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Platelet Aggregation Inhibitors from Jyu-yaku (Houttuyniae Herb)

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Two potent inhibitors of platelet aggregation were isolated from the chloroform extract of jyu-yaku (Houttuyniae herb), a traditional medicine in Japan and China, by a combination of high-performance liquid chromatography and other techniques. These compounds were identified as *cis*- and *trans*-*N*-(4-hydroxystyryl)benzamide by proton nuclear magnetic resonance spectrometry.

Keywords——jyu-yaku; Houttuyniae herb; *Houttuynia cordata*; *cis*-*N*-(4-hydroxystyryl)benzamide; *trans*-*N*-(4-hydroxystyryl)benzamide; rabbit platelet aggregation *in vitro*

Introduction

Jyu-yaku (Houttuyniae herb; *Houttuynia cordata* THUNBERG in the flowering season) has many pharmacological activities.[1] However the only components so far reported are several kinds of flavone glycosides and decanoyl acetaldehyde, a component responsible for the peculiar odor of the herb.[2]

In our present study on jyu-yaku, attention has been focused on the isolation of certain constituents affecting platelet function.

Results and Discussion

Jyu-yaku (Houttuyniae herb, 5.5 kg) was extracted with 10 l of methanol. The extract was partitioned with 4 l of chloroform (yield, 216 g). Since the inhibitory activity on platelet aggregation emerged mainly in the chloroform extract, this fraction was subjected to preparative reversed-phase high-performance liquid chromatography (HPLC) to yield a highly active fraction (20.2 g). A portion (2 g) of this fraction was further subjected to semi-preparative reversed-phase HPLC to yield an active fraction (1.5 mg) emerging as a single peak.

However, two kinds of active constituents (compound I, 1.0 mg; compound II, 0.1 mg) were obtained from this fraction by preparative silica gel thin layer chromatography (TLC).

Electron ionization mass spectra (EI-MS) of I and II produced the same mass fragmentation pattern, and peaks were observed at *m/z* 77 (10%), 105 (100%) and 239 (50%). The infrared (IR) spectrum of I exhibited amide (1650 cm^{-1}), benzene ring (1605, 1580 and 1500 cm^{-1}) and hydroxy (3400 cm^{-1}) absorptions. The two-dimensional shift correlation proton nuclear magnetic resonance (^{1}H-NMR) spectrum of I exhibited monosubstituted benzene signals at δ 7.98 (2H, d), 7.62 (1H, t) and 7.55 (2H, t), *para*-disubstituted benzene signals at δ 7.36 and 6.93 (each 2H, d), –NH–CH=CH– signals at δ 9.00 (1H, br), 7.07 (1H, t) and 5.85 (1H, d), and a phenolic –OH proton signal at δ 8.42 (1H, s). The ^{1}H-NMR spectrum of II showed different signals assignable to –NH–CH=CH– at δ 9.65 (1H, br), 7.65 (1H, t)

and 6.45 (1H, d) as compared with the spectrum of I, while the other signals were almost the same as those of I. The coupling constants of –CH=CH– were 9.5 Hz (I) and 14.7 Hz (II). From these spectra, I and II were assumed to be *cis*- and *trans*-*N*-(4-hydroxystyryl)benzamide, respectively.

Compounds I and II were synthesized to confirm their structures and inhibitory activities on platelet aggregation. The EI-MS and ^{1}H-NMR spectra of synthetic I and II were the same as those of the compounds obtained from jyu-yaku.

Both I and II showed similar dose-dependent inhibitory effects on platelet aggregation induced by arachidonic acid (the IC_{50} values were both about 5×10^{-6} M). Compounds I and II were about twenty times more potent than acetylsalicylic acid and were about a half as potent as indomethacin. However, they did not inhibit the platelet aggregation induced by adenosine diphosphate at the concentration of 2×10^{-3} M.

In conclusion, we isolated two constituents from jyu-yaku and identified them as *cis*- and *trans*-*N*-(4-hydroxystyryl)benzamide, which have potent inhibitory actions on platelet aggregation. Although *cis*- and *trans*-*N*-(4-hydroxystyryl)benzamide were reported as intermediates in the synthesis of organic compounds,[4] no previous report on their presence in plants has appeared. Furthermore, the pharmacological activities of these compounds have not been evaluated yet. Further studies are continuing.

Experimental

The melting points were determined on a Mettler FP 800 apparatus and are uncorrected. The following spectrometers were used: ^{1}H-NMR, JEOL GX-400 with tetramethylsilane ($\delta=0$) as an internal standard; EI-MS, Hitachi RMU-6MG; IR, Bio Rad DIGILAB QUALIMATIC. Elemental analysis was done on a Yanagimoto MT-3. HPLC was performed with CAPCELL PAK C_{18} (70—230 mesh, 100 mm i.d. × 1000 mm; S-15 μ, 30 mm i.d. × 500 mm; S-5 μ, 10 mm i.d. × 250 mm; Shiseido), and a ultraviolet (UV) detector was used at 254 nm. Kieselgel 60 F_{254} plates (Merck) were used for TLC and preparative TLC. Kieselgel 60 (70—230 mesh, Merck) was used for column chromatography. Sodium arachidonate, adenosine diphosphate, indomethacin and acetylsalicylic acid were purchased from Sigma.

