

## Online Structural Elucidation of Alkaloids and Other Constituents in Crude Extracts and Cultured Cells of *Nandina domestica* by Combination of LC-MS/MS, LC-NMR, and LC-CD Analyses

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The combination of NMR, MS, and CD data permitted the structural elucidation including the absolute configuration of the known alkaloids and unknown components in the extract matrix solution of *Nandina domestica* without isolation and sample purification prior to the coupling experiments. Unstable natural stereoisomers were identified by LC-NMR and LC-MS. Five known alkaloids, (*S*)-isoboldine, (*S*)-domesticine, (*S*)-nantenine, sinoacutine, and menispermene, were identified from *N. domestica*. *O*-Methylpallidine and (*E,E*)-, (*E,Z*)-, and (*Z,Z*)-terrestribisamide were also characterized for the first time from this plant. Known jatrorrhizine, palmatine, and berberine and unknown (*R*)-carnegine and (*E,E*)-, (*E,Z*)-, and (*Z,Z*)-terrestribisamide were identified in the callus of *N. domestica*.

*Nandina domestica* Thumb. grows wild in Japan and China.<sup>1</sup> In Japan, the fruits of this plant have long been used to treat asthma, whooping cough, pharyngeal tumors, and uterine bleeding.<sup>2</sup> In China, other parts of the plant, e.g., stems and leaves, have also been used as medicines.<sup>3</sup> Shoji et al.<sup>2</sup> reported that the alkaloid nantenine is a serotonergic receptor antagonist. The fruits, seeds, roots, and stem bark contain aporphine-, promorphine-, protoberberine-, and protopine-type alkaloids.<sup>4–18</sup> Twelve protoberberines and magnoflorine have been identified from tissue cultures of *N. domestica*.<sup>19–22</sup>

Application of LC-NMR combined with LC-MS to drug metabolism, identification of natural products in crude plant extracts and the characterization of isomeric mixtures prepared by chemical reactions have been summarized.<sup>23–25</sup> The coupling of HPLC to a CD detector is one of the most powerful hyphenated techniques for stereochemical investigation.<sup>26</sup> The combination of three online coupling methods, LC-NMR, LC-MS, and LC-CD, permitted determination of the structures including absolute configuration of the natural products in crude plant extracts without the necessity of isolation and purification.<sup>27,28</sup>

In this report, we describe the characterization of components, particularly alkaloids, in crude extracts of intact plants and tissue cultures of *N. domestica* using LC-MS-MS, LC-NMR, and LC-CD analyses.

### Results and Discussion

The ground parts of *N. domestica* were extracted to give Plant Fr. E and Plant Fr. C (see Experimental Section), which were subjected to LC-NMR, LC-MS, and LC-CD analyses. In the LC-MS chromatogram of Plant Fr. E (Figure 1), peaks a<sub>1</sub>, b<sub>1</sub>, c<sub>1</sub>, and d<sub>1</sub> showed protonated molecular ions [M + 1]<sup>+</sup> at *m/z* 328 (a<sub>1</sub> and b<sub>1</sub>), 326 (c<sub>1</sub>), and 340 (d<sub>1</sub>), respectively. The stopped-flow <sup>1</sup>H NMR spectrum of peak a<sub>1</sub> showed three aromatic proton singlets at δ 8.02, 6.83, and 6.77, two methoxy groups at δ 3.85 and 3.82, and an *N*-methyl group at δ 2.99. From these data, the compound associated with peak a<sub>1</sub> was identified as isoboldine (Figure 2, A).

NOESY spectroscopic data (Figure 2) also supported this structure. Similarly, the compounds associated with peaks b<sub>1</sub> and c<sub>1</sub> were identified as sinoacutine and domesticine, respectively (Figure 2, B and C), on the basis of their stopped-flow <sup>1</sup>H NMR [peak b<sub>1</sub> displayed two aromatic proton doublets (*J* = 8.0 Hz) at δ 6.89 and 6.71, two olefinic proton singlets at δ 7.78 and 6.46, two methoxy groups at δ 3.76 and 3.66, an *N*-methyl group at δ 2.70; c<sub>1</sub> showed three aromatic proton singlets at δ 7.85, 6.84, and 6.76, a methylenedioxy group at δ 5.93, a methoxy group at δ 3.81, and an *N*-methyl group at δ 2.95] and NOESY data (Figure 2). The stopped-flow <sup>1</sup>H NMR spectrum of peak d<sub>1</sub> displayed three aromatic proton singlets at δ 7.77, 6.86, and 6.81, a methylenedioxy group at δ 5.96, two methoxy groups at δ 3.81 and 3.58, and an *N*-methyl group at δ 2.87. The compound corresponding to peak d<sub>1</sub> was identified as nantenine by comparison of its LC-NMR spectrum (Figure 2, D) with that of domesticine (Figure 2, C). Isoboldine, sinoacutine, domesticine, and nantenine have been isolated previously from *N. domestica*.<sup>4–8,10–18</sup>

The absolute configuration at C-6a of the isolated aporphine alkaloids (isoboldine, domesticine, and nantenine) were determined from LC-CD analysis. Initially, CD spectra of commercially available (*S*)-boldine and (*S*)-isocorydine in the stopped-flow mode were recorded at 220–420 nm to find a suitable wavelength for the measurement of LC-CD spectra. (*S*)-Boldine and (*S*)-isocorydine showed a positive CD sign near 240 nm (Figure 3). A wavelength of 236 nm was used in subsequent LC-CD measurements. In an LC-CD spectrum of Plant Fr. E measured under the same conditions, peaks a<sub>1</sub>, c<sub>1</sub>, and d<sub>1</sub> corresponding to isoboldine, domesticine, and nantenine, respectively, had a positive CD sign (Figure 4). Therefore, the absolute configuration at C-6a of these alkaloids was identified as *S*. The proaporphine-type alkaloid sinoacutine (peak b<sub>1</sub>) showed a negative CD sign.

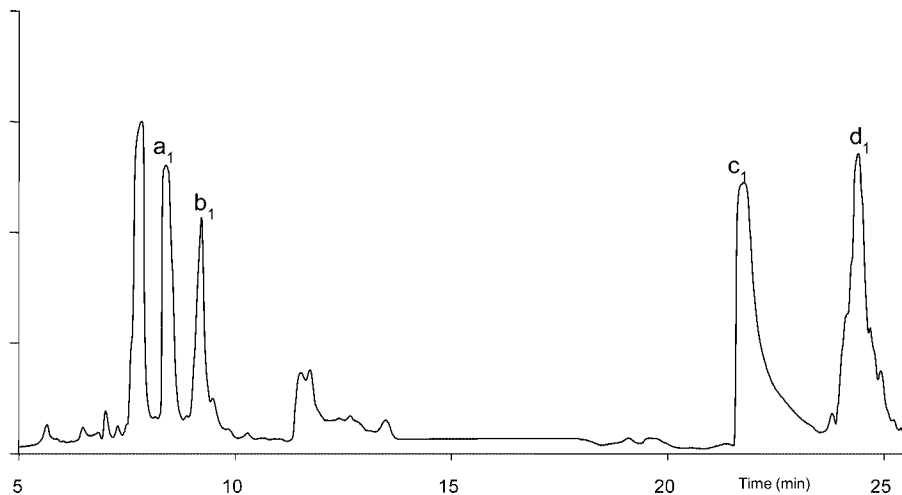
LC peaks a<sub>2</sub>–e<sub>2</sub> of Plant Fr. C (Figure 5) exhibited protonated molecular ions [M + 1]<sup>+</sup> or molecular ions [M]<sup>+</sup> of *m/z* 356 (a<sub>2</sub>), *m/z* 342 (b<sub>2</sub>), *m/z* 328 (c<sub>2</sub>), *m/z* 441 (d<sub>2</sub>), and *m/z* 336 (e<sub>2</sub>). The stopped-flow <sup>1</sup>H NMR spectrum of peak a<sub>2</sub> contained a singlet aromatic proton at δ 7.02, two aromatic proton doublets (*J* = 8.0 Hz) at δ 7.04 and 6.99, three methoxy groups at δ 3.86, 3.80, and 3.60, and two *N*-methyl groups at δ 3.28 and 2.89 (Figure 6, A). The compound associated with peak a<sub>2</sub> was identified as menispermene by comparison of LC-NMR data with those of magnoflorine. NOESY data (Figure 6) supported this structure.

