Identification of Magnolol and Honokiol as Anxiolytic Agents in Extracts of Saiboku-to, an Oriental Herbal Medicine

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The principal active anxiolytic components in Saiboku-to, an Oriental herbal medicine, have been isolated and identified as magnolol (5,5′-di-2-propenyl-1,1′-biphenyl-2,2′-diol) and honokiol (3′,5-di-2-propenyl-1,1′-biphenyl-2,4′-diol). Evaluation by means of an elevated plus-maze test showed that honokiol was at least 5000 times more potent than Saiboku-to when mice were treated orally for 7 days.

In traditional Chinese medicine, called Kampo medicine in Japan, herbal remedies composed of specified mixtures of dried plant materials have been utilized empirically for thousands of years for the treatment of a wide variety of clinical disorders.[1] Preparations such as Hange-Koboku-to, Yoku-Kan-san, Saiboku-to, and Kami-Kihi-to, for example, have been historically prescribed for clinical depression; anxiety-related disorders such as anxiety neurosis, insomnia, and anxiety hysteria, as well as for thrombotic stroke and gastrointestinal complaints. Saiboku-to, one of the most popular of these Chinese herbal medicines, has been shown in laboratory experiments to be effective for treatment of asthma.[2,3] Evidence has also been presented from nonplacebo controlled studies that Saiboku-to is able to relieve anxiety and/or nervous tension.[1,4]

One of the goals in our laboratory has been to characterize the anxiolytic properties of Kampo medicines through more rigorous experimentation.[5,6] But because anxiety-related responses are so subjective in nature, it is very difficult (if not impossible) to conduct this type of investigation with human subjects. For this reason, animal models have been developed to study anxiety and to evaluate the anxiolytic potential of new therapeutic agents. In common among the 20 to 30 models described in the literature is the use of fear to mimic human anxiety.[7] Many of these animal models, however, are quite time consuming, generally requiring the use of sophisticated instrumentation, prior training of the animals, and noxious stimuli to elicit anxiety in the animal. In contrast, the "elevated plus-maze," originally developed by Pellow and co-workers,[8] is a simple yet sensitive test that assesses anxiety in rodents through monitoring of a fear−avoidance conflict whereby an animal chooses to enter either the closed or open-sided arms of an elevated maze that is in the shape of a "plus." Validation of this model, which is now in

Table 1. Anxiolytic Activity of Saiboku-to Fractions Obtained by Solvent Extraction

test substance[a]	yield (g)	recovery (%)	corresponding dose (mg/kg)[b]	anxiolytic effect (%)[c]
Saiboku-to	60.0	100	2000	100.0
F1	8.3	14	280	15.8 ± 7.7
F2	17.7	30	600	37.8 ± 13.1
F3	26.3	44	880	33.3 ± 12.2
F4	0.6	1	20	117.6 ± 42.8

[a] Unfractionated Saiboku-to or fractions obtained by solvent extraction, as described in the Experimental Section. [b] Dosages were determined by using the percent recovery of each fraction to calculate the contribution by weight of each fraction in a 2000-mg/kg dose of unfractionated Saiboku-to. [c] Values represent the mean ± standard error for 10 animals, as determined by the elevated plus-maze test described in the Experimental Section. For animals treated with 2000 mg/kg of Saiboku-to, the meantime spent in the open-sided arms was 22.2 ± 6.0 s.

widespread use,[7,9] has been obtained by the demonstration that treatment with clinically effective anxiolytic drugs such as benzodiazepines results in an increase in the time an animal spends in the open-sided arms of the plus-maze. We have now constructed an improved version of the elevated plus-maze in which the floor of the open-sided arms is transparent.[5] We have employed this device in our initial studies of Saiboku-to and found that Saiboku-to is able to produce a statistically significant, dose-dependent reduction in anxiety in mice after 7 days of oral administration.[6,10] The current report describes our efforts to identify the active anxiolytic components in Saiboku-to.

