Constituents of the Roots of *Boerhaavia diffusa* L. III. Identification of Ca$^{2+}$ Channel Antagonistic Compound from the Methanol Extract

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Two known ligans, liriodendrin and syringaresinol mono-β-D-glucoside, have been isolated from the methanol extract of the roots of *Boerhaavia diffusa* L. (Nyctaginaceae), and the former compound was found to exhibit a significant calcium (Ca$^{2+}$) channel antagonistic effect in frog heart single cells using the whole-cell voltage clamp method. Reexamination of the carbon-13 nuclear magnetic resonance ($^{13}$C-NMR) spectra of these compounds was also carried out by the use of two-dimensional NMR techniques including the $^1$H-detected heteronuclear multiple bond connectivity (HMBC) experiment, and it was found that the previous signal assignments for C-1' and C-4' have to be revised.

Keywords: calcium channel antagonistic action; *Boerhaavia diffusa*; Nyctaginaceae; liriodendrin; syringaresinol mono-β-D-glucoside; HMBC

In previous papers, we reported the isolation and structure determination of 3-O-(6′-palmitoyl-β-D-glucopyranosyl)isotestrol and three new rotenoids named boeravinones A, B, and C from the ether extract of the roots of *Boerhaavia diffusa* L. (Nyctaginaceae), which is used as a traditional medicine in Nepal, Sri Lanka, India, and East Africa. In a further study, we have examined the constituents of the methanol extract, which showed a calcium (Ca$^{2+}$) channel antagonistic activity in frog heart single cells. This paper deals with the isolation and identification of two known ligans, liriodendrin (I) and syringaresinol mono-β-D-glucoside (2), and the Ca$^{2+}$ channel antagonistic action of compound 1.

The methanol extract of *B. diffusa*, obtained as described in a previous paper, was separated roughly into the chloroform-soluble, butanol-soluble, ethanol-soluble, and ethanol-insoluble fractions (Chart 2). Among these fractions, three (not the ethanol-insoluble one) showed significant Ca$^{2+}$ channel antagonistic effect in an electrophysiological bioassay.

The ethanol-soluble fraction was further separated by a combination of Iatrobeads column chromatography and preparative thin layer chromatography (TLC) to give compound 1 (I) along with glycerol and a complex mixture of sugars. On the other hand, the butanol-soluble fraction was extracted with 1.5% hydrochloric acid solution at room temperature and the insoluble material, which contained a mixture of phenolic compounds, was separated by filtration. Then the aqueous layer was treated as shown in Chart 2, giving an additional crop of compound 1 together with sucrose and a mixture of sugars. The chloroform-soluble fraction was also separated in a similar manner (Chart 2) to give compound 2 (2) together with mixtures of fatty acids and sterol glucosides.

Compound 1 (I) was obtained as colorless needles, mp 256–259°C, and showed [α]$^28_D$ $-13.5^\circ$ (pyridine). It showed characteristic absorption bands at 272 and 281 nm (log ε 3.15 and 3.27, respectively) in the ultraviolet (UV) spectrum, and a strong hydroxyl absorption at 3450 cm$^{-1}$ and aromatic absorptions at 1597, 1510, and 1465 cm$^{-1}$ in the infrared (IR) spectrum. The positive ion fast atom bombardment mass spectrum (FAB-MS) of I revealed the quasi-molecular ion peak at $m/z$ 743 [M+H]$^+$, while the negative ion FAB-MS showed the [M−H]$^-$ peak at $m/z$ 741. These MS data coupled with the combustion analysis data indicated the molecular formula of I to be C$_{34}$H$_{44}$O$_{18}$.

The proton nuclear magnetic resonance ($^1$H-NMR) spectrum of I gave a rather simple pattern, showing singlet signals due to methoxy methyl(s) and aromatic proton(s) at δ$_H$ 3.76 and 6.65 ppm, respectively, along with signals in the 3.03–4.95 ppm region, which could be interpreted as being due to the protons of glucose and propane units based on the results of proton-proton shift correlation spectroscopy ($^1$H→$^1$H COSY) and $^1$H→$^1$C COSY (Table I).