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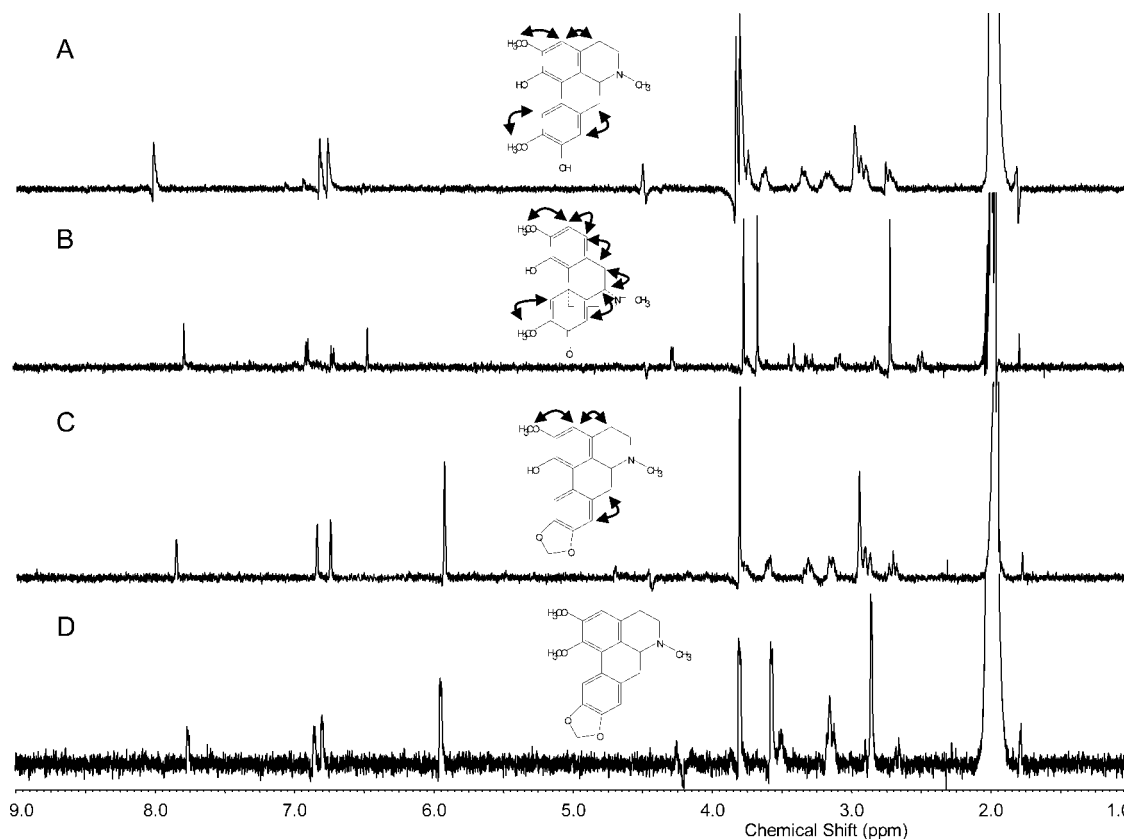
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**Figure 1.** LC data of the alkaloid fraction (Plant Fr. E) obtained from *N. domestica*. Column: Cosmosil 5C-AR-II (4.6 × 150 mm). Eluent: A: 0.1 M NH<sub>4</sub>OAc/D<sub>2</sub>O (0.05% TFA); B: MeCN (0.05% TFA). Gradient A/B: initial 90/10, 5 min 75/25, 15 min 75/25, 30 min 0/100. Flow rate: 1.0 mL/min. UV detector: 280 nm.

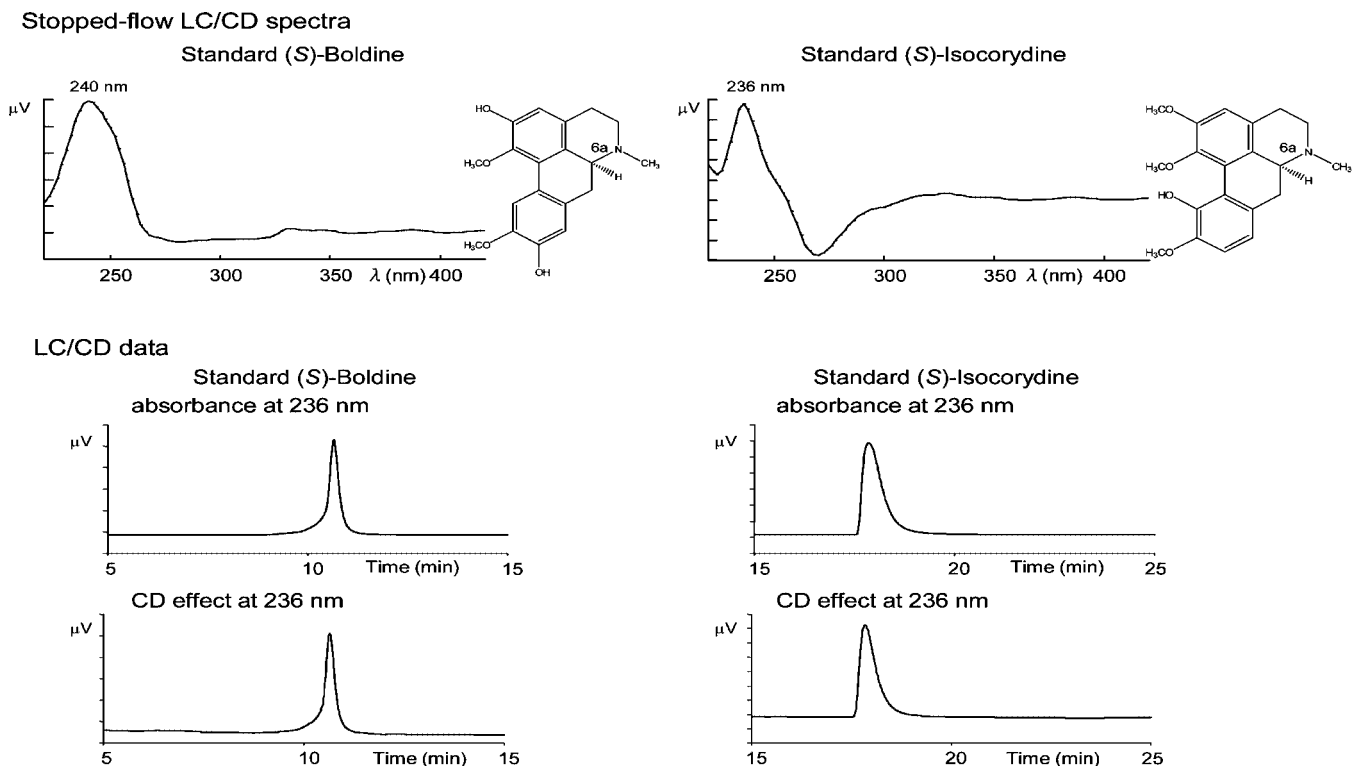


**Figure 2.** Stopped-flow LC-<sup>1</sup>H NMR spectra and NOESY data of major components in the alkaloid fraction (Plant Fr. E) obtained from *N. domestica*. (A) <sup>1</sup>H NMR spectrum of peak a<sub>1</sub> (isoboldine). (B) <sup>1</sup>H NMR spectrum of peak b<sub>1</sub> (sinoacutine). (C) <sup>1</sup>H NMR spectrum of peak c<sub>1</sub> (domesticine). (D) <sup>1</sup>H NMR spectrum of peak d<sub>1</sub> (nantenine).

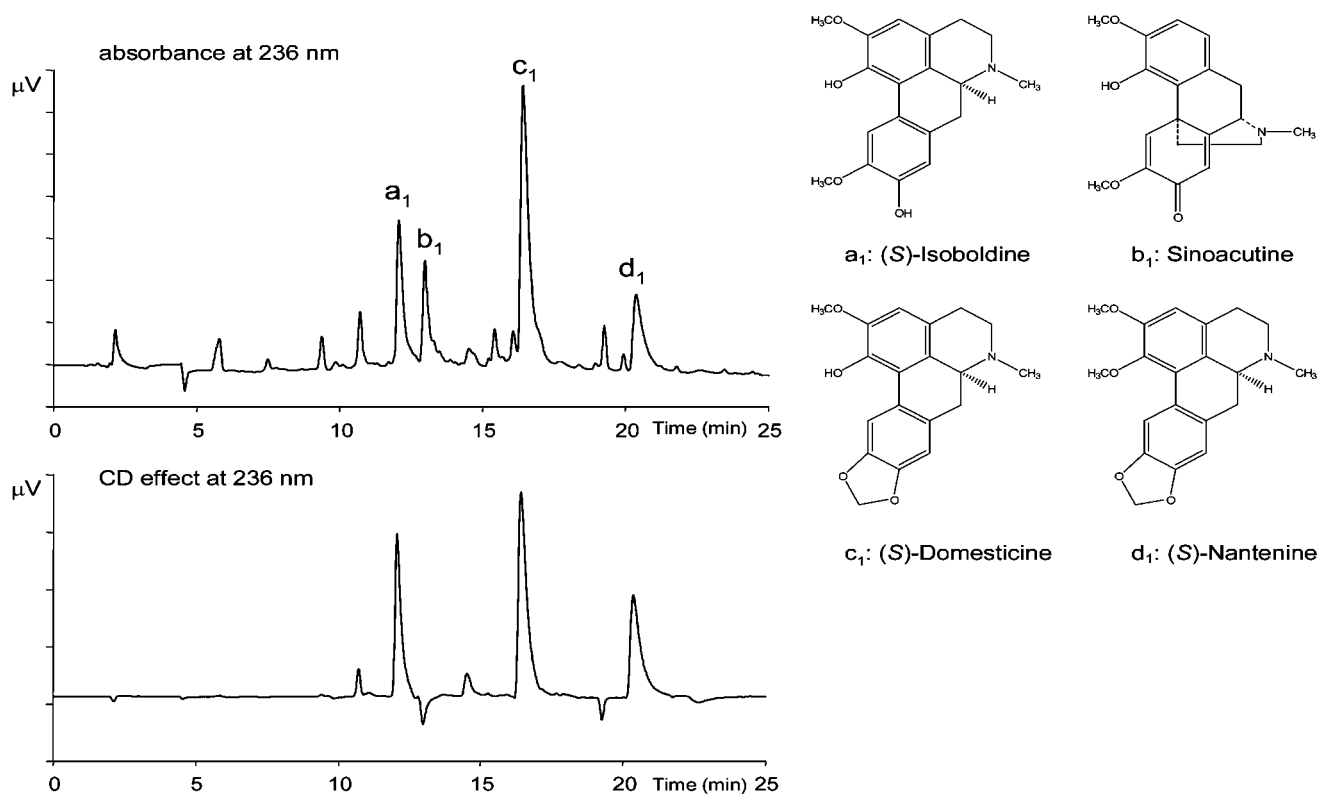
The LC-NMR spectrum of peak b<sub>2</sub> exhibited two aromatic proton singlets at δ 7.07 and 6.83, two singlet olefinic protons at δ 6.93 and 6.50, three methoxy groups at δ 3.80, 3.79, and 3.67, and one *N*-methyl group at δ 2.74 (Figure 6, C). The compound associated with peak b<sub>2</sub> was recognized as *O*-methylpallidine by comparison of LC-NMR data with those of sinoacutine. NOESY data (Figure 6) supported this structure.