Saiboku-to was fractionated by sequential solvent extraction, as described in the Experimental Section, and the anxiolytic effectiveness of each fraction was assessed in the elevated plus-maze test. So that we could directly compare our results with those we obtained previously by treatment with 2000 mg/kg of Saiboku-to,[6] each test dose represented the contribution by weight of that fraction in 2000 mg/kg of Saiboku-to. The results are summarized in Table 1, showing that fraction F4 exhibited the greatest anxiolytic potency of the fractions isolated by solvent extraction. Fraction F4 was, therefore, further fractionated by Si gel column chromatography, eluting with increasing proportions of

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**Table 2.** Anxiolytic Activity of Components in Saiboku-to Fraction 4 Obtained by Column Chromatography

test substance[a]	yield from F4 fraction (g)	recovery (%)	corresponding dose (mg/kg)[b]	anxiolytic effect (%)[c]
Saiboku-to F4	5.50	100	20.4	117.6 ± 42.8
F4-1	0.63	11.5	3.6	74.8 ± 28.8
F4-2	1.29	23.5	7.2	38.3 ± 15.3
F4-3	2.79	50.9	15.8	28.4 ± 21.2
F4-4	0.30	5.5	1.1	9.0 ± 4.5

[a] Saiboku-to fraction F4 or sub-fractions of 5.5 g of F4 obtained by Si gel column chromatography, as described in the Experimental Section. [b] Doses calculated as described in the legend to Table 1 to correspond to the proportion of fraction F4 in 2000 mg/kg of Saiboku-to and the contributions of the sub-fractions in 3000 mg/kg of Saiboku-to. [c] Values represent the mean ± standard error for 10 animals, as determined by the elevated plus-maze test described in the Experimental Section. For animals treated with 2000 mg/kg of Saiboku-to, the mean time spent in the open-sided arms was 22.2 ± 6.0 s.

MeOH in $CHCl_3$. The yields and activities of these subfractions are summarized in Table 2, where it can be seen that fraction F4-1 exhibited the most potent anxiolytic activity, producing 78% of the response of 2000 mg/kg of Saiboku-to at a dose of only 3.6 mg/kg of F4-1. Based on the assumption that there is a linear dose-dependent response for fraction F4-1, similar to the one we observed for Saiboku-to,[6,10] from the results in Table 1 it can be estimated that treatment with 4.6 mg/kg of F4-1 would lead to a reduction in anxiety equivalent to 2000 mg/kg of unfractionated Saiboku-to. This indicates that a greater than 400-fold increase in anxiolytic potency was gained by fractionation of Saiboku-to.

GC−EIMS was then utilized to identify the principal components in fraction F4-1. There were three major peaks in the reconstructed total ion current chromatogram. From a search of the Wiley mass spectral library included with the mass spectrometer data system software, the spectra for peak numbers 1 (t_R 2.8 min) and 2 (3.3 min) were found to match those of magnolol (5,5′-di-2-propenyl-1,1′-biphenyl-2,2′-diol) and honokiol (3′,5-di-2-propenyl-1,1′-biphenyl-2,4′-diol), respectively. Further confirmation of the identities of these neolignan derivatives was obtained by comparison with authentic magnolol and honokiol, which exhibited the same GC retention times and mass spectral fragmentation patterns as their respective components in fraction F4-1. Although present in the Wiley mass spectral database, details of the EI mass spectra of magnolol and honokiol have not been reported in the literature. Full elucidation of the unusual fragmentation observed in these spectra is currently underway. Regarding peak number 3 (5.6 min), the mass spectrum was composed predominantly of ions at m/z 284 (55% rel int), m/z 269 (100%), m/z 241 (25%), and m/z 139 (25%). This component does not appear to share any structural similarities with either magnolol or honokiol and remains unidentified at this time.

Quantification of magnolol and honokiol in fraction F4-1 was obtained by HPLC, through use of calibration curves generated with the authentic analytes. The standard curves were linear over the range examined (0.9 to 18 nmole injected; $r = 0.9996$) with an estimated detection limit of 0.18 nmole for each of the substances. Under the conditions described in the Experimental Section, the HPLC retention times were 9.4 min for
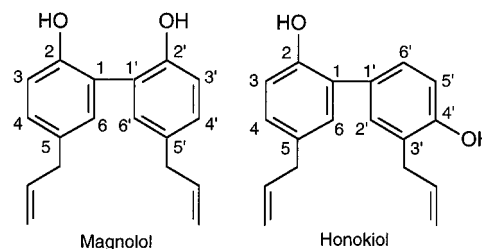
Magnolol   Honokiol

