Analgesic Component of *Notopterygium incisum* TING

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Notopterol was identified as the analgesic component of *Notopterygium incisum* TING by using the acetic acid-induced writhing method. Notopterol also indicated an anti-inflammatory activity by its inhibitory effect in the vascular permeability test. The intensive prolongation of pentobarbital-induced hypnosis was possibly caused by its inhibitory effect on the drug metabolism in liver. Pharmacological differences between the analgesic components of *N. incisum*, *Aralia cordata* and *Angelica pubescens* were also discussed.

Keywords notopterol; analgesics; *Notopterygium incisum*; Tou-kyokatsu; Qiang-huop; anti-inflammatory activity

In Kampoh-therapy (a traditional Japanese medical system), "Kyohkatsu" and "Dokukatsu" have been widely used as prescription analgesics. Both are the Kampoh names of medicinal plants which came from China more than 1000 years ago. The Chinese names are "Qiang-huo" and "Du-huo", respectively. However, their usage and plant origins have long been confused.[1] Now, in Japan, the rhizomes and roots of *Notopterygium incisum* TING (Umbelliferae) and the closed species are applied as "(Tou-)Kyohkatsu" ("Tou" means China in Japanese), the roots of *Aralia cordata* THUNB. (Araliaceae) as "Wa-kyohkatsu" or "(Wa-) dokukatsu" ("Wa" means Japan) and the roots of *Angelica pubescens* MAXIM. (Umbelliferae) and the closed species as "Tou-dukukatsu".[2] The analgesic components of *A. cordata*, (*ent*)-kaur-16-en-19-oic acid and (*ent*)-pimara-8(14),15-dien-19-oic acid, and the pharmacological properties were reported in our previous paper.[3] Osthol was isolated as the active component from *A. pubescens*.[4] This paper will describe the isolation and identification of the analgesic principle of *N. incisum*, and the pharmacological effects were examined to make clear the differences between it and those of *A. cordata* and *A. pubescens*.

The acetic acid-induced writhing method in mice[5] was

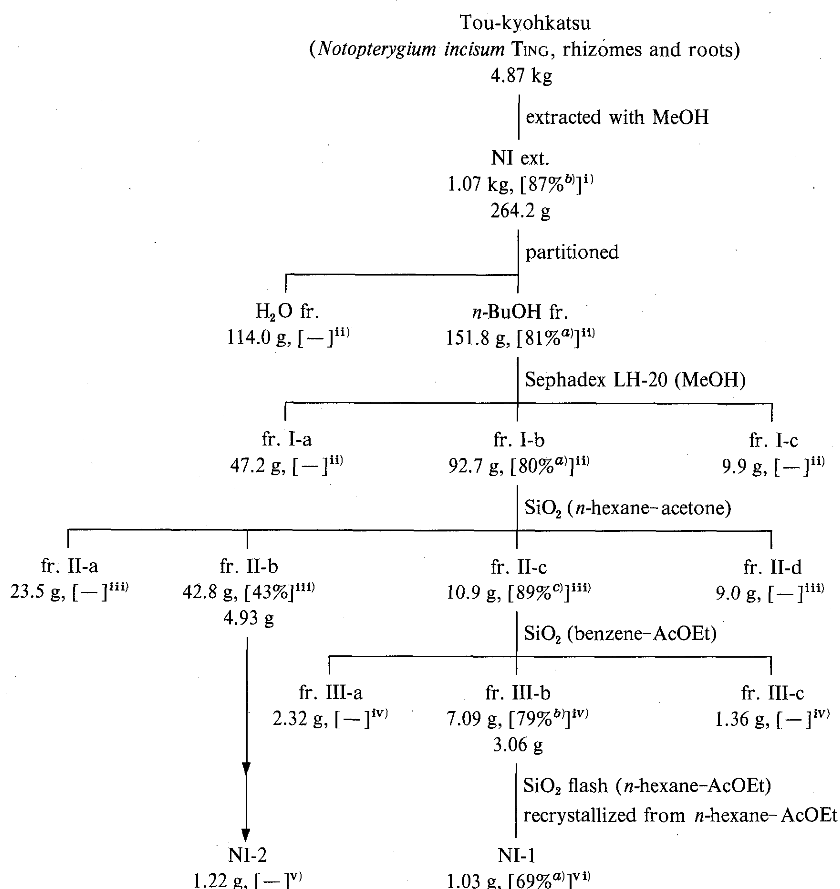

Chart 1. Isolation of an Analgesic Component from *N. incisum* TING

i) 3.0 g/kg, ii) 2.0 g/kg, iii) 1.0 g/kg, iv) 500 mg/kg, v) 300 mg/kg, vi) 100 mg/kg. *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$, $n=4$—5.