Peak d<sub>2</sub> displayed a protonated molecular ion at *m/z* 441 and product ion at *m/z* 177. The stopped-flow <sup>1</sup>H NMR spectrum of peak d<sub>2</sub> showed an aromatic proton singlet at δ 7.12, two aromatic proton doublets (*J* = 8.0 Hz) at δ 7.04 and 6.82, two olefinic proton

doublets (*J* = 16.0 Hz) at δ 7.35 and 6.41, a methoxy group at δ 3.80, and two methylene proton singlets at δ 3.23 and 1.53 (Figure 7, Table 1). After preparative HPLC of Plant Fr. C, HR-SIMS analysis of the compound corresponding to peak d<sub>2</sub> gave a molecular formula of C<sub>24</sub>H<sub>28</sub>N<sub>2</sub>O<sub>6</sub> with a molecular ion at *m/z* 440 and fragment ions at *m/z* 265 and 177. <sup>13</sup>C NMR and DEPT spectra showed only 12 carbons: a methyl at δ 56.36, two methylenes at δ 40.14 and 27.92, five methines at δ 142.02, 123.15, 118.73, 116.46, and 111.53, three quaternary carbons at δ 149.82, 149.27, and 128.25, and a carbonyl at δ 169.23 (Figure 8 and Table 2). Thus, on the basis of HR-SIMS and <sup>13</sup>C NMR data, this compound is



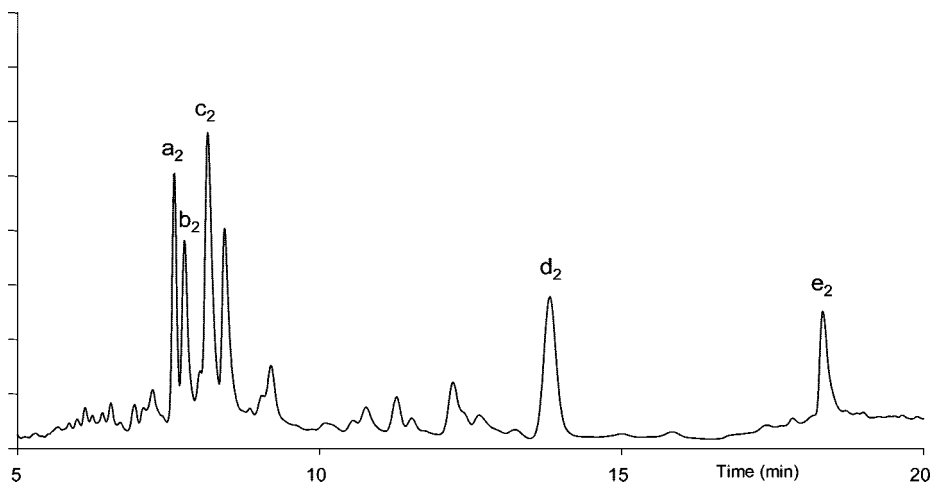
**Figure 3.** Stopped-flow LC-CD spectra and LC-CD data of (S)-boldine and (S)-isocorydine. Column: Cosmosil 5C<sub>18</sub>-AR-II (6.0 × 150 mm). Gradient: A: 0.1 M NH<sub>4</sub>OAc (0.05% TFA); B: MeCN (0.05% TFA). A/B: initial 100/0, 40 min 0/100. Flow rate: 2.0 mL/min. Temp: 40 °C.



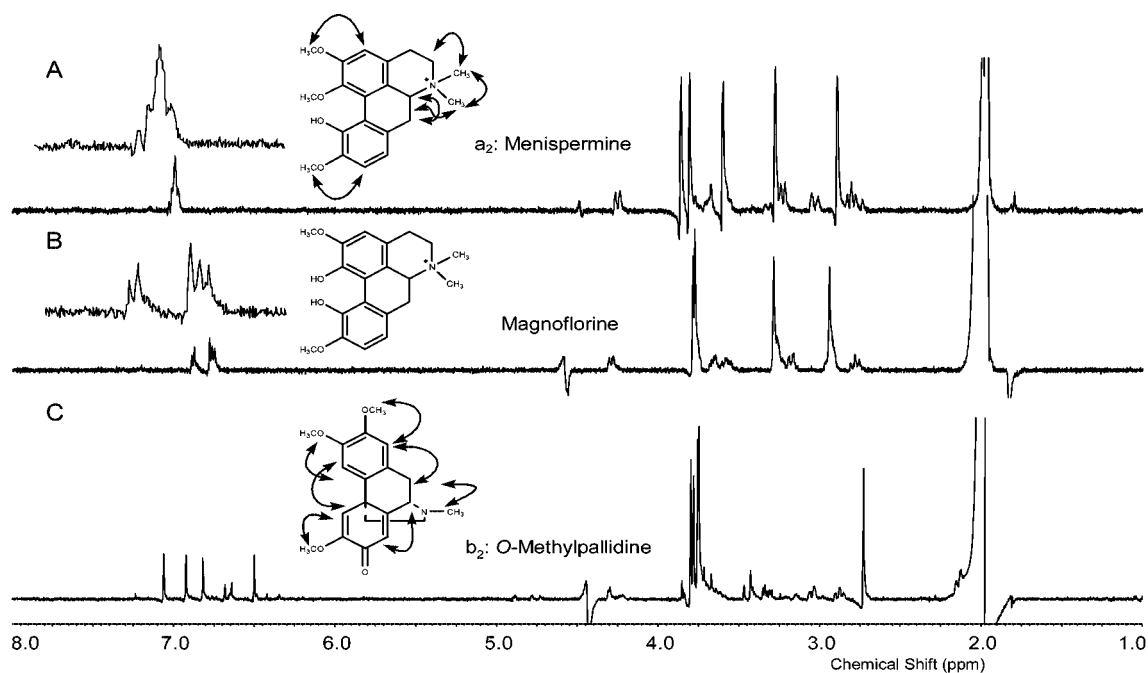
**Figure 4.** LC-CD data of the alkaloid fraction (Plant Fr. E) obtained from *N. domestica*. Column: Cosmosil 5C<sub>18</sub>-AR-II (6.0 × 150 mm). Gradient: A: 0.1 M NH<sub>4</sub>OAc (0.05% TFA); B: MeCN (0.05% TFA). A/B: initial 100/0, 40 min 0/100. Flow rate: 2.0 mL/min. Temp: 40 °C. UV detector: 236 nm.

dimeric. The structure corresponding to peak d<sub>2</sub> was identified as terretribisamide. Compositions of C<sub>14</sub>H<sub>21</sub>N<sub>2</sub>O<sub>3</sub> and C<sub>10</sub>H<sub>9</sub>O<sub>3</sub> for

HR-SIMS fragment ions *m/z* 265 and 177, respectively, supported this structure (Scheme 1). The coupling constant (16 Hz) of the



**Figure 5.** LC data of the alkaloid fraction (Plant Fr. C) obtained from *N. domestica*. Column: Cosmosil 5C<sub>18</sub>-AR-II (4.6 × 150 mm). Eluent: A: 0.1 M NH<sub>4</sub>OAc/D<sub>2</sub>O (0.05% TFA); B: MeCN (0.05% TFA). Gradient A/B: initial 90/10, 5 min 75/25, 15 min 75/25, 30 min 0/100. Flow rate: 1.0 mL/min. UV detector: 280 nm.



**Figure 6.** Stopped-flow LC-<sup>1</sup>H NMR spectra of peaks a<sub>2</sub> and b<sub>2</sub> in LC of the alkaloid fraction (Plant Fr. C) obtained from *N. domestica* and magnoflorine. (A) <sup>1</sup>H NMR spectra of peak a<sub>2</sub> (menispermine). (B) <sup>1</sup>H NMR spectra of standard magnoflorine. (C) <sup>1</sup>H NMR spectra of peak b<sub>2</sub> (*O*-methylpallidine).

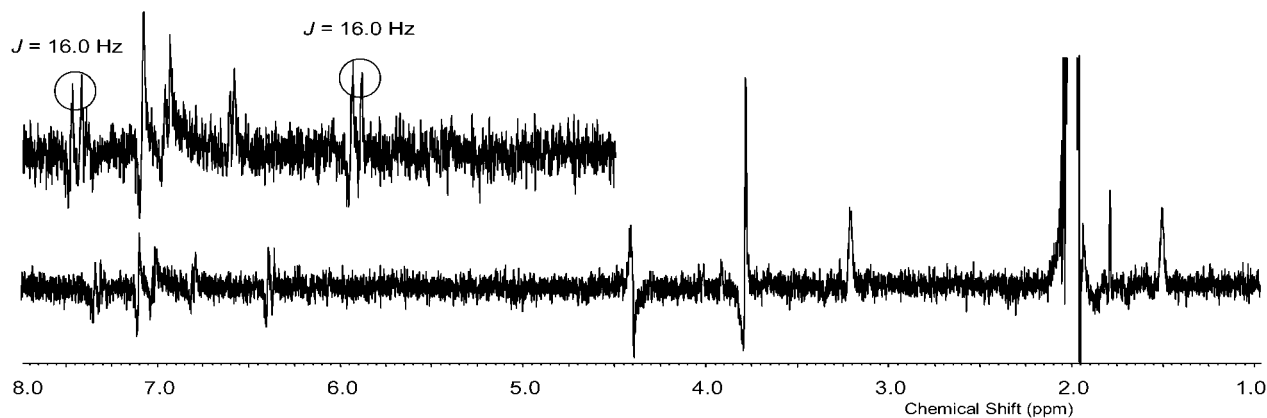
olefinic protons indicated that both double bonds have an *E* configuration. HMBC data (Table 2) were also consistent with identification of this compound (peak d<sub>2</sub>) as (*E,E*)-terrestribisamide.