magnolol and 14.2 min for honokiol. The results of the HPLC analyses revealed that 9.5 mg of magnolol and 3.8 mg of honokiol were recovered in fraction F4-1 derived from 60 g of Saiboku-to, representing 0.016% and 0.006%, respectively, of the starting Saiboku-to mixture. From examination of the list of herbs that comprise Saiboku-to, it can be deduced that the source for the magnolol and honokiol in the preparation is the magnolia bark. We calculate that only about 2% of the amount of magnolol and 4% of honokiol that is expected to be present in the starting Saiboku-to mixture was recovered by our hot H_2O extraction and fractionation protocol.

Isolation of magnolol and honokiol directly from Saiboku-to was then undertaken according to published procedures[11] in order to obtain sufficient quantities for NMR spectroscopy. Both the ^{1}H- and ^{13}C-NMR spectra of the components isolated from Saiboku-to matched the corresponding spectra of authentic magnolol and honokiol in addition to the spectra reported in the literature.[11]

We then began a study of these biologically active anxiolytic components of Saiboku-to, treating mice separately with authentic magnolol and honokiol for 7 days and evaluating their behavior by means of the elevated plus-maze test. The doses used in these initial experiments (0.49 and 0.19 mg/kg, respectively) correspond to 1.5 times the level of magnolol and honokiol in our standard 2000-mg/kg dose of Saiboku-to—a dose of Saiboku-to that consistently produces a significant anxiolytic effect.[6,10] The results presented in Figure 1 clearly show that both magnolol and honokiol are active anxiolytic agents in mice, with honokiol substantially more effective than magnolol. From the results in Figure 1 it can be estimated that honokiol is at least 5000 times more potent than Saiboku-to in reducing anxiety in mice, as assessed by the elevated plus-maze test. The impressive oral anxiolytic potency of honokiol is particularly noteworthy in view of the report by Watanabe and co-workers[12] in which high doses of magnolol and honokiol (63 to 500 mg/kg), administered intraperitoneally, were required to obtain CNS depressant effects in mice.

Our preliminary experiments employed a seven-day administration of magnolol and honokiol in order to mimic the traditional use of Kampo medicines for chronic therapy. In view of our intriguing results, we then followed the time course for the anxiolytic activity of honokiol, comparing it to Saiboku-to and fraction F4-1. The results are shown in Table 3, where the acute activity of diazepam is included for reference. While honokiol was not found to be an effective anxiolytic agent after either 1 or 3 days of treatment, substantial activity was measured when honokiol was administered for at least 5 days.

Studies are now underway in which BALB/c mice (an inbred strain) are being utilized to evaluate more fully
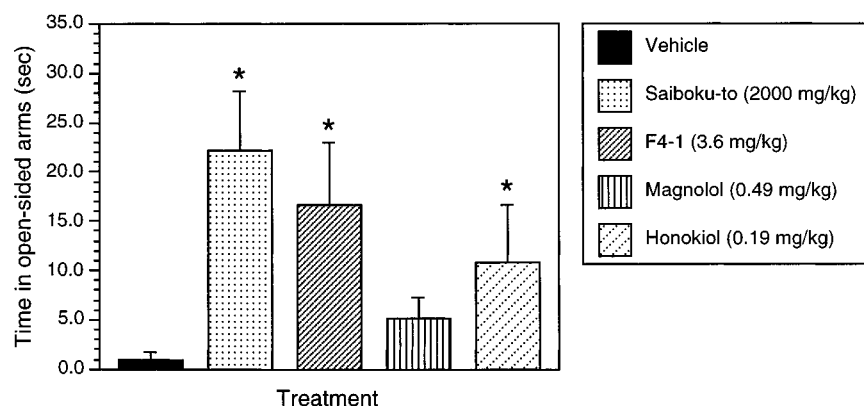
Figure 1. Anxiolytic activity of magnolol and honokiol. Treatment with each agent was for seven days, as described in the Experimental Section. Values represent the mean ± standard error for 10 animals. *, statistically significant ($p < 0.05$) as compared to the vehicle-treated group.