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used as a guide analgesia during the isolation procedure shown in Chart 1. The methanol extract of *N. incisum* produced 87% inhibition of the writhing at an oral dose of 3 g/kg. The extract was partitioned with water and *n*-butanol. As it demonstrated inhibitory activity, the latter was further separated by Sephadex LH-20, then by silica gel chromatography. The active fraction was finally purified by crystallization with a mixture of *n*-hexane and ethyl acetate to give the analgesic component, NI-1.

NI-1 is in the form of colorless needles with a mp of 93.5—95 °C. The molecular weight, 354, was obtained by MS, and the ^{1}H- and ^{13}C-NMR spectra indicated a molecular formula of $C_{21}H_{22}O_5$. The IR in chloroform showed absorption at 3450 cm^{-1} (hydroxyl group) and at 1736 cm^{-1} (ester carbonyl). The UV spectrum having maximum absorptions at 221, 250, 259, 268 and 310 nm is similar to those of furanocoumarins which were isolated from this plant.[6] The proton signals of the coumarin part in the ^{1}H-NMR were observed at 6.28 (1H, d, $J=9.9$ Hz), 7.16 (1H, dd, $J=1.1$, 0.6 Hz) and 8.15 ppm (1H, dd, $J=9.9$, 0.6 Hz), and the furan part at 6.96 (1H, dd, $J=2.4$, 1.1 Hz) and 7.60 ppm (1H, d, $J=2.4$ Hz), indicating that NI-1 contains a mono-substituted furanocoumarin skeleton. A substituent of 5-hydroxy-3,7-dimethyl-2,6-octadienyloxy group (the NMR data: see Experimental) was also established from the ^{1}H–^{1}H and ^{13}C–^{1}H correlation spectra (COSY) and from correlation spectroscopy *via* long-range couplings (COLOC). The position of this substituent at the furanocoumarin ring was obtained from the cross peak between 4.97 (2H, d, $J=6.8$ Hz; 1′-H of the substituent) and 148.83 ppm (C-5 of the ring) in the COLOC. Therefore, NI-1 was identified as notopterol.[6] As NI-1 has no optical activity, the *dl*-form was estimated.

During the isolation procedure, fr. II-b, which showed a major spot on TLC, seemed to slightly inhibit the writhing, although it was not significant. Further purification of fr. II-b was done to make clear the origin of the activity. NI-2 obtained as a colorless oil from this fraction was not very stable, and contained the characteristic absorption, 2245 and 2145 cm^{-1}, due to acetylenic chromophores in the IR spectrum. The assignment of the NMR spectrum by COSY and COLOC lead us to identify NI-2 as falcarindiol,[7] which has already been isolated from this plant.[6] NI-2, however, did not indicate any effect on acetic acid-induced writhing or on pentobarbital hypnosis in mice by oral administration of 300 mg/kg. It was concluded that the analgesic property of *N. incisum* belonged mostly to NI-1 (notopterol).

The effect of NI-1 on acetic acid-induced writhing in mice is shown in Fig. 2. Oral administration (*p.o.*) of NI-1 indicated an analgesic effect in a dose-dependent manner. NI-1 also inhibited the writhing at the similar level by subcutaneous injection (60%, $p<0.01$, 100 mg/kg).

Some pharmacological experiments on NI-1 were then carried out.

NI-1 strongly prolonged the pentobarbital-hypnosis shown in Fig. 3. On the other hand, oral doses of 300 and