These spectral data suggested that compound 1 may be a ligan glucoside having a highly symmetric structure. Then, we measured the $^1$H-detected heteronuclear multiple bond connectivity (HMBC)$^{29}$ spectrum to establish the connectivities of these benzene, glucose, and propane units. As can be seen in Fig. 1, the quaternary carbon at δ$_C$ 133.7 (C-4') showed long-range correlations with the protons at δ$_H$ 6.65 (2′,6′-H) and 4.88 (G1-H), indicating the connection of the glucose moiety with the benzene ring at the C-4' position. On the other hand, the quaternary carbon at δ$_C$ 137.1 (C-1') showed long-range correlations with the protons at δ$_H$ 6.65 (2′,6′-H), 4.67 (2-H), and 3.08 (1-H), and the tertiary carbons at δ$_C$ 104.6 (C-2',6') were correlated only with the proton at δ$_H$ 4.67 (2-H). In turn, the carbon at δ$_C$ 85.1 (C-2) showed correlations with 2′,6′-H (δ$_H$ 6.65) and 8-H$_3$ (or 4-H$_3$) (δ$_H$ 3.83 and 4.21). Further, the carbon at δ$_C$ 152.6 (C-3',5') showed correlations with the methoxy protons (δ$_H$ 3.76) and with 2′,6′-H (δ$_H$ 6.65). These data disclosed the connectivities of the propane unit and methoxy group(s) with the benzene ring at the C-1' and C-3',5' positions.

Eventually, compound 1 was identified as liriodendrin (I), a known symmetric lignan, by direct spectral com-
parisons with an authentic sample. It should be noted that the previous $^{13}$C-signal assignments$^5$ for C-1' (and 1") and C-4' (and 4") were revised (Table I).

Compound 2 (2), is a minor constituent obtained as an amorphous solid, and showed $[\alpha]_D^{28} -10^\circ$ (MeOH). It gave UV and IR spectra similar to those of 1. The positive ion FAB-MS of 2 showed the quasi-molecular ion peak at $m/z$ 603 [M + Na]$^+$, corresponding to the molecular formula $C_{28}H_{36}O_{13}$, which differed by one glucose unit from that of 1.

The $^1$H- and $^{13}$C-NMR spectra of 2, analyzed with the aid of $^1H-^1H$ COSY, $^1H-^{13}$C COSY, and HMBC, indicated the presence of one glucose, two benzene rings, two propane units and four methoxy groups in the molecule (Table I), and the overall spectral patterns resembled those of 1, except for the lack of signals corresponding to one glucose unit.

On the basis of the above spectral evidence, compound 2 was concluded to be syringaresinol mono-$\beta$-d-glucoside (2).$^5$ The physico-chemical and spectral data were in good agreement with those published.$^5$ Further, the previous assignments of $^{13}$C-NMR signals for C-1' and C-4' were revised on the basis of the HMBC experimental result.$^5$

Next, the Ca$^{2+}$ channel antagonistic activity of liriodendrin (1) was examined. In frog atrium, a regenerative action potential can be obtained even after the transient inward sodium current has been inhibited completely by tetrodotoxin (TTX, $3 \times 10^{-6}$ M). The peak height and the duration of the slow response are both modulated strongly by a transmembran calcium current which we designate $I_{Ca}$. Fig. 2 illustrates a control 'slow response', as well as a response obtained 15 min after bath application of liriodendrin ($10^{-5}$ M). Liriodendrin produces a significant decrease in both the height and the duration of the slow response, but causes no measurable change in either the resting membrane potential ($-90$ mV) or the rate of negative repolarization of $-70$ mV. These findings suggest...
that liriodendrin (I) reduces $I_{Ca}$ quite selectively, but no conclusive information could be obtained at this stage.

