Nitric Oxide (NO) Production Inhibitory Constituents of *Tabebuia avellanedae* from Brazil

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From the water extract of Brazilian *Tabebuia avellanedae*, two new iridoids (1, 2) and a new phenylethanoid glycoside (3) have been isolated together with twelve known compounds (4—15). Their structures were determined based on the spectroscopic data. The isolated compounds inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophage-like J774.1 cells. Compounds 1, 3, 10, 11, and 12 showed inhibitory activities more potent (IC_{50}, 13.8—26.1 μg/ml) than a positive control N^G-monomethyl-L-arginine (L-NMMA; IC_{50}, 27.4 μg/ml).

Key words *Tabebuia avellanedae*; iridoid; nitric oxide (NO) production inhibitory activity; phenylethanoid glycoside; anti-inflammatory

Tabebuia avellanedae LORENZ [syn.: *T. impetiginosa* MART. ex DC, Bignoniaceae] is a popular tree distributed throughout the tropical rain forests of Central and South America. Its inner bark is commonly known as "taheebo", "lapacho", "pau d'arco", and "ipe roxo" and used as an antifungal,[1] antibacterial,[2] anti-inflammatory,[3] antinociceptive,[4] and anticancer[5] medicine in Latin America. It has been reported to contain furanonaphthoquinones,[6—8] quinones,[9] naphthoquinones,[10] benzoic acid, benzaldehyde derivatives,[11] cyclopentene dialdehyde,[12] flavonoids,[13] iridoids,[14] and phenolic glycosides.[15] Recent pharmacological study indicated that constituents of the bark of this plant (*e.g.* β-lapachone) inhibit cell growth and induce apoptosis in human colon carcinoma and prostate tumor cell lines. A number of compounds from "taheebo" were reported as effective antipsoriatic, antinociceptive, and antiedematogenic agents.[16] Recently, as a part of our continued study on the nitric oxide (NO) production inhibitory medicinal plants,[17—20] we found that a water extract of the inner bark of "taheebo" from Brazil showed significant inhibitory activity on NO production in lipopolysaccharide (LPS)-activated J774.1 macrophage-like cells (IC_{50}, 64.7 μg/ml). Thus, further separation of the water extract was carried out to isolate the active constituent and isolated three new compounds (**1**—**3**), together with twelve known compounds. In this paper, we report the isolation and structure elucidation of the new compounds together with their NO inhibitory activity.

Results and Discussion

The dried inner bark of *T. avellanedae* from Brazil was extracted with water at 90 °C and the water suspension of the extract was successively partitioned into EtOAc-soluble and H_2O-soluble fractions. The EtOAc-soluble fraction (IC_{50}, 48.1 μg/ml), which showed stronger activity than the EtOAc-insoluble fraction (IC_{50}, 54.5 μg/ml), was subjected to a series of chromatographic separation and preparative TLC to afford two new iridoids (**1**, **2**) and a new phenylethanoid glycoside (**3**), together with twelve previously-reported compounds (**4**—**15**). The known compounds were identified by analysis of their spectroscopic data and comparison with literature data to be 2-(4-hydroxyphenyl)ethyl-1-*O*-β-D-[5-*O*-(3,4-dimethoxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**4**), 2-(4-hydroxyphenyl)ethyl-1-*O*-β-D-[5-*O*-(4-methoxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**5**), 3,4-dimethoxyphenyl 1-*O*-β-D-[5-*O*-(4-hydroxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**6**), 3,4-dimethoxyphenyl 1-*O*-β-D-[5-*O*-(3,4-dimethoxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**7**), 3,4,5-trimethoxyphenyl 1-*O*-β-D-[5-*O*-(4-methoxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**8**), 3,4-dimethoxyphenyl 1-*O*-β-D-[5-*O*-(4-methoxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**9**), 6-*O*-(3,4-dimethoxybenzoyl)-ajugol[14] (**10**), 6-*O*-(4-methoxybenzoyl)-ajugol[14] (**11**), 6-*O*-(4-hydroxybenzoyl)-ajugol[14] (**12**), 4-methoxybenzoic acid[11] (**13**), 3,4-dimethoxybenzoic acid[11] (**14**), and 4-hydroxybenzoic acid[11] (**15**).

Compounds **1** and **2** were both isolated as colorless amorphous solid having $[\alpha]_D^{25}$ −110.2° and −56.4° in MeOH. Both of them exhibited the same molecular formula $C_{18}H_{25}O_7$ as determined by high-resolution FAB-MS (HR-FAB-MS). The IR spectrum of **1** showed absorptions of hydroxyl (3400 cm^{-1