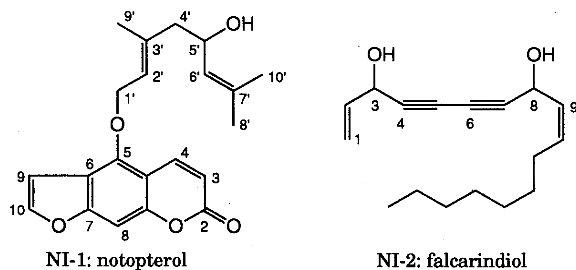

Fig. 1. Structures of NI-1 and 2

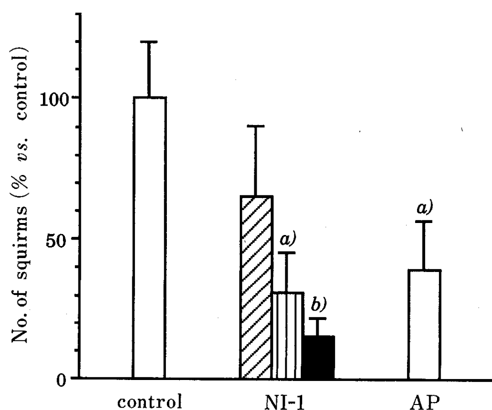

Fig. 2. Effect of NI-1 on Acetic Acid-Induced Writhing in Mice

a) $p<0.05$, *b*) $p<0.01$. Each bar represents the mean ± S.E. The number of squirms in control (20.3) is set as 100%. $n=6$. AP, aminopyrine 50 mg/kg, *p.o.* ▨, 30 mg/kg; ▥, 100 mg/kg; ■, 300 mg/kg.

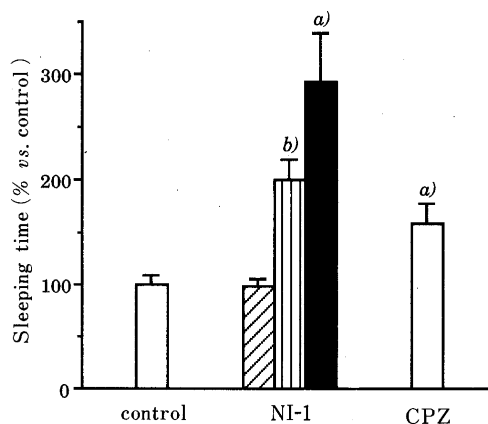

Fig. 3. Effect of NI-1 on Hypnosis Induced by Sodium Pentobarbital in Mice

a) $p<0.01$, *b*) $p<0.001$. Each bar represents the mean ± S.E. The sleeping time (50.5 min in control) induced by sodium pentobarbital is set as 100%. $n=6$. CPZ: chlorpromazine 5 mg/kg, *p.o.* ▨, 3 mg/kg; ▥, 10 mg/kg; ■, 30 mg/kg.

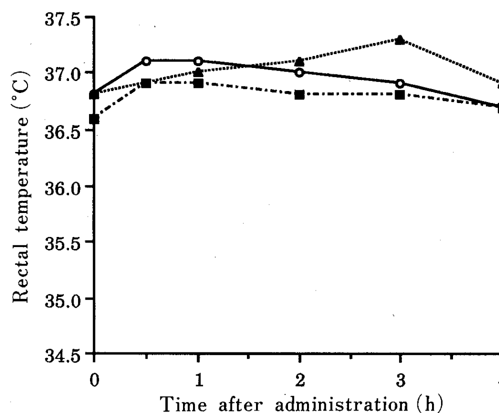

Fig. 4. Hypothermic Effect of NI-1 in Mice

$n=4$. —○—, control; ---▲---, NI-1 300 mg/kg; —·—■—·—, NI-1 500 mg/kg.

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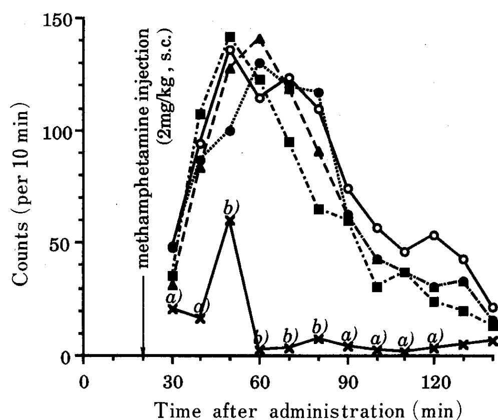

Fig. 5. Effect of NI-1 on Locomotor Activity Enhanced by Methamphetamine in Mice

a) $p<0.05$, *b)* $p<0.01$, $n=6$. CPZ: chlorpromazine. —○—, control; ---●---, NI-1 30 mg/kg; --▲--, NI-1 100 mg/kg; --·■--·, NI-1 300 mg/kg; —×—, CPZ 10 mg/kg.