The LC-NMR spectrum of peak e<sub>2</sub> exhibited four aromatic proton singlets at  $\delta$  9.60, 8.55, 7.54, and 6.94, two aromatic proton doublets ( $J = 9.0$  Hz) at  $\delta$  8.03 and 7.95, a methylenedioxy group at  $\delta$  6.06, and two methoxy groups at  $\delta$  4.06 and 4.04. The compound associated with peak e<sub>2</sub> was determined to be berberine (identical with the <sup>1</sup>H NMR spectrum of C in Figure 15) by comparison of LC-NMR data with those of an authentic sample.

By using LC-NMR, LC-MS/MS, and LC-CD techniques, we have identified four known alkaloids, (*S*)-isoboldine, (*S*)-domesticine, (*S*)-nantenine, sinoacutine, and menispermine, from *N. domestica*. *O*-Methylpallidine and (*E,E*)-, (*E,Z*)-, and (*Z,Z*)-terrestribisamide were also characterized for the first time from this plant.

Calli of *N. domestica* were derived from the stems of wild plants grown in Kobe (Japan) and cultured in the dark on Murashige and Skoog's medium. Methanol extracts of callus cultured for 5–6

weeks were extracted to give Callus Frs. E and C (see Experimental Section), which were subjected to LC-NMR, LC-MS, and LC-CD analyses. In the LC of Callus Fr. E (Figure 9), peaks a<sub>3</sub>, b<sub>3</sub>, c<sub>3</sub>, and d<sub>3</sub> exhibited protonated molecular ions at  $m/z$  222 (a<sub>3</sub>) and 441 (b<sub>3</sub>, c<sub>3</sub>, and d<sub>3</sub>). The stopped-flow <sup>1</sup>H NMR spectrum of peak a<sub>3</sub> showed the presence of two aromatic proton singlets at  $\delta$  6.82 and 6.76, two methoxy groups at  $\delta$  3.77 (6H, s), and one methyl doublet ( $J = 6.0$  Hz) at  $\delta$  1.56. The compound associated with peak a<sub>3</sub> was determined to be carnegine by comparison of its LC-NMR data with those of an authentic sample (Figure 10). The absolute configuration at C-1 of carnegine was predicted from LC-CD analysis. (*R*)- and (*S*)-Carnegine were resolved with L-(+)- and D-(-)-tartaric acid and showed negative and positive CD signs, respectively, in LC-CD spectra on Chiralcel OD-RH (Figure 11). An LC-CD spectrum of Callus Fr. E was measured under the same conditions. The peak corresponding to carnegine had a negative CD sign (Figure 11); therefore, the absolute configuration at C-1 of the isolated alkaloid was determined to be *R*.



**Figure 7.** Stopped-flow LC- $^1\text{H}$  NMR spectrum of peak  $d_2$  in LC of the alkaloid fraction (Plant Fr. C) obtained from *N. domestica*.

**Table 1.** Stopped-Flow  $^1\text{H}$  NMR and HMBC Data of (*E,E*)- and (*Z,Z*)-Terrestribisamide

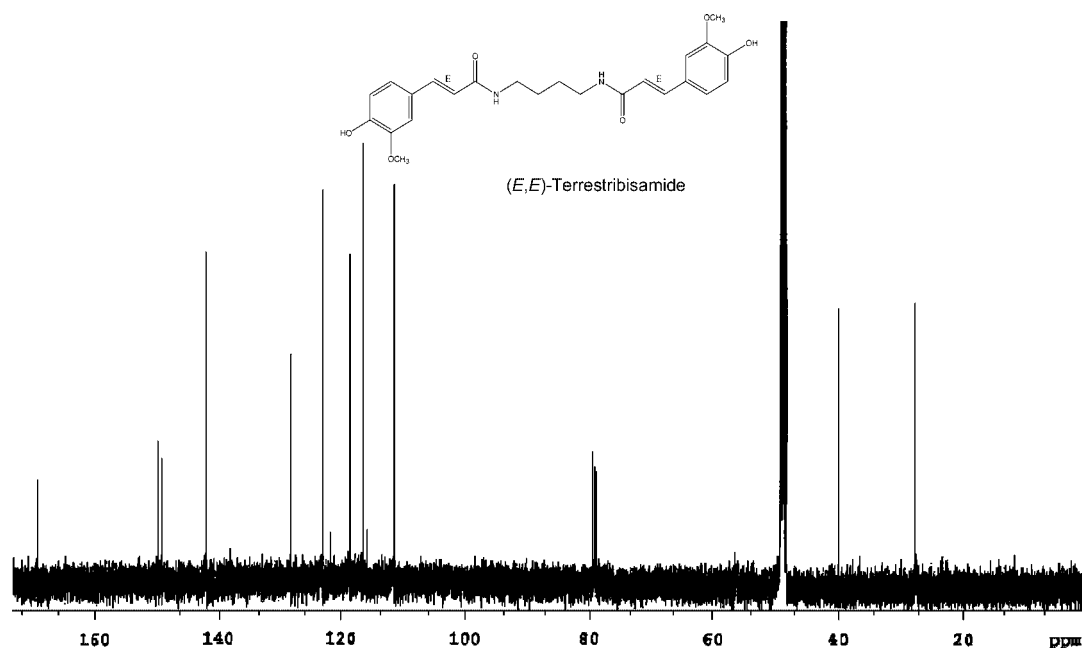
protons	( <i>E,E</i> )-terrestribisamide	( <i>Z,Z</i> )-terrestribisamide
2-H	7.12	7.08
5-H	6.82 <sup>a</sup>	6.75 <sup>c</sup>
6-H	7.04 <sup>a</sup>	6.83 <sup>c</sup>
7-H	7.35 <sup>b</sup>	6.63 <sup>d</sup>
8-H	6.41 <sup>b</sup>	5.83 <sup>d</sup>
10-H	3.23	3.07
11-H	1.53	1.28
3-OCH <sub>3</sub>	3.80	3.73

<sup>a</sup>  $d J = 8.0$  Hz. <sup>b</sup>  $d J = 16.0$  Hz. <sup>c</sup>  $d J = 7.5$  Hz. <sup>d</sup>  $d J = 12.0$  Hz.

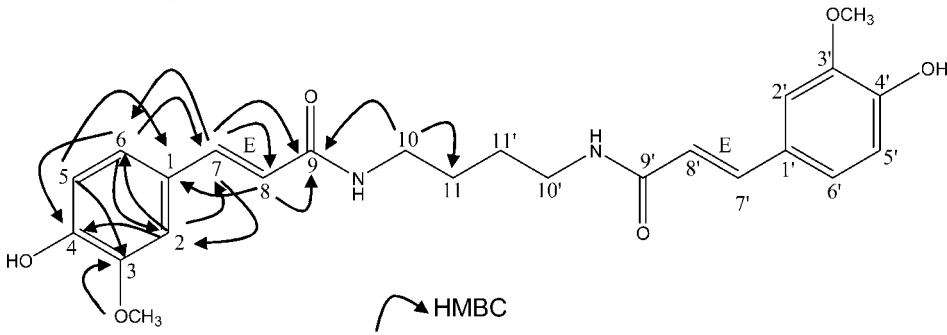
The compounds associated with peaks  $b_3$ – $d_3$  had the same protonated molecular ion ( $m/z$  441) and, thus, were isomers of terrestribisamide with different double-bond configurations. The compound corresponding to peak  $d_3$  had identical data to those described above for (*E,E*)-terrestribisamide. The stopped-flow  $^1\text{H}$  NMR spectrum of peak  $b_3$  showed an aromatic proton singlet at  $\delta$  7.08, two aromatic proton doublets ( $J = 7.5$  Hz) at  $\delta$  6.83 and 6.75, two olefinic proton doublets ( $J = 12.0$  Hz) at  $\delta$  6.63 and 5.83, a methoxy group at  $\delta$  3.73, and two methylene proton singlets at  $\delta$  3.07 and 1.28 (Figure 12). The coupling constant (12 Hz) of the olefinic protons indicated that the configuration of both double bonds was *Z*. Thus, the compound responsible for peak  $b_3$  was

identified as (*Z,Z*)-terrestribisamide. The stopped-flow  $^1\text{H}$  NMR spectrum of peak  $c_3$  displayed olefinic signals for both *E* and *Z* double bonds (Figure 13). Therefore, peak  $c_3$  was identified as (*E,Z*)-terrestribisamide.

