–100 spectrometers and chemical shifts are given in parts per million (δ) downfield from the internal standard, TMS (δ=0.00 ppm). Abbreviations: s=singlet, d=doublet. The mass spectra were taken

by Hitachi mass spectrometer RMU-6M. Optical rotation was determined by Carl Zeiss LEPA2. pKa' value was measured by Metrohm Herisau Potentiograph E436.

Method of assay for activity against COMT:

The reaction mixture consisted of distilled water 0.125 ml, 0.1 M phosphate buffer (pH 8.0) 0.05 ml, 0.1 M $MgCl_2$ 0.1 ml, 0.05 M adrenaline 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 and the enzyme solution 0.05 ml. The reaction mixture except for the enzyme was mixed at 0°C and the reaction was started after addition of the enzyme solution. The incubation was carried out at 37°C for 20 minutes. The reaction was stopped by addition of 1.0 ml of 0.5 M borate buffer (pH 10.0) and the reacted mixture was extracted with 3 ml of toluene - isoamylalcohol (3: 2). The radioactivity in the solvent layer (1.5 ml) was counted by Aloka LSC-653 liquid scintillation counter.

The enzyme solution was prepared from rat liver. It was homogenized with 3 volumes of cold 0.25 M sucrose and centrifuged at 105,000 *g* for 30 minutes. The supernatant was divided into 0.3-ml fractions and kept frozen. Before use, it was diluted 10 times with cold distilled water and employed. Adrenaline solution was prepared as follows: 832.5 mg of adrenaline bitartarate (purchased from Tokyo Kasei Co.) was dissolved in 50 ml of 0.1 N HCl and the pH was adjusted to 5.0. It was kept frozen. The percent inhibition was calculated from radioactivity determination as follows: $[1-(A-C/B-C)]\times100$, where B is the run without inhibitor, A is that with inhibitor and C is that without the enzyme.

For kinetic studies, COMT was purified as follows: Rat liver (31.25 g) was homogenized with 2 volumes of cold 0.25 M sucrose and centrifuged at 105,000 *g* for 1 hour. The supernatant was adjusted to 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 (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 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 subjected to Sephadex G-100 column (3×70 cm) chromatography developed with the same buffer. The effluent was cut into 5-g fractions, and the active effluent (from 20th to 28th fractions) was passed through a CM-Sephadex column (2×6 cm) equilibrated with 2 mM phosphate buffer (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. The enzyme was unstable when purified further. This partially purified enzyme solution was employed for the kinetic study.

Extraction and isolation of compounds A, B-1 and B-2:

Inonotus sp. K-1410 from an agar slant (2% glucose, 0.5% dried yeast and 1.5% agar) was inoculated into 500-ml flasks which contained 125 ml of medium (4% glucose, 0.5% Polypepton, 0.3% yeast extract, 0.3% KH_2PO_4 and 0.1% $MgSO_4\cdot7H_2O$, pH 5.6~5.8). After 3-day standing at 27°C, shaking culture was continued for 4 days at 27°C. Portions of 10 ml of the cultured broth were inoculated into 100 flasks (same volume and medium described above) and the culture was shaken at 27°C for 10 days after 3-day standing at 27°C. The cultured broth was filtered through a filter cloth under reduced pressure. Addition of 0.05 ml of the twice diluted filtrate to the reaction mixture for assay of inhibition of COMT showed about 50% inhibition. The filtrate (10 liters) was extracted with 10 liters of butyl acetate at pH 2.0. The butyl acetate extract was concentrated to dryness, yielding 5.0 g of brown powder ($ID_{50}=30$ μg/ml). After the powder was dissolved in 100 ml of methanol and 15 g of silica gel (Wakogel C-200) was added to the solution, this mixture was dried under reduced pressure. This powder was placed on the top of a silica gel column (4.0×22 cm), and the column was developed with chloroform (1 liter), chloroform - methanol (100: 1) (500 ml) and chloroform - methanol (40: 1) (1 liter). Compounds A, B-1 and B-2 appeared together in chloroform - methanol (40: 1) fractions (900 ml). The fractions were combined and evaporated to dryness, yielding a brown powder (1.5 g, $ID_{50}=16$ μg/ml). This powder was dissolved in 10 ml of methanol and passed through a Sephadex LH-20 column (2.5×100 cm). Compound A was obtained from the first active fractions (70