To test further the mechanism of liriodendrin action(s) the whole-cell voltage clamp method was employed. Fig. 3 shows the effect of liriodendrin (I) on the calcium current, $I_{Ca}$, which generates the slow response, in comparison with that of verapamil. In this experiment, the isolated atrial cell was voltage-clamped at its normal resting membrane potential ($-90\, \text{mV}$), and a series of graded depolarizing or hyperpolarizing clamp steps, each 100 ms in duration, were applied. Liriodendrin (I) at $10^{-5}\, \text{M}$ markedly decreased the amplitude of $I_{Ca}$ (approx. 40% of the control). However, it has no significant effect on the activation threshold or the apparent reversal potential for $I_{Ca}$ (Fig. 3). These actions of liriodendrin (I) on $I_{Ca}$ were dose-dependent and
Table I. 400 MHz $^1$H- and 100 MHz $^{13}$C-NMR Data for 1 and 2 in DMSO-$d_6$ (Coupling Constants in Parenthesis)

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_H$</th>
<th>$\delta_C$</th>
<th>$\delta_H$</th>
<th>$\delta_C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.08 m</td>
<td>53.6 d</td>
<td>3.08 m</td>
<td>53.6 d</td>
</tr>
<tr>
<td>2</td>
<td>4.67 d (4.0)</td>
<td>85.1 d</td>
<td>4.66 d (4.5)</td>
<td>85.0 d</td>
</tr>
<tr>
<td>4</td>
<td>4.21 d (9.0, 6.0)</td>
<td>71.4 t</td>
<td>4.19 d (6.0, 2.0)</td>
<td>71.1 t</td>
</tr>
<tr>
<td>5</td>
<td>3.83 d (9.0, 3.5)</td>
<td>3.79 m</td>
<td>3.06 m</td>
<td>53.5 d</td>
</tr>
<tr>
<td>6 (same as those at the 1', 2', and 4-positions)</td>
<td>4.61 d (4.5)</td>
<td>85.3 d</td>
<td>4.10 d (6.5, 2.0, 71.2 t)</td>
<td>3.79 m</td>
</tr>
<tr>
<td>7</td>
<td>152.6 s</td>
<td>152.6 s</td>
<td>133.6 s</td>
<td>131.3 s</td>
</tr>
<tr>
<td>8</td>
<td>133.7 s</td>
<td>133.6 s</td>
<td>131.3 s</td>
<td>131.3 s</td>
</tr>
</tbody>
</table>

$^a$ values in ppm and coupling constants in Hz. Multiplicities of carbon signals were determined by means of the DEPT method and are indicated as $s$, $t$, $d$, and $q$. Assignments may be interchanged.

It is now well known that Ca$^{2+}$ channel antagonists act on cardiac muscles to cause a relaxation and a reduction of heart rate. Thus, some of them are used effectively for the treatment of cardiovascular diseases such as hypertension, ischemia, and arrhythmia. In recent years, there have been many reports concerning the screening of traditional herbal medicines for Ca$^{2+}$ antagonistic activity.8) Ichikawa et al.8c and Chen et al.8e) reported that some ligands such as trachelogenin, pinoresinol, pinoresinol dimethyl ether, and fargesones A and B have a Ca$^{2+}$ antagonistic activity, as determined by measuring the mechanical contraction of the taenia coli of the guinea pig. Only tetrandrine,3,9) a bisbenzylisoquinoline alkaloid from a plant of Stephania species, and tanshinone,3,10) a diterpene from a plant of Salvia species, have so far been reported as naturally occurring Ca$^{2+}$ channel antagonists which have been studied by electrophysiological techniques. Apparently our present result provides the first example of a water-soluble ligan glucoside having a Ca$^{2+}$ antagonistic action, as found by the electrophysiological method. It must be emphasized that liroindin (1) is a member of the fused tetrahydrofuran-type ligands, which are a very common class of ligands in the plant kingdom. Further studies on the activities of related ligands and ligan glucosides should be interesting.