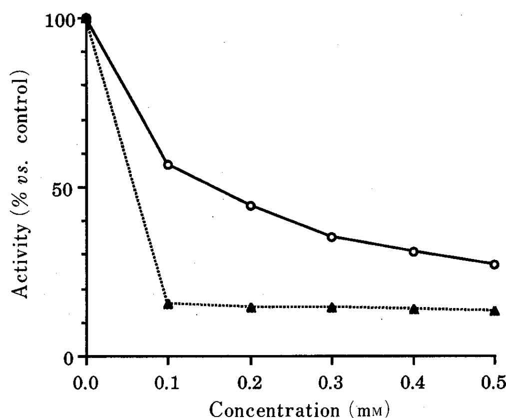

Fig. 7. Effect of NI-1 on Aminopyrine *N*-Demethylase Activity in Liver Microsomes of Mice

—○—, SKF 525A; ---▲---, NI-1.

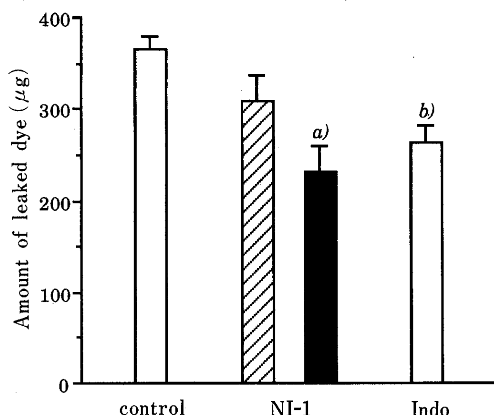

Fig. 6. Effect of NI-1 on Increased Vascular Permeability in Acetic Acid-Treated Mice

a) $p<0.01$, *b)* $p<0.001$. Each bar represents the mean±S.E. *n* (control and Indo) = 8, *n* (sample) = 7. Indo: Indomethacin 10 mg/kg, *p.o.* ▨, 30 mg/kg; ■, 100 mg/kg.

500 mg/kg did not decrease rectal temperature in mice (Fig. 4). The locomotor activity in methamphetamine-treated mice was not affected by the administration of NI-1 up to 300 mg/kg, *p.o.* (Fig. 5).

The anti-inflammatory activity of NI-1 was examined by a vascular permeability test in acetic acid-treated mice.[5] NI-1 indicated a significant inhibition of increased permeability at a dose of 100 mg/kg, *p.o.* (Fig. 6).

The effect of NI-1 on drug metabolism in mouse liver was investigated to understand why NI-1 had such a strong effect on pentobarbital-induced hypnosis, but no effect on body temperature or locomotor activity. NI-1 showed significantly potent inhibition on aminopyrine *N*-demethylase activity in the liver microsomes as compared to SKF-525A, a typical inhibitor of drug metabolism (Fig. 7). The liver slice preparations did not indicate denaturation under a microscope when 200 mg/kg, *p.o.* of NI-1 was administered to mice in the pentobarbital-induced hypnosis test.

In conclusion, the analgesic component of *N. incisum* is identified as notopterol. It seemed to have anti-inflammatory activity, but a neurological effect, such as sadation, may not be involved. Notopterol also inhibits drug metabolism in the liver to cause an intensive prolongation of pentobarbital-induced hypnosis. The anti-peroxidative effect of *N. incisum* reported by Yang *et al.*[8] is possibly derived from the same reason.

In comparison with (*ent*)-kaur-16-en-19-oic acid and (*ent*)-pimara-8(14),15-dien-19-oic acid and osthol, the analgesic components of *Aralia cordata* and *Angelica pubescens*, respectively, by the writhing method (*p.o.*), notopterol showed a slightly stronger effect than those active components [osthol[9]: 38% ($p<0.01$) and 77% ($p<0.001$) inhibitions at doses of 100 and 300 mg/kg, respectively, the former compounds: see our previous paper[3]]. However, this fact does not fully illustrate the analgesic potentials of these medicinal plants because many other components in the plants may have some role in the effect, such as solubilization, absorption, *etc.* Notopterol and the analgesic components of *A. cordata* prolonged the anesthesia induced by pentobarbital, although osthol did not. The prolongation was caused by an inhibition of drug metabolizing enzymes in the former, as mentioned above, and mostly by sedation in the latter.[3] Osthol did not indicate an effect on normal body temperature in mice up to 300/mg/kg, *p.o.* The anti-inflammatory activity of osthol was previously reported,[4] and the pharmacological properties of notopterol and osthol seemed to be rather similar as anti-inflammatory analgesics, except for the effect on drug metabolism. The analgesic principles of the traditional medicines, "Kyohkatsu" and "Dokukatsu" are now made clear, and those compounds have different pharmacological properties.