ml). These fractions were concentrated to 10 ml and 20 ml of butyl acetate was added. After 3 days at room temperature, 340 mg of pale yellowish needles were obtained (ID_{50}=15 μg/ml). The second active fraction (180 ml) containing compounds B–1 and B–2 was concentrated to dryness and applied to a silica gel (Wakogel C–200) column (2×15 cm) developed with benzene - acetone (10: 1). The evaporation of the active fractions (300 ml) gave a pale yellowish powder (280 mg, ID_{50}=16 μg/ml). This powder was a racemic mixture of compounds B–1 and B–2. The powder was dissolved in 3 ml of methanol and subjected to Sephadex LH–20 column (2.5×140 cm). The first peak (70 ml) contained compound B–1; the second fraction (40 ml) was a mixture of compounds B–1 and B–2 and compound B–2 was obtained from the third peak (110 ml). Compound B–1 (125 mg, ID_{50}=14 μg/ml) and compound B–2 (120 mg, ID_{50}=18 μg/ml) were obtained as white micro-crystals from benzene-acetone solution.

Compound A showed m.p. 214~220°C (dec) and pKa′ 5.5 and 9.9 (50% ethanol). $UV\lambda_{max}^{EtOH}$ (ε): 219 nm (14,700), 235 nm (10,100), 244 nm (10,500), 300 nm (13,500), 329 nm (17,100). ν_{max} cm^{-1}: 3410, 3220, 2910, 2550, 2310, 1660 (shoulder), 1645, 1620, 1600, 1450, 1280, 1215, 1115, 970, 895, 845, 815. PMR (DMSO-d_6) δ: 6.19 (1H, d, J=16 Hz), 6.60~7.10 (3H), 7.44 (1H, d, J=16 Hz), 8.60~10.50 (2H, broad). MS: M^+, *m/e* 180.

Anal. Found: C, 60.40: H, 4.45: O, 35.35.
Calcd. for $C_9H_8O_4$: C, 60.00; H, 4.48; O, 35.53.

Both compounds B–1 and B–2 showed the same melting point (199~201°C, dec). PMR (DMSO-d_6) δ: 4.08 (2H, broad s, C–1 and C–5), 5.72 (2H, broad s, C–2 and C–6), 6.60~7.00 (6H, arom. C–2′, C–5′ and C–6′), 9.14 (4H, broad s OH at C–3′ and C–4′). CMR (MeOH-d_4) δ: 50.4 (d), 85.5 (d), 115.8 (d), 118.9 (d), 120.4 (d), 133.5 (s), 149.3 (s), 149.7 (s), 180.4 (s).

Anal. Found: Compound B-1; C, 60.53; H, 4.03; O, 35.71.
Compound B-2; C, 60.14; H, 3.98; O, 35.68.
Calcd. for $C_{18}H_{14}O_8$: C, 60.34; H, 3.94; O, 35.73.

Methylation of compounds B–1 and B–2:

An etheral solution of diazomethane (3 ml) was added to a solution of compound B–1 (100 mg) in methanol (10 ml). After evaporation, the residue was dissolved in methanol (10 ml) and diazomethane (3 ml) was added to this solution. This procedure was repeated again and the solution was dried *in vacuo*, the residue was crystallized from chloroform-methanol yielding colorless needles (85 mg), m.p. 206~207°C. Methylation of compound B–2 was carried out by the same procedure, yielding colorless needles (79 mg), m.p. 206~207°C. The IR, pmr and mass spectra of methylated compounds B–1 and B–2 were identical. $[\alpha]_D^{20}$ (methylated B–1)=+48° (*c* 0.5, $CHCl_3$), $[\alpha]_D^{20}$ (methylated B–2)=−48° (*c* 0.5, $CHCl_3$). ν_{max} cm^{-1}: 3450, 2950, 1780, 1610, 1595, 1520, 1460, 1385, 1340, 1265, 1230, 1200, 1165, 1145, 1020, 995, 960, 870, 805, 765, 715. PMR ($CDCl_3$): δ 3.58 (2H, broad s), δ 3.87 (6H, s), δ 3.89 (6H, s), δ 5.87 (2H, broad s), δ 6.75~6.90 (6H, arom.). MS: M^+, *m/e* 414.

Anal. Found (Methylated B-1): C, 64.24; H, 5.45; O, 31.10.
Found (Methylated B-2): C, 63.95; H. 5.41; O, 31.25.
Calcd. for $C_{22}H_{22}O_8$: C, 63.76; H, 5.35; O, 30.89.