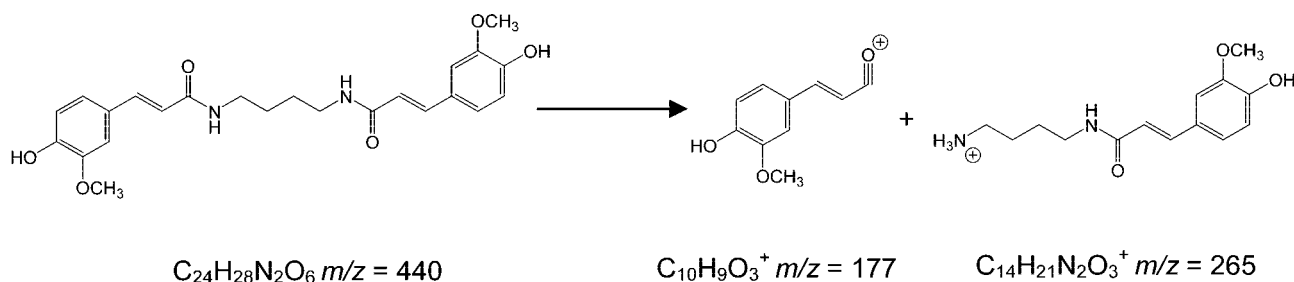
Similarly, in the LC-MS/MS of Callus Fr. C, peaks  $a_4$ ,  $b_4$ , and  $d_4$  (Figure 14) showed the same protonated molecular ion at  $m/z$  441 and product ion at  $m/z$  177. Peaks  $a_4$ ,  $b_4$ , and  $d_4$  were identified as (*Z,Z*)-, (*E,Z*)-, and (*E,E*)-terrestribisamide, respectively. Peaks  $c_4$ ,  $e_4$ , and  $f_4$  in Figure 14 displayed molecular ions at  $m/z$  338, 352, and 336 and product ions at  $m/z$  322, 336, and 320, respectively, in the LC-MS/MS. The stopped-flow  $^1\text{H}$  NMR spectrum of peak  $c_4$  showed four aromatic proton singlets at  $\delta$  9.57, 8.61, 7.55, and 6.88, two aromatic proton doublets ( $J = 9.0$  Hz) at  $\delta$  8.02 and 7.94, and three methoxy groups at  $\delta$  4.05, 4.03, and 3.93 (Figure 15, A). The compound associated with peak  $c_4$  was determined to be jatrorrhizine by comparison of LC-NMR data with those of an authentic sample. The LC-NMR spectrum of peak  $e_4$  exhibited four aromatic proton singlets at  $\delta$  9.60, 8.64, 7.55, and 7.03, two aromatic proton doublets ( $J = 9.5$  Hz) at  $\delta$  8.03 and 7.95, and four methoxy groups at  $\delta$  4.06, 4.03, 3.92, and 3.87 (Figure 15, B). The compound related to peak  $e_4$  was confirmed to be palmatine by comparison of LC-NMR data with those of an authentic sample. The compound corresponding to peak  $f_4$  was



**Figure 8.**  $^{13}\text{C}$  NMR spectrum of (*E,E*)-terrestribisamide.

**Table 2.**  $^{13}\text{C}$  NMR HMBC Data of (*E,E*)-Terrestribisamide


C-1	C	128.25	C-7	CH	142.02
C-2	CH	111.53	C-8	CH	118.73
C-3	C	149.27	C-9	C=O	169.23
C-4	C	149.82	C-10	CH <sub>2</sub>	40.14
C-5	CH	116.46	C-11	CH <sub>2</sub>	27.92
C-6	CH	123.15	C(3)-OCH <sub>3</sub>	CH <sub>3</sub>	56.36

**Scheme 1**

identical with berberine in LC-NMR (Figure 15, C), also recognized in Plant Fr. C.

Accordingly, jatrorrhizine, palmatine, berberine, (*R*)-carnegine, and (*E,E*)-, (*E,Z*)-, and (*Z,Z*)-terrestribisamide have been identified in the callus of *N. domestica*. (*R*)-Carnegine and (*E,E*)-, (*E,Z*)-, and (*Z,Z*)-terrestribisamide were characterized herein for the first time. Identification of unstable (*E,Z*)- and (*Z,Z*)-terrestribisamide formed by *trans*-*cis* isomerization of (*E,E*)-terrestribisamide indicates the usefulness of LC-NMR for structural analysis.

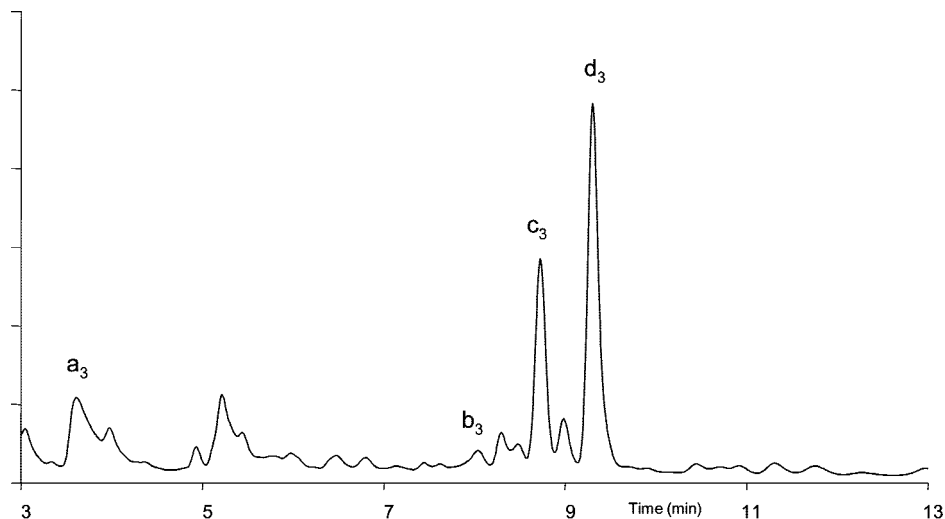
**Experimental Section**

**General Experimental Procedures.** Conventional  $^1\text{H}$  NMR and NOESY spectra were obtained on a Varian VXR-500 spectrometer (500 MHz) in  $\text{CD}_3\text{OD}$  as solvent.  $^{13}\text{C}$  NMR and DEPT spectra were

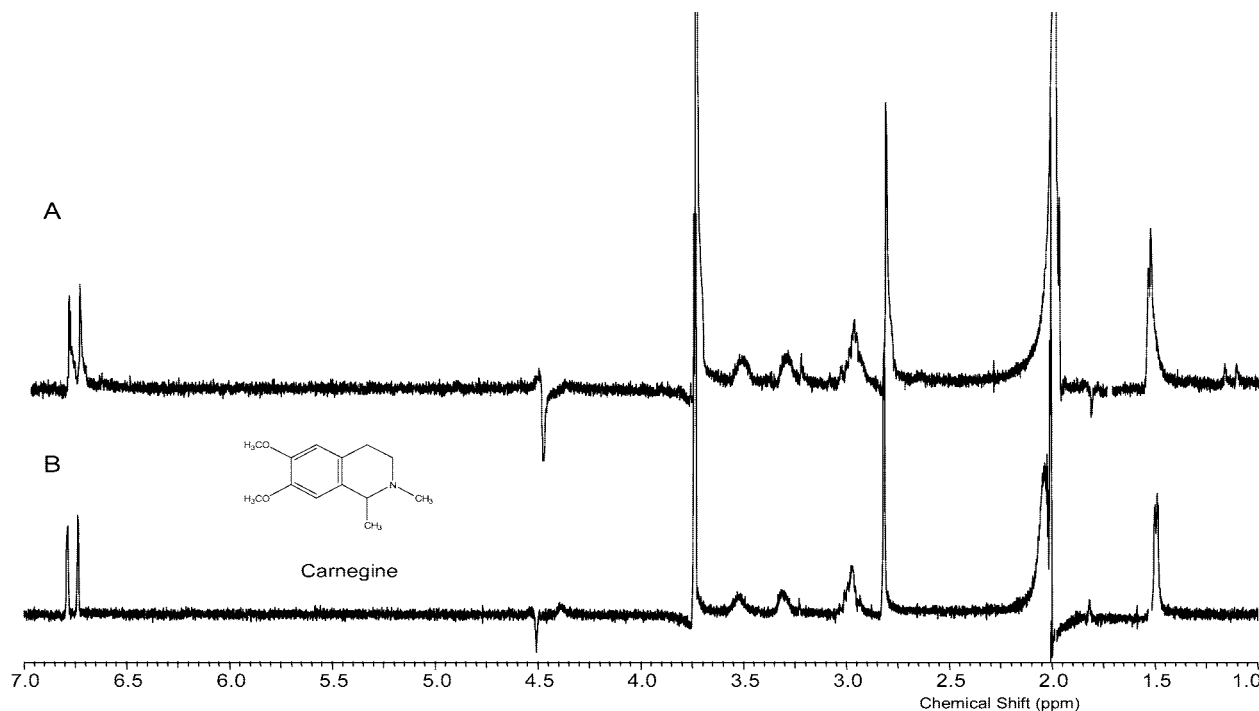
measured on a Varian VXR-500 spectrometer (125 MHz). Mass spectra were determined on a Hitachi M 80 instrument at 75 eV.

**Materials.** In 2003, calli of *N. domestica* were derived from the stems of wild plants grown in Kobe (Japan) on Murashige and Skoog's medium containing 2,4-dichlorophenoxyacetic acid (1 mg/L), kinetin (0.1 mg/L), yeast extract (0.1%), and agar (1%). The callus tissues were subcultured every four or five weeks on fresh medium at 25 °C in the dark.