Experimental

Melting points were determined with a Kofler-type apparatus and are uncorrected. Optical rotations were measured in MeOH solutions on a JASCO DIP-140 digital polarimeter at 28°C. UV spectra were taken with a Shimazu 202 UV spectrometer in MeOH solutions and IR spectra with a JASCO IRA-2 or a Nicolet DX FT-IR spectrometer in KBr. 1H-NMR and 13C-NMR spectra were taken on a JEOL JNM-GX 400 spectrometer with tetramethylsilane as an internal standard. Chemical shifts are recorded in $\delta$ values and coupling constants in hertz (Hz). Multiplicities of $^{13}$C-NMR signals were determined by means of the distortionless enhancement by polarization transfer (DEPT) method. 1H-1H COSY, 1H-13C COSY, and HMBC spectra were obtained with the JEOL standard pulse sequences and data processing was performed with JEOL standard software. FAB-MS was employed with a JEOL SX-102 spectrometer (matrix: glycerol or m-nitrobenzyl alcohol). Column chromatography was done with Iatrobeads. Preparative TLC was carried out on Merck Kieselgel GF$254$ plates and the plates were examined under UV light. Extraction of substances from silica gel was done with MeOH-CHCl$_3$ (3:7) and solutions were concentrated in vacuo. TLC analyses were done on Merck Kieselgel GF$254$ plates and spots were detected by the use of 1% Ce(SO$_4$)$_2$aq.-aqueous H$_2$SO$_4$ (10%) reagent. For drying organic solutions, anhydrous MgSO$_4$ was used.

Isolation of Liroindin (1) and Syringaresinol Mono-β-D-glucoside (2)

The MeOH extract (130 g) obtained from the air-dried roots of B. diffusa (1.2 kg), which was described in a previous paper,2a) was employed in this experiment. The extract was dissolved again in MeOH (about 500 mL) and the insoluble crystalline precipitate (7.1 g) was separated by filtration. This precipitate was identified as sucrose by direct 1H-NMR comparison with an authentic sample. The MeOH filtrate was evaporated to dryness and the residue (122 g) was suspended in water (650 mL) and fractionated by chromatography on a column with CHCl$_3$ (500 mL × 3) and BuOH (500 mL × 4) to give the CHCl$_3$ fraction (7.5 g), BuOH fraction (9.4 g), and water fraction (ca. 100 g).

The water fraction was concentrated in vacuo. The residue (ca. 100 g) was dissolved in EtOH (500 mL) and the insoluble material (77 g) was separated by filtration. The EtOH solution was concentrated in vacuo, giving the EtOH-soluble fraction (23 g). This was subjected to column chromatography on Iatrobeads (250 g) and eluted successively with MeOH-CHCl$_3$ (1:2, 1:1, and 2:1; 1900, 760, and 650 mL, respectively), MeOH (700 mL), and MeOH-H$_2$O (8:1 and 8:3, 900 mL each). A fraction (1.2 g) eluted with MeOH-CHCl$_3$ (1:2) was triturated with MeOH to give liroindin (1) (24 mg). The mother liquor was further purified by preparative TLC with EtOAc- MeOH-H$_2$O (6:2:1) to give an additional crop of liroindin (1) (11 mg) and glycerol (500 mg). On the other hand, fractions eluted with MeOH-CHCl$_3$ (2:1 and 1:1) contained sucrose and a mixture of sugars, respectively.