Experimental

Melting points were determined on a Yanagimoto melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-140 polarimeter. IR spectra were recorded on a Hitachi EPI-G3 spectrometer, UV spectra on a Hitachi U-3200 spectrometer, and MS spectra on a Hitachi M-60 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GSX 400 spectrometer with tetramethylsilane as an internal standard. Column chromatographies were performed on Sephadex LH-20, Wakogel C-200, Nakalai Silica gel 60 and Chromatorex ODS (100—200 mesh). A pre-packed column (Kusano CPO-HS-221-10) was used for medium pressure liquid chromatography (MPLC).

Isolation "Tou-kyohkatsu" (*N. incisum*) was purchased from Uchida Wakanyaku Co., Ltd.

The dried rhizomes and roots (4.97 kg) were extracted with methanol to obtain a methanol extract (1.07 kg). The extract (264.2 g) was parti-

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tioned with *n*-butanol/water. The *n*-butanol fraction (151.8 g), which inhibited acetic acid writhing in mice, was chromatographed on Sephadex LH-20 (methanol) to get the active fraction, fr. I-b (92.7 g). Fraction I-b was separated by silica gel column chromatography, and fr. II-c (10.9 g) was obtained from a *n*-hexane–ethyl acetate (1 : 1) eluent. Further separation of fr. II-c silica gel column chromatography eluted with benzene–ethyl acetate (1 : 1) gave the active fraction, fr. III-b (7.09 g), which was purified by silica gel flash column chromatography (*n*-hexane–ethyl acetate 1 : 1) and by recrystallization from *n*-hexane–ethyl acetate. An analgesic component, NI-1 (1.03 g), was obtained.

Fraction II-b (4.93 g), which showed a major spot on TLC, was separated by silica gel column chromatography (benzene–ethyl acetate 6 : 1). Reversed phase flash column chromatography (methanol–water 4 : 1) and MPLC (silica gel, benzene–ethyl acetate 6 : 1) were used for further separation to give a colorless oil, NI-2 (1.22 g).

NI-1 Colorless needles from *n*-hexane–AcOEt, mp 93.5—95 °C, $[\alpha]_D^{25}$ *ca.* 0° (c=1.99, $CHCl_3$). IR ν_{max}^{KBr} cm^{-1}: 3530, 3450 br, 3125, 1736, 1628, 1606, 1581, 1458, 1359, 1162, 1095, 817, 750. UV λ_{max}^{MeOH} nm (log ε): 220.8 (4.37), 250.4 (4.23), 259.2 (4.18), 267.6 (4.18), 309.6 (4.12). EI-MS *m/z* (%): 354 (M^+, 0.3), 286 (17), 202 (100), 174 (51), 145 (37), 135 (17), 118 (13). ^{1}H-NMR ($CDCl_3$) δ: 1.43 (1H, d, J=2.6 Hz, 5′-OH), 1.70 (3H, d, J=1.3 Hz, 10′-H), 1.72 (3H, d, J=1.3 Hz, 8′-H), 1.76 (3H, d, J=0.7 Hz, 9′-H), 2.22 (1H, dd, J=13.7, 4.9 Hz, 4′-H_a), 2.30 (1H, dd, J=13.7, 8.4 Hz, 4′-H_b), 4.49—4.54 (1H, m, 5′-H), 4.97 (2H, d, J=6.8 Hz, 1′-H), 5.16—5.20 (1H, m, 6′-H), 5.65 (1H, td-like, J=6.8, 1.1 Hz, 2′-H), 6.28 (1H, d, J=9.9 Hz, 3-H), 6.96 (1H, dd, J=2.4, 1.1 Hz, 9-H), 7.16 (1H, dd, J=1.1, 0.6 Hz, 8-H), 7.60 (1H, d, J=2.4 Hz, 10-H), 8.15 (1H, dd, J=9.9, 0.6 Hz, 4-H). ^{13}C-NMR ($CDCl_3$) δ: 17.06 (C-9′), 18.23 (C-10′), 25.73 (C-8′), 47.68 (C-4′), 66.42 (C-5′), 69.47 (C-1′), 94.27 (C-8), 105.00 (C-9), 107.41 (C-4a), 112.65 (C-3), 114.05 (C-6), 122.06 (C-2′), 127.37 (C-6′), 135.56 (C-7′), 139.46 (C-4), 139.57 (C-3′), 144.97 (C-10), 148.83 (C-5), 152.67 (C-1a), 158.14 (C-7), 161.23 (C-2).