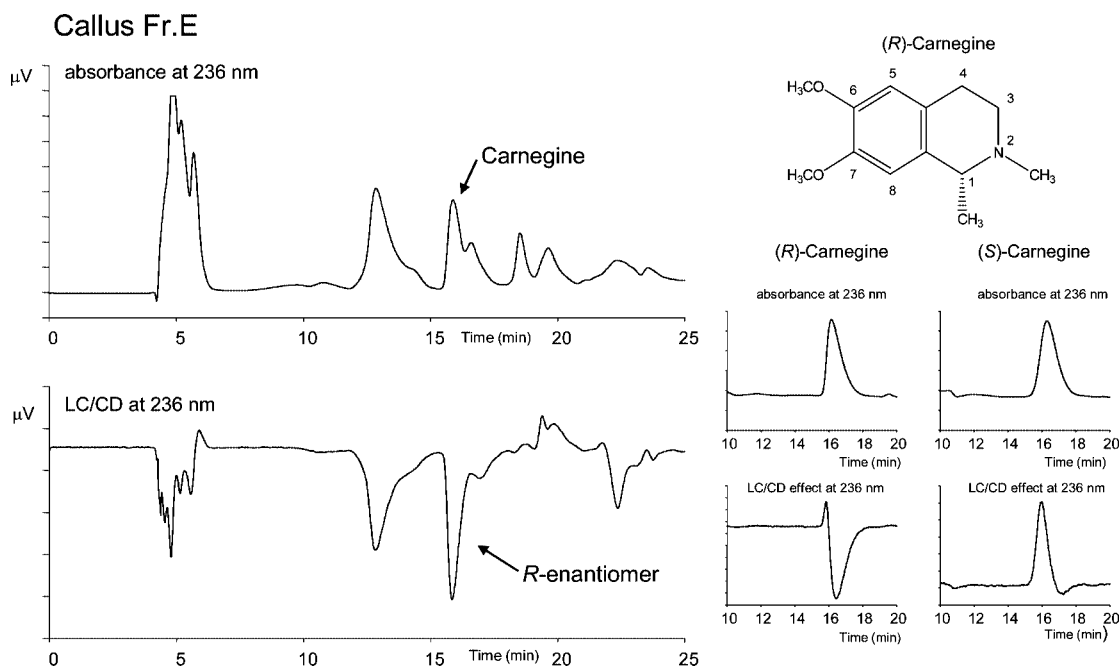
**Extraction of *N. domestica*.** Ground parts of *N. domestica* (217 g) were cut into pieces, extracted several times with MeOH under reflux, and concentrated after addition of  $\text{H}_2\text{O}$ . Methanol extracts were extracted with 2% HCl several times. The extracts were washed with  $\text{Et}_2\text{O}$ , made basic with  $\text{NH}_4\text{OH}$ , and extracted with  $\text{Et}_2\text{O}$  and then  $\text{CHCl}_3$  to give Plant Frs. E (11.0 mg) and C (10.2 mg), which were subjected to LC-NMR, LC-MS, and LC-CD measurements.



**Figure 9.** LC data of the alkaloid fraction (Plant Fr. E) obtained from *N. domestica*. Column: Cosmosil 5C<sub>18</sub>-AR-II (4.6 × 150 mm). Eluent: A: 0.1 M  $\text{NH}_4\text{OAc}/\text{D}_2\text{O}$  (0.05% TFA); B: MeCN (0.05% TFA). Gradient A/B: initial 80/20, 10 min 60/40, 20 min 60/40, 30 min 0/100. Flow rate: 1.0 mL/min. UV detector: 280 nm.



**Figure 10.** Stopped-flow LC- $^1\text{H}$  NMR spectrum of peak  $a_3$  in LC of the alkaloid fraction (Callus Fr. E) obtained from *N. domestica* and carnegine. (A)  $^1\text{H}$  NMR spectrum of peak  $a_3$  (carnegine). (B)  $^1\text{H}$  NMR spectrum of standard carnegine.



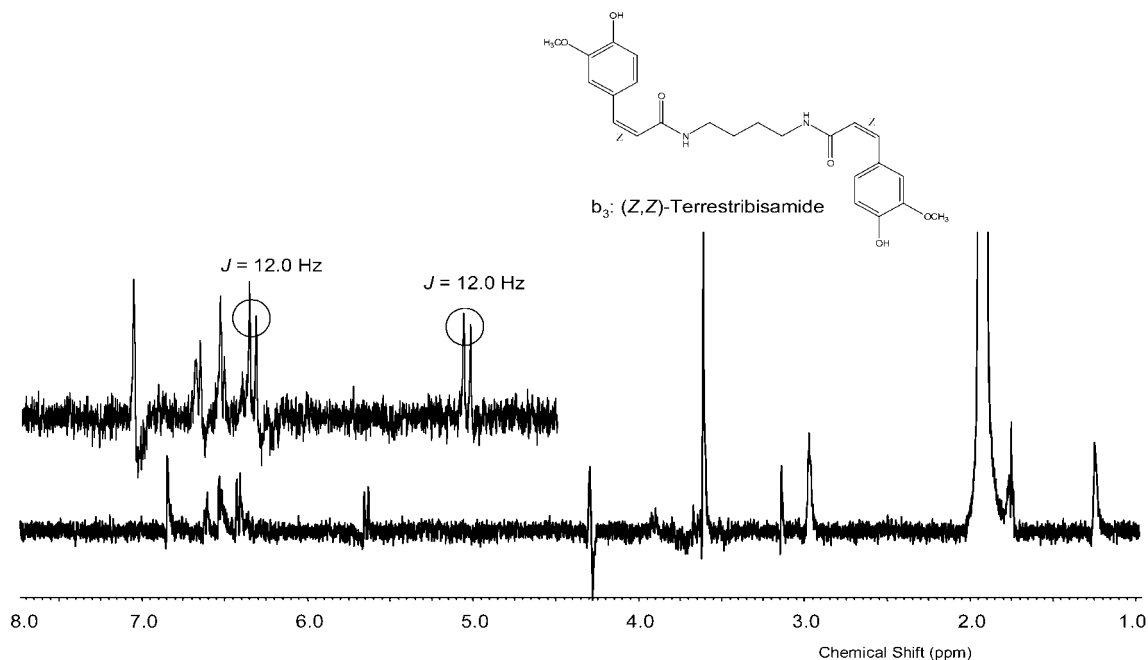
**Figure 11.** LC-CD data of the alkaloid fraction (Callus Fr. E) obtained from *N. domestica* and (*R*) or (*S*)-carnegine. Column: Chiralcel OD-RH ( $4.6 \times 150$  mm). Eluent: A: 0.1 M  $\text{NH}_4\text{OAc}$  (0.05% TFA); B: MeCN (0.05% TFA). Gradient A/B: initial 100/0, 5 min 95/5, 15 min 75/25, 25 min 75/25, 35 min 0/100. Flow rate: 0.5 mL/min. Temp: 40 °C. UV detector: 236 nm.

**Extraction of Callus of *N. domestica*.** After being subcultured for 5–6 weeks, calli (441 g) and medium were freeze-dried. Water was separated, and callus and medium were extracted several times with hot MeOH.  $\text{H}_2\text{O}$  and MeOH extracts were concentrated. The extracts were washed with  $\text{Et}_2\text{O}$  after acidification, made basic with  $\text{NH}_4\text{OH}$ , and extracted with  $\text{Et}_2\text{O}$  and then  $\text{CHCl}_3$  to give Callus Frs. E (15.2 mg) and C (20.6 mg), which were subjected to LC-NMR, LC-MS, and LC-CD measurements.

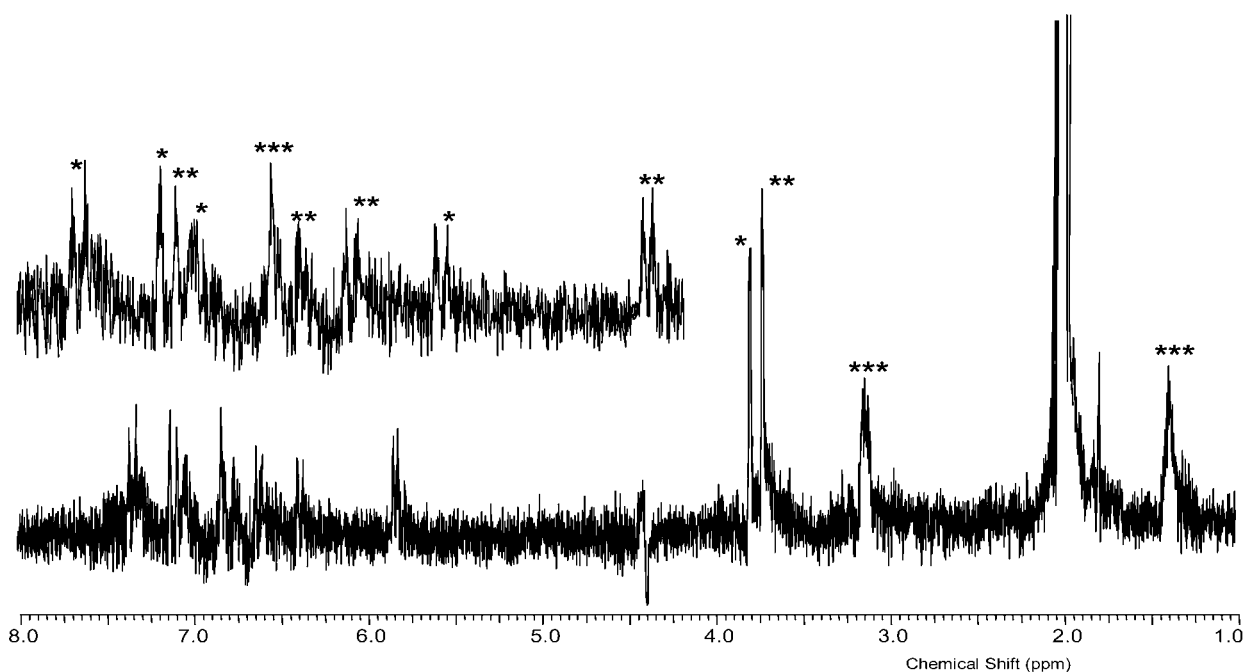
Peak  $d_4$  (*E,E*-terrestribisamide) in LC of Callus Fr. C (Figure 14) was separated by preparative HPLC [Cosmosil 5  $\text{C}_{18}$ -AR ( $20 \times 250$  mm), (A) 0.1 M  $\text{NH}_4\text{OAc}$  (0.05% TFA) and (B)  $\text{CH}_3\text{CN}$  (0.05% TFA):

A/B initial 80/20, 75/25 (5 min), 75/25 (15 min), 0/100 (30); 6 mL/min; 280 nm].