The BuOH fraction (8.9 g) was extracted with 1.5% aqueous HCl (160 mL) and the room temperature filtrate was the insoluble material (1 g), which contained a mixture of phenolic compounds,11) which was separated by filtration. The aqueous acidic solution was slightly alkalized by the addition of 10% NH$_4$OH and washed with EtOAc (200 mL × 3). The alkaline aqueous solution was then neutralized by the careful addition of 5% HCI and evaporated to dryness. The residue (9 g) was subjected to column chromatography on Iatrobeads (140 g) and eluted with EtOAc- MeOH-CHCl$_3$ (6:2:1, 2500 mL), MeOH (600 mL), and MeOH-H$_2$O (1:1, 500 mL). A fraction (ca. 4 g) eluted with EtOAc- MeOH-H$_2$O (6:2:1) was again chromatographed on Iatrobeads (200 g) with EtOAc- MeOH-CHCl$_3$ (6:2:0.5) fractions which showed a dark spot on TLC, examined under UV light, were combined (ca. 290 mg) and further purified by preparative TLC with EtOAc- MeOH-H$_2$O (6:2:0.5) to give syringaresinol mono-β-D-glucoside (2) (20 mg) as an amorphous solid.

Liroindin (1): White needles from (MeOH), mp 256—259°C, $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$ $\Delta x^2_0$.


Assay of Ca2+ Current Inhibitory Activity Cell Dispersion: Adult bullfrogs (Rana catesbeiana) were pithed and their hearts were removed and transferred to a dissection dish containing standard Ringer’s solution. The right atrium was separated and several large pieces of tissue were cut off and placed in a second dish containing calcium-free Ringer’s solution. Single atrial cells were obtained by using an enzymatic dispersion procedure (0.15% collagenase, 0.1% bovine pancreatic trypsin, and 0.1% bovine albumin) as described by Hume and Giles.12 The cell suspension was taken into a chamber (1 ml) on the stage of an inverted bipolar phase-contrast microscope (Olympus, IMT-2, Tokyo, Japan). After the single cells had settled at the bottom of the chamber, the frog Ringer’s solution was perfused with 95% O2 and 5% CO2 mixed gas. Ringer’s solution had the following composition (mm): NaCl, 90.6; NaHCO3, 20; KCl, 2.5; MgCl2, 5.0; CaCl2, 2.5; and glucose, 10 (pH 7.4, adjusted with NaOH). The temperature of the perfusate in the experimental chamber was maintained at 20–22°C, and perfusion was started at a rate of 4 ml/min.

Electrophysiology: The membrane potentials were measured by using a conventional current clamp method. A glass microelectrode (Radnoti Glass Technology, CA, U.S.A.) was constructed with a two-stage vertical microelectrode puller (Narishige, PP-83, Tokyo, Japan). The electrode resistance resistance was 3–5 megohm when filled with 150 mm KCl. A single cell was impaled by a glass microelectrode by suction, after positioning by using a hydraulic micromanipulator (Narishige, MO-102, Tokyo, Japan). The potential difference between the intracellular microelectrode and a bath reference electrode was measured using a micro-electrode amplifier (Nihon Kohden, MEZ-8201, Tokyo, Japan). An action potential was elicited by an intracellular stimulus (15–20 nA, 1 ms, 0.2 Hz). The current pulse was controlled by an electronic stimulator (Nihon Kohden, SEN-7203, Tokyo, Japan). The whole-cell voltage clamp technique was employed to measure the transmembrane Ca2+ current through Ca2+ channels in frog atrial single cells. A patch amplifier (Yale MK V, CT, USA) was used to record the whole cell membrane currents. Data were displayed on a storage oscilloscope (Cos 5020-ST, Kikusui, Tokyo, Japan) and simultaneously recorded on a PCM data recording system (RP-880, NF Electronic Instruments, Tokyo, Japan) at a bandwidth of DC to 10 kHz.

Drugs and Solutions: The drugs used were as follows: collagenase (Sigma), trypsin (Sigma), albumin (Sigma), and tetrodotoxin (Sankyo). Liriodendrin and drugs were applied by changing the perfusion solution to one which contained the substance at a desired concentration.

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