Platelet Aggregation Study——Blood samples were collected into plastic tubes containing 0.1 volume of 3.8% (w/v) trisodium citrate through a canula inserted into the carotid artery of male Japanese White rabbits weighing 2.5—3.5 kg. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood samples at 150 *g* for 15 min at room temperature. The upper white suspension was used as PRP having platelet counts between 3 and 4×10^8/ml. Aggregation of PRP was monitored turbidimetrically by the method of Born and Cross[3] with a slight modification on an aggregometer (AUTORAM-31, Rikadenki Kogyo) connected to a recorder. PRP (250 μl) was placed in the aggregometer and then 12.5 μl of sample or vehicle (20% MeOH) was added. After preincubation of the mixture of PRP and sample or vehicle at 37 °C for 2 min, 25 μl of sodium arachidonate and adenosine diphosphate (final concentration of 200 and 2 μM, respectively) was added to induce platelet aggregation.

Isolation——Jyu-yaku obtained from a market in Tokyo was extracted with methanol. The methanol extract was extracted with chloroform. The chloroform extract was fractionated by HPLC (100 mm i.d. × 1000 mm) with methanol. The active fraction was injected into the HPLC apparatus (30 mm i.e. × 500 mm and 10 mm i.d. × 250 mm) repeatedly, to give compounds I and II. The mixture was applied to a preparative TLC silica gel plate and developed with chloroform–ethyl acetate (8 : 2) to give compounds I and II.

Synthesis and Purification of Compound I and II——A solution of 10 g of octopamine hydrochloride (98%, Aldrich) in 50 ml of pyridine was prepared, then 7.4 g of benzoyl chloride was added dropwise with stirring in an ice bath. After addition of 150 ml of distilled water, the mixture was extracted with 100 ml of chloroform (× 3) to give 9 g of *N*-benzoyloctopamine. A mixture of this with adding 800 ml of xylene and 2 g of aluminum oxide was refluxed for 30 h, then filtered. After evaporation of the filtrate, the residue was chromatographed over silica gel to separate the *cis* and *trans* isomers. The *cis* isomer (I) was eluted first with chloroform. Then, the *trans* isomer (II) was eluted with chloroform and ethyl acetate mixture (9 : 1). The crude I and II isomers were recrystallized repeatedly from benzene and chloroform, respectively. The final yields of I and II were 0.5 and 2.0 g.

Compound I: mp 179.3—182.2 °C. *Anal.* Calcd for $C_{15}H_{13}NO_2$: C, 75.28; H, 5.48; N, 5.86; O, 13.38. Found: C, 75.0; H, 5.4; N, 5.8; O, 13.5. UV λ_{max}^{MeOH} nm (ε): 231 (17000), 301 (6900). IR ν_{max}^{KBr} cm^{-1}: 3400, 1640, 1610, 1580, 1505, 1480, 1230, 1075. ^{1}H-NMR (in acetone-d_6) δ: 5.85 (1H, d, J=9.5 Hz), 6.93 (2H, d, J=8.5 Hz), 7.07 (1H, t, J=10 Hz), 7.36 (2H, d, J=8.5 Hz), 7.54 (2H, t, J=7.3 Hz), 7.61 (1H, t, J=7.3 Hz), 7.98 (2H, d, J=7.3 Hz), 8.41 (1H, s), 9.00 (1H, br). ^{13}C-NMR (in acetone-d_6) δ: 165.8 (s), 157.7 (s), 135.4 (s), 133.1 (d), 130.9 (d), 129.9 (d), 128.8 (d and s), 122.2

(d), 117.2 (d), 112.7 (d). EI-MS *m/z*: 239 (M^+), 105, 77.

Compound II: mp 204.8—206.1 °C. *Anal.* Calcd for $C_{15}H_{13}NO_2$: C, 75.28; H, 5.48; N, 5.86; O, 13.38. Found: C, 75.2; H, 5.3; N, 5.8; O, 13.4. UV λ_{max}^{MeOH} nm (ε): 224 (17000), 313 (23000). IR ν_{max}^{KBr} cm^{-1}: 3350, 1640, 1610, 1530, 1505, 1490, 1330, 1295, 1240, 945. ^{1}H-NMR (in acetone-d_6) δ: 6.46 (1H, d, J=14.7 Hz), 6.86 (2H, d, J=8.5 Hz), 7.29 (2H, d, J=8.5 Hz), 7.54 (2H, t, J=7.3 Hz), 7.60 (1H, d, J=7.3 Hz), 7.65 (1H, dd, J=14.7, 10.1 Hz), 8.04 (2H, d, J=7.3 Hz), 8.26 (1H, s), 9.70 (1H, br d, J=10 Hz). ^{13}C-NMR (in acetone-d_6) δ: 165.3 (s), 157.8 (s), 135.6 (s), 133.0 (d), 129.8 (d and s), 128.8 (d), 128.0 (d), 123.1 (d) 117.1 (d), 114.7 (d). EI-MS *m/z*: 239 (M^+), 105, 77.

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