**Optical Resolution of ( $\pm$ )-Carnegine.** A solution of the free base obtained from ( $\pm$ )-carnegine hydrochloride (4.12 g)<sup>29</sup> in  $\text{H}_2\text{O}$  (10 mL) was added to a solution of L-(+)-tartaric acid (2 g) in  $\text{H}_2\text{O}$  (5 mL). The mixture was left in the refrigerator overnight. The resulting white crystals were filtered and washed with cold water to provide the salt [(+)-carnegine $\cdot$ (+)-tartaric acid] (2.72 g, *R/S*: 70/30). This salt (910 mg) was dissolved in  $\text{H}_2\text{O}$  (5 mL) and left in the refrigerator for 3 days. The resulting crystals were filtered, and the collected salt (216 mg, *R/S*: 93/7) was dissolved in  $\text{H}_2\text{O}$ , basified with aqueous  $\text{NH}_3$ , and



**Figure 12.** Stopped-flow LC- $^1\text{H}$  NMR spectrum of peak  $b_3$  in LC of the alkaloid fraction (Callus Fr. E) obtained from *N. domestica*.



**Figure 13.** Stopped-flow LC- $^1\text{H}$  NMR spectrum of peak  $c_3$  in LC of the alkaloid fraction (Callus Fr. E) obtained from *N. domestica*. \*Signals attributed to (*E,E*)-terrestribisamide. \*\*Signals attributed to (*Z,Z*)-terrestribisamide. \*\*\*Signals attributed to (*E,E*)-terrestribisamide and (*Z,Z*)-terrestribisamide.

extracted with  $\text{Et}_2\text{O}$ . The ether extract was dried and evaporated to give (+)-carnegine as an oil (118 mg). Similarly, a solution of the free base obtained from ( $\pm$ )-carnegine hydrochloride (7.50 g) in  $\text{H}_2\text{O}$  (10 mL) was added to a solution of D-(−)-tartaric acid (2.7 g) in  $\text{H}_2\text{O}$  (14 mL). The mixture was left in the refrigerator for 4 days. The resulting white crystals were filtered and washed with cold  $\text{H}_2\text{O}$  to give the salt [(−)-carnegine·(−)-tartaric acid] (3.34 g, *R/S*: 40/60). This salt was dissolved in  $\text{H}_2\text{O}$  (5 mL) and left in the refrigerator for 2 days. The resulting crystals were filtered, and the resulting salt (1.01 g, *R/S*: 4/96) was converted to the free base [(−)-carnegine].

**LC-APCI-MS Method.** LC-APCI-MS (*MS*) was measured on an Applied Biosystems API 3000 triple quadrupole mass spectrometer (*MS/MS*) with a heated nebulizer interface as described in a previous paper.<sup>30</sup>

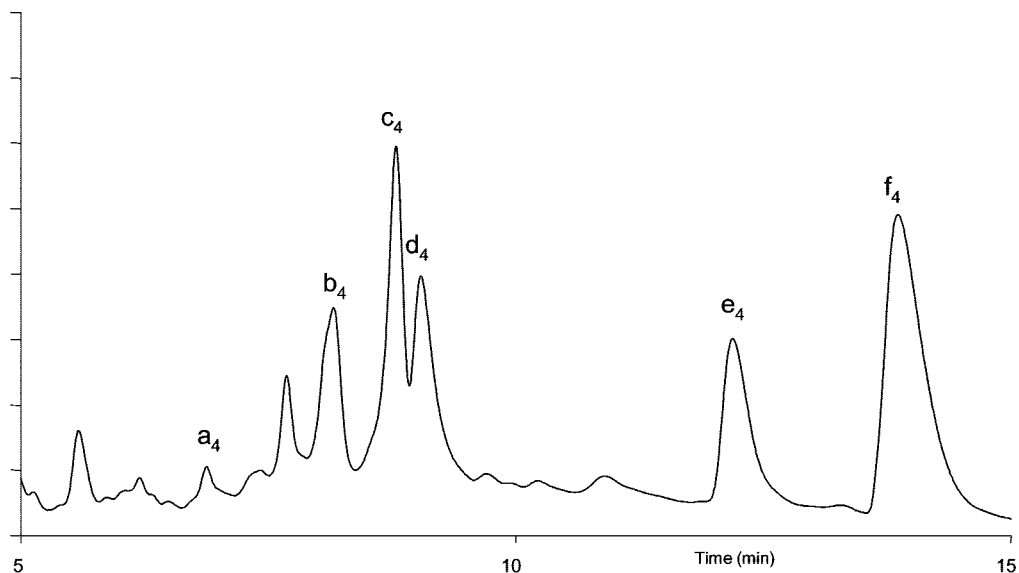
**LC-CD Method.** LC-CD analyses were carried out on a chiral phase column at 236 nm. Chromatographic separations were performed using

a Jasco PU-2080Plus intelligent pump with a column oven (Jasco 860-CO), Jasco Brown NT, HSS-2000 data processor, and Jasco CD-2095Plus CD chiral detector (Hg–Xe lamp), simultaneously monitoring the CD and UV signals at one specific wavelength (range 220–420 nm).

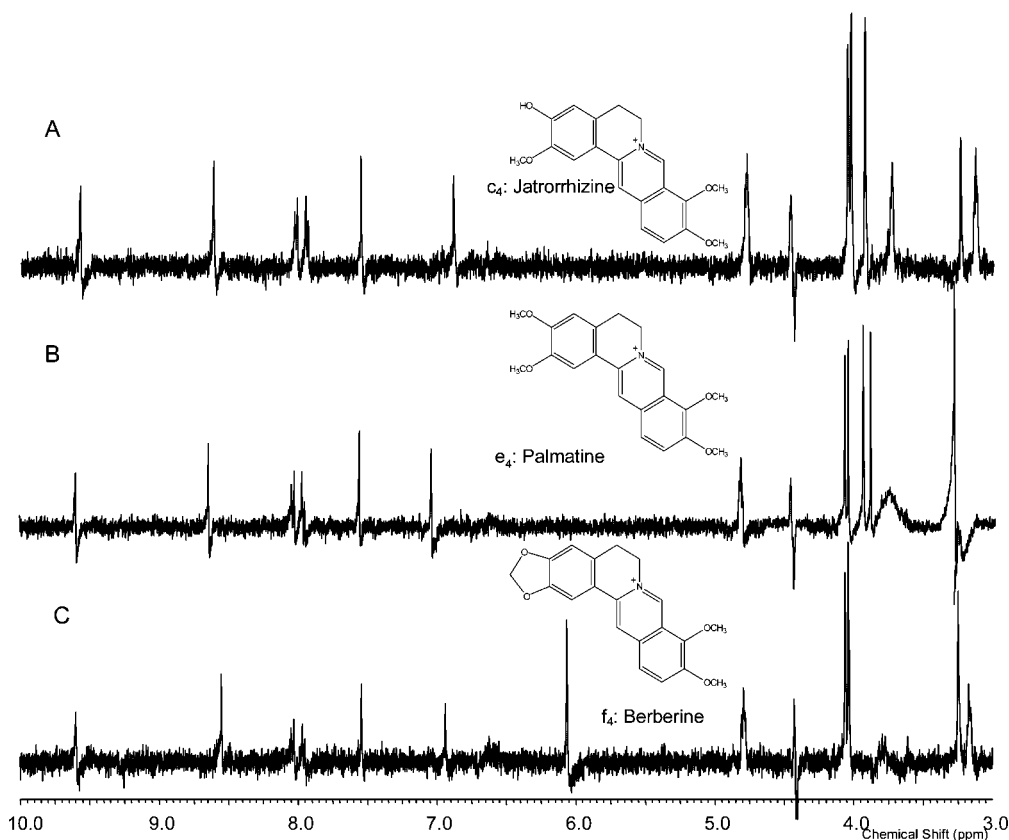
**LC-NMR Method.** LC-NMR data were acquired using a Varian UNITY-INOVA-500 spectrometer ( $^1\text{H}$ : 499.83 MHz) equipped with a 60  $\mu\text{L}$  triple-resonance microflow NMR probe. 1D  $^1\text{H}$  NMR spectra were obtained in stopped-flow mode as described in the previous paper.<sup>30</sup>

LC- $^1\text{H}$  NMR: isoboldine  $\delta$  2.74 (1H, t,  $J$  = 13.5 Hz, H-7), 2.94 (1H, m, H-4), 2.99 (3H, s,  $\text{NCH}_3$ ), 3.18 (1H, m, H-7), 3.22 (1H, m, H-4), 3.36 (1H, m, H-5), 3.65 (1H, m, H-5), 3.82 (3H, s,  $\text{OCH}_3$ -10), 3.85 (3H, s,  $\text{OCH}_3$ -2), 4.12 (1H, m, H-6a), 6.77 (1H, s, H-3), 6.83 (1H, s, H-8), 8.02 (1H, s, H-11); sinoacutine  $\delta$  1.95 (overlap with  $\text{H}_2\text{O}$ ,





**Figure 14.** LC data of the alkaloid fraction (Callus Fr. C) obtained from *N. domestica*. Column: Cosmosil 5C<sub>18</sub>-AR-II (4.6 × 150 mm). Gradient: A: 0.1 M NH<sub>4</sub>OAc/D<sub>2</sub>O (0.05% TFA), B: MeCN (0.05% TFA). A/B: initial 80/20, 5 min 70/30, 15 min 70/30, 25 min 0/100. Flow rate: 1.0 mL/min. UV detector: 280 nm.