Table 3. Time Course for Observation of Anxiolytic Activity

| test substance[a] | anxiolytic effect | | | | |
| | time (sec) spent in open-sided arms[b] | | | | |
	1 day	3 days	5 days	7 days	14 days
control[c]	2.8 ± 2.8	0.2 ± 0.2	0.8 ± 0.5	0.9 ± 0.9	1.3 ± 0.6
Saiboku-to (2000 mg/kg)	2.4 ± 1.3	6.9 ± 4.6	11.6 ± 6.0	22.2 ± 5.0	23.2 ± 4.7
F4-1 (3.6 mg/kg)	8.5 ± 5.4	9.0 ± 5.8	10.6 ± 3.6	16.6 ± 6.4	16.1 ± 4.9
honokiol (0.19 mg/kg)	3.7 ± 2.1	4.0 ± 1.9	9.1 ± 4.9	10.9 ± 5.7	12.3 ± 4.5
diazepam (1.0 mg/kg)[d]	22.1 ± 5.9	nt	nt	nt	nt

[a] Oral treatment, as described in the Experimental Section, once per day for the indicated number of days. [b] Values represent the mean ± standard error for 10 animals, as determined by the elevated plus-maze test described in the Experimental Section; nt, not tested. [c] Treated with the vehicle, Tween-80. [d] Diazepam administered orally, 10 min prior to evaluation by the elevated plus-maze test.

the anxiolytic effects of magnolol and honokiol. Our preliminary experiments have indicated that the BALB/c mice exhibit increased times spent in the open-sided arms of the elevated plus-maze, with decreased data variability, as compared to the more genetically heterogeneous ddY mice. Current efforts are aimed at elucidating the dose−response and structure−function relationships of these compounds in more detail.

Experimental Section

General Experimental Procedures. EIMS was performed on a Kratos Analytical Ltd. Concept 32 1S magnetic sector mass spectrometer in combination with a Shimadzu Mach 3 data system. Mass spectrometry conditions were: ion source temperature, 200 °C; accelerating voltage, 8 kV; ionization energy, 70 eV; resolution, 5000. Sample introduction was by means of a Shimadzu model 14A gas chromatograph fitted with a DB-5MS fused silica capillary column (30 m × 0.25 mm; J & W Scientific). GC conditions were: carrier gas, helium; injection split ratio, 50:1; column temperature, 290 °C; injector temperature, 320 °C; interface temperature, 320 °C.

HPLC was accomplished with the following instrumentation: pump, Shimadzu model LC-6A LC; detector, Shimadzu model SPD-6AN; wavelength, 254 nm; LC data processor, Waters model 741 data module. Analytical reversed-phase fractionation was obtained through use of a YMC ODS A-312 column (5 μm, 4.6 mm × 150 mm; YMC Co., Japan) with an isocratic mobile phase of MeCN−H_2O−glacial HOAc (50:50:1, v/v/v) at a flow rate of 2.0 mL/min. The column temperature was maintained at 40 °C.

^{1}H- and ^{13}C-NMR analyses were conducted on a Bruker AM-500 spectrometer. Samples were dissolved in $CDCl_3$ at a concentration of approximately 20 mg/mL. Chemical shifts were expressed as parts per million relative to TMS (for ^{1}H) or solvent (for ^{13}C), which were used as internal standards.

Materials. Saiboku-to was obtained from Tsumura & Company Ltd. (Tokyo, Japan). This oriental herbal medicine is composed of the following dried raw materials: bupleurum root, 7.0 g [*Bupleurum falcatum* Linné (Umbelliferae)]; hoelen, 5.0 g [*Poria cocos* Wolf (Polyporaceae)]; pinellia tuber, 5.0 g [*Pinellia ternata* Breitenbach (Araceae)]; ginseng root, 3.0 g [*Panax ginseng* C. A. Meyer (Araliaceae)]; jujube fruit, 3.0 g [*Zizyphus jujuba* Miller var. *inermis* Rehder (Rhamnaceae)]; magnolia bark, 3.0 g [*Magnolia obovata* Thunberg (Magnoliaceae)]; scutellaria root, 3.0 g [*Scutellaria baicalensis* Georgi (Labiatae)]; glycyrrhiza root, 2.0 g [*Glycyrrhiza uralensis* Fisher (Leguminosae)]; perilla herb, 2.0 g [*Perilla frutescens* Britton var. *acuta* Kudo (Labiatae)]; ginger rhizome, 1.0 g [*Zingiber officinale* Roscoe (Zingiberaceae)]. Authentic magnolol and honokiol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). TMS and deuterated $CHCl_3$ were purchased from Merck, KGaA (Darmstadt, Germany). All other reagents were of the highest purity available from commercial sources. Precoated Si gel plates (60F$_{254}$; 0.25 mm and 0.5 mm in thickness) for TLC and Si gel 60 (70−230 mesh) for column chromatography were obtained from Merck, KGaA.