Synthesis of 2,6-bis-(3′,4′-dimethoxyphenyl)-3,7-dioxabicyclo-[3,3,0]-octane 4,8-dione:

A solution of 4-hydroxy-3-methoxycinnamic acid (20 g) in methanol (220 ml) was added to a solution of ferric chloride (40 g) in water (1,500 ml) under stirring by a rapid stream of air. A brown precipitate gradually separated, and after 6 hours the air stream was stopped and the reaction mixture was kept overnight. The precipitate was collected as a paste, suspended in water (250 ml), heated to 70~80°C and 12 N hydrochloric acid (50 ml) was added with vigorous shaking. After cooling, the precipitate was collected and crystallized from acetone - methanol, yielding white plates (6 g), m.p. 210~213°C (dec). An ethereal solution of diazomethane (5 ml) was added to a solution of these crystals (500 mg) in acetone (50 ml). After concentration to dryness, the residue was dissolved in acetone (50 ml) and diazomethane (5 ml) was added to this solution. The solution was dried *in vacuo;* the residue was crystallized from chloroform-methanol, yielding colorless needles (360 mg), m.p. 204~205°C. The IR, pmr and mass spectra were identical with those of the tetramethyl ether derivatives of com-

pounds B–1 and B–2.

Anal. Found: C, 63.52; H, 5.37; O, 31.10.
Calcd. for $C_{22}H_{22}O_8$: C, 63.76; H, 5.35; O, 30.89.

Method of assay for the activity to inhibit dopamine β-hydroxylase:

Dopamine β-hydroxylase was prepared from beef adrenal medullae. The adrenal medullae (20 g) were homogenized in 200 ml of 0.02 M phosphate buffer (pH 6.5) containing 8.5% sucrose. The homogenate was centrifuged at 700 *g* for 10 minutes and the supernatant was centrifuged at 10,000 g for one hour. The precipitate was collected and suspended in 200 ml of the same buffer. The suspension was diluted 40 times with the same buffer and the diluted solution (the crude enzyme) was used for the enzymatic reaction.

The activity to inhibit dopamine β-hydroxylase was determined by addition of a sample solution (0.1 ml) to a reaction mixture containing 0.2 ml of 1 M potassium phosphate buffer (pH 6.5), 0.1 ml of 0.1 M ascorbic acid, 0.05 ml of 0.02 M fumaric acid (neutralized with 0.2 N NaOH), 0.05 ml of 4 mg/ml of catalase (Tokyo Kasei Kogyo Co.), 0.1 ml of 0.1 M tyramine, 0.1 ml of 0.1 M N-ethylmaleimide, 0.1 ml of the crude enzyme, and 0.2 ml of distilled water. After incubation under shaking at 37°C for 25 minutes, 0.2 ml of 50% trichloroacetic acid was added to stop the reaction and the reaction mixture was centrifuged at 2,000 rpm for 5 minutes. The supernatant was passed through a column (0.6×5 cm) of Dowex 50–X8 (H^+, 1 ml). After washing the column with distilled water (10 ml), the reaction product (norsynephrine) was eluted with 3 ml of 4 N NH_4OH. The product in the eluate was oxidized to *p*-hydroxybenzaldehyde with 0.3 ml of 2% sodium metaperiodate for 6 minutes. To the oxidized solution, 0.3 ml of 1% sodium metabisulfite was added and the optical density was measured at 330 nm.

Method of assay for the activity to inhibit dopa decarboxylase:

The reaction mixture consisted of L-dopa 1×10^{-3} M, pyridoxal phosphate 7.5×10^{-5} M, iproniazid phosphate 1×10^{-3} M, phosphate buffer (pH 6.9) 0.03 M, 0.05 ml of dopa decarboxylase solution, 0.1 ml of a test material, in a total volume of 1.5 ml with distilled water. The reaction was started by addition of the L-dopa solution after preincubation for 5 minutes at 37°C. 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 (0.5×2.5 cm) and the adsorbed dopamine was eluted with 1.5 ml of 1 N acetic acid. The optical density of the eluate was read at 279 nm.

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 so addition of 0.05 ml gave an optical density of 0.3 with dopamine. Generally, the protein content of this enzyme solution was 1.0 mg/ml.

Testing the hypotensive effect:

Two spontaneously hypertensive rats (male) of 15~20 weeks age were employed for each dose level. The racemic mixture of compounds B–1 and B–2 was administrated intraperitoneally in doses of 50 mg/kg, 12.5 mg/kg or 3.1 mg/kg, once a day for 3 days. One, 3, 6 and 24 hours after the first injection, 3, 6 and 24 hours after the second and the third injection of each dose, the blood pressure was measured by the plethysmographic tail method. Percent decrease from the pressure before injection was calculated (Fig. 4).

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