**Figure 15.** Stopped-flow LC-<sup>1</sup>H NMR spectra of protoberberine-type alkaloids in the alkaloid fraction (Callus Fr. C) obtained from *N. domestica*. (A) <sup>1</sup>H NMR spectrum of peak c<sub>4</sub> (jatrorrhizine). (B) <sup>1</sup>H NMR spectrum of peak e<sub>4</sub> (palmatine). (C) <sup>1</sup>H NMR spectrum of peak f<sub>4</sub> (berberine).

H-15), 2.49 (1H, m, H-15), 2.70 (3H, s, NCH<sub>3</sub>), 2.81 (1H, m, H-16), 3.07 (1H, m, H-16), 3.29 (1H, dd, *J* = 19.0, 5.5 Hz, H-10), 3.42 (1H, d, *J* = 19.0 Hz, H-10), 3.67 (3H, s, OCH<sub>3</sub>-6), 3.77 (3H, s, OCH<sub>3</sub>-3), 4.26 (1H, d, *J* = 5.5 Hz, H-9), 6.47 (1H, s, H-8), 6.72 (1H, d, *J* = 8.0 Hz, H-1), 6.90 (1H, d, *J* = 8.0 Hz, H-2), 7.78 (1H, s, H-5); domesticine δ 2.73 (1H, t, *J* = 14.0 Hz, H-4), 2.91 (1H, m, H-7), 2.97 (3H, s, NCH<sub>3</sub>), 3.16 (1H, m, H-7), 3.20 (1H, m, H-4), 3.33 (1H, m, H-5), 3.62 (1H, m, H-5), 3.83 (3H, s, OCH<sub>3</sub>), 4.09 (1H, s, H-6a), 5.94 (2H, s, OCH<sub>2</sub>O), 6.76 (1H, s, H-3), 6.86 (1H, s, H-8), 7.87 (1H, s, H-11); nanténine δ 2.66 (1H, t, *J* = 14.0 Hz, H-4), 2.87 (3H, s, NCH<sub>3</sub>), 2.89

(1H, m, H-7), 3.13 (1H, m, H-7), 3.16 (1H, m, H-4), 3.18 (1H, m, H-5), 3.50 (1H, br s, H-5), 3.58 (3H, s, OCH<sub>3</sub>-1), 3.81 (3H, s, OCH<sub>3</sub>-2), 5.96 (2H, s, OCH<sub>2</sub>O), 6.81 (1H, s, H-3), 6.86 (1H, s, H-8), 7.77 (1H, s, H-11); menispermine δ 2.83 (1H, t, *J* = 13.0 Hz, H-7), 2.92 (3H, s, NCH<sub>3</sub>), 3.06 (1H, m, H-4), 3.25 (1H, m, H-7), 3.30 (3H, s, NCH<sub>3</sub>), 3.34 (1H, m, H-4), 3.59 (1H, m, H-5), 3.62 (3H, s, OCH<sub>3</sub>-1), 3.71 (1H, m, H-5), 3.83 (3H, s, OCH<sub>3</sub>-10), 3.88 (3H, s, OCH<sub>3</sub>-2), 4.27 (1H, d, *J* = 13.0 Hz, H-6a), 7.01 (1H, d, *J* = 8.5 Hz, H-8), 7.02 (1H, s, H-3), 7.04 (1H, d, *J* = 8.5 Hz, H-9); *O*-methylpallidine δ 1.95 (overlap with H<sub>2</sub>O, H-15), 2.15 (1H, m, H-15), 2.74 (3H, s, NCH<sub>3</sub>),

2.88 (1H, m, H-16), 3.06 (1H, m, H-16), 3.33 (1H, dd,  $J = 19.0$ , 5.5 Hz, H-10), 3.45 (1H, d,  $J = 19.0$  Hz, H-10), 3.75 (3H, s, OCH<sub>3</sub>-6), 3.76 (3H, s, OCH<sub>3</sub>-2), 3.80 (3H, s, OCH<sub>3</sub>-3), 4.31 (1H, d,  $J = 5.5$  Hz, H-9), 6.50 (1H, s, H-8), 6.83 (1H, s, H-1), 6.93 (1H, s, H-5). 7.07 (1H, s, H-4).

**HPLC Parameters for LC-NMR, LC-MS, and LC-CD.** Chromatographic preparations were performed using a Cosmosil 5 C<sub>18</sub>-AR (4.6 i.d. × 150 mm) reversed-phase column. The mobile phases [(A) 0.1 M NH<sub>4</sub>OAc (0.05% TFA, D<sub>2</sub>O for LC-NMR) and (B) CH<sub>3</sub>CN (0.05% TFA) or CH<sub>3</sub>OH (0.05% TFA)] were used by nonlinear gradient elution. Chiral analytical separation was carried out on a chiral OJ-RH column (4.6 i.d. × 150 mm, Daicel Chemical Ltd.) at 40 °C for LC-CD.

## References and Notes

- (1) *The Wealth of India, Raw Materials*, Vol. VVII; Publication & Information Directorate, CSIR: New Delhi, 1966; p 1.
- (2) Shoji, N.; Umeyama, A.; Takemoto, T.; Ohizumi, Y. *J. Pharm. Sci.* **1984**, *73*, 568–570.
- (3) Ching Su New Medical College. *The Encyclopedia of Chinese Materia Medica*; Shanghai Scientific & Technological Publisher: Shanghai, 1978; p 1564.
- (4) Guinaudeau, H.; Leboeuf, M.; Cave, A. *Lloydia* **1975**, *38*, 275–338.
- (5) Guinaudeau, H.; Leboeuf, M.; Cave, A. *J. Nat. Prod.* **1979**, *42*, 325–60.
- (6) Guinaudeau, H.; Leboeuf, M.; Cave, A. *J. Nat. Prod.* **1983**, *46*, 761–835.
- (7) Moriyasu, M.; Ichimaru, M.; Sawada, Y.; Izutsu, K.; Nishiyama, Y.; Kato, A. *Shoyakugaku Zasshi* **1992**, *46* (2), 143–149.
- (8) Chikamatsu, H.; Tomita, M.; Kotake, M. *Nippon Kagaku Zasshi* **1961**, *82*, 1708–1712.
- (9) Zhu, M.; Xiao, P. *Zhongcaoyao* **1991**, *22* (5), 207–208.
- (10) Kunitomo, J.; Juichi, M.; Yoshikawa, Y.; Masada, Y.; Hashimoto, K.; Inoue, T.; Fujioka, M. *Yakugaku Zasshi* **1974**, *94* (9), 1149–1153.
- (11) Kunitomo, J.; Juichi, M.; Yoshikawa, Y.; Chikamatsu, H. *Yakugaku Zasshi* **1974**, *94* (1), 97–100.
- (12) Kunitomo, J.; Juichi, M.; Yoshikawa, Y.; Chikamatsu, H. *Experientia* **1973**, *29* (5), 518–519.
- (13) Kunitomo, J.; Juichi, M.; Ando, Y.; Yoshikawa, Y.; Nakamura, S.; Shingu, T. *Yakugaku Zasshi* **1975**, *95* (4), 445–447.
- (14) Tomita, M.; Kitamura, T. *Yakugaku Zasshi* **1959**, *79*, 1092–1093.
- (15) Tomita, M.; Fujie, M. *Yakugaku Zasshi* **1962**, *82*, 1457–1458.
- (16) Tomita, M.; Sugamoto, M. *Yakugaku Zasshi* **1961**, *81*, 1090–1093.
- (17) Tomita, M.; Inubushi, Y.; Ishii, S.; Yamagata, M. *Yakugaku Zasshi* **1951**, *71*, 381–385.
- (18) Wu, T.; Shi, L.; Kuo, S. *Phytochemistry* **1999**, *50* (8), 1411–1415.
- (19) Johns, S. R.; Lambertson, J. A.; Sioumis, A. *Aust. J. Chem.* **1966**, *19* (12), 2331–2338.
- (20) Ikuta, A.; Itokawa, H. *Plant Tissue Cell Cult.* **1982**, 315–316.
- (21) Ikuta, A.; Itokawa, H. *Phytochemistry* **1988**, *27* (7), 2143–2145.
- (22) Ikuta, A. *Biotechnol. Agric. For.* **1994**, *26*, 259–268.
- (23) Hostettmann, K.; Potterat, O.; Wolfender, J.-L. *Pharm. Ind.* **1977**, *59*, 339–347.
- (24) Wolfender, J.-L.; Ndjoko, K.; Hostettmann, K. *Curr. Org. Chem.* **1998**, *2*, 575–596.
- (25) Wolfender, J.-L.; Ndjoko, K.; Hostettmann, K. *Phytochem. Anal.* **2001**, *12*, 2–22.
- (26) Berova, N.; Nakanishi, K.; Woody, R. W. *Circular Dichroism*, 2nd ed.; Wiley: New York, 2000.
- (27) Bringmann, G.; Wohlfarth, M.; Rischer, H.; Heubes, M.; Saeb, W.; Diem, S.; Herderich, M.; Schlauer, J. *Anal. Chem.* **2001**, *73*, 2571–2577.
- (28) Kanazawa, H.; Tsubayashi, A.; Nagata, Y.; Matsushima, Y.; Mori, C.; Kizu, J.; Higaki, M. *J. Chromatogr. A* **2002**, *948*, 303–308.
- (29) Cui, W.; Iwasa, K.; Harukuni, T.; Kashihara, A.; Mitani, Y.; Asegawa, T.; Nishiyama, Y.; Moriyasu, M.; Nishino, H.; Hanaoka, M.; Mukai, C.; Takeda, K. *Phytochemistry* **2006**, *67*, 70–79.
- (30) Iwasa, K.; Cui, W.; Sugiura, M.; Takeuchi, A.; Moriyasu, M.; Takeda, K. *J. Nat. Prod.* **2005**, *68* (7), 992–1000.

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