Fractionation of Saiboku-to. Saiboku-to (60 g) was suspended in 500 mL of hot H_2O (60 °C) for 30 min and centrifuged at 3000 rpm (precipitate, fraction F1; yield, 8.3 g, 14%). Sufficient EtOH was then added to the aqueous layer to produce a 60% EtOH solution. The insoluble material that precipitated (F2; yield 17.7 g, 30%) was separated by centrifugation and dried *in vacuo*. Residual EtOH was removed from the solution by a stream of nitrogen, and the aqueous layer was extracted with one-fourth volume of $CHCl_3$. Separation of the two phases followed by solvent evaporation *in*

vacuo gave the following fractions: aqueous layer (F3; yield 26.3 g, 44%); organic layer (F4; yield, 0.61 g, 1.02%).

From TLC on Si gel plates (CHCl$_3$−MeOH, 4:1, v/v) with visualization by 10% H$_2$SO$_4$, four distinct components (R_f 0.00, 0.10, 0.42, and 0.60) were detected in F4. Isolation of these components was accomplished by Si gel column chromatography starting with 5.5 g of Saiboku-to fraction F4 and using 500-mL portions of the following solvents: CHCl$_3$ (F4-1; yield 0.63 g, 11.5%); CHCl$_3$−MeOH, 30:1 (F4-2; 1.29 g, 23.5%); CHCl$_3$−MeOH, 10:1 (F4-3; 2.79 g, 50.9%); and CHCl$_3$−MeOH, 3:2 (F4-4; 0.30 g, 5.5%).

EIMS analyses−magnolol: m/z [M]$^+$ 266 (100), 247 (14), 237 (18), 225 (15), 207 (11), 197 (17), 184 (19), 165 (7), 115 (7), 77 (6), 41 (8). **Honokiol**: 266 (100), 237 (13), 224 (8), 210 (5), 197 (8), 184 (10), 165 (4), 152 (3), 115 (3), 77 (2), 41 (2).

Animals. Male ddY mice, 6 weeks of age, weighing 25−28 g were obtained from Japan Laboratory Animals (Tokyo). Randomly chosen mice were housed 10 to a polycarbonate cage (20 cm × 25 cm × 15 cm) and maintained on a 12-h light cycle starting at 6:00 am. The mice were allowed free access to water and solid food (MF; Oriental Yeast, Tokyo). No anxiolytic response was detected in the vehicle-treated animals.

Treatment. Test substances were dissolved in 0.1 mL of EtOH and diluted with the appropriate volume of 0.1% aqueous Tween-80 so that doses would be administered in 0.1 mL per 10 g of body weight. Mice were treated orally (between 9 am and 12 pm) for 7 days with either vehicle or test substance. Anxiolytic activity was assessed on day 8.

Measurement of Anxiolytic Activity. The elevated plus-maze for mice used in this study was constructed in our laboratory[6,10] through modification of the design initially described by Pellow and associates[8] for use with rats. Briefly, the plus-maze, which was maintained at a 40-cm elevation, consisted of four arms (6 cm × 30 cm, each) that extended from a central platform (8 cm × 8 cm). Two of the arms had side walls, with both the floor and side walls nontransparent and painted gray. The other two arms had no side walls and the floor was transparent. The central platform was nontransparent gray. For testing, a mouse was placed on the central platform, oriented randomly toward one of the walled arms. During the ensuing 5-min period, the cumulative time that the mouse entered either of the open-sided arms was recorded. The mouse was considered to have entered an open-sided arm if all four paws crossed the border between the central platform and the open arm. Statistical significance was assessed by means of the Mann−Whitney U-test.

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