NI-2 Colorless oil. IR $\nu_{max}^{CHCl_3}$ nm^{-1}: 3580, 3385 br, 2245, 2145, 1377, 1010, 987, 938. UV λ_{max}^{MeOH} nm (log ε): 233.0 (3.01), 245.4 (2.99), 259.0 (2.76). ^{1}H-NMR ($CDCl_3$) δ: 0.88 (3H, t-like, J=6.9 Hz, 17-H), 1.28—1.33 (8H, m, 13, 14, 15, 16-H), 1.36—1.40 (2H, m, 12-H), 1.91 (1H, d, J=5.2 Hz, 8-OH), 2.00 (1H, d, J=6.6 Hz, 3-OH), 2.09—2.13 (2H, m, 11-H), 4.94 (1H, dd, J=6.6, 5.2 Hz, 3-H), 5.21 (1H, dd, J=8.5, 5.2 Hz, 8-H), 5.26 (1H, ddd, J=10.2, 1.4, 0.8 Hz, 1-H_{cis}), 5.47 (1H, dt, J=17.1, 0.8 Hz, 1-H_{trans}), 5.51 (1H, dd, J=10.7, 8.5 Hz, 9-H), 5.61 (1H, dt, J=10.7, 7.4 Hz, 10-H), 5.94 (1H, dddd, J=17.1, 10.2, 5.2, 0.6 Hz, 2-H). ^{13}C-NMR ($CDCl_3$) δ: 14.10 (C-17), 22.64 (C-16), 27.70 (C-11), 29.12, 29.17, 29.29 (C-12, 13, 14), 31.80 (C-15), 58.56 (C-8), 63.43 (C-3), 68.75, 70.30 (C-5, 6), 78.28 (C-4), 79.85 (C-7), 117.33 (C-1), 127.67 (C-9), 134.62 (C-10), 135.82 (C-2).

Pharmacological Assay Male ddY mice weighing 24—32 g were used. The animals were propagated at Shizuoka Agricultural Cooperative Association (Hamamatsu, Japan), and were housed under a 12-h light/dark cycle at 20—25 °C. Food was withheld for 2 h before and during the experiments. Test samples were suspended in saline with 5% arabic gum, 2% Tween 80 and/or 20% olive oil.

Analgesic Activity: An acetic acid-induced writhing method[5] was used. Samples were given 30 min (in the cases of the extract and the partitioned fractions) or 40 min (in the other cases) prior to an i.p. injection of 0.7% acetic acid (0.1 ml/10 g). The number of squirms was counted in each mouse for 15 min beginning 5 min after the acetic acid injection.

Effect on Pentobarbital-Induced Hypnosis: Samples were administered 40 min before i.p. injection of 0.5% sodium pentobarbital (Tanabe Pharmaceutical Co., Ltd.). The time required to the righting reflex was measured.

Hypothermic Effect: Rectal temperatures were measured up to 4 h after sample injection by a thermistor (Takara Instrumental Co., Ltd.).

Effect on Locomotor Activity: Locomotor activity was measured by a Tilting-type Ambulometer AMB-10 (O'hara & Co., Ltd.) Samples were administered at 20 min before the subcutaneous injection of methamphetamine (Dainippon Pharmaceutical Co., Ltd.) at a dose of 2 mg/kg. The movements were counted for every 10 min up to 120 min. One mouse at a time was put in a cage.

Effect on Vascular Permeability: The procedure was based on Whittle's method.[5] Before 10 min, 4% Pontamine sky blue in saline (0.1 ml/10 g) was intravenously given to each mouse. At 30 min after administration of the samples, 1% acetic acid was injected intraperitoneally. After 20 min the mice were sacrificed, and the abdominal wall was cut to expose the viscera. The viscera were washed with saline and the washings were filtrated. Each washing produced up to 20 ml in a test tube after the addition of 0.1 ml of 1 N NaOH solution. The absorption was read at 590 nm in a spectrophotometer (model 200-10, Hitachi).

Effect on Drug Metabolism in Liver: Liver microsomes of the mice were prepared as described previously.[10] A reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (0.33 mM NADP, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase and 6 mM $MgCl_2$), liver microsomes (0.3—1.0 mg of protein) and aminopyrine (5 mM) in a final volume of 1.0 ml. The activity of aminopyrine *N*-demethylase was estimated by determination of formaldehyde by the method of Nash.[11]

Statistics: Statistical significance was evaluated by the Student's *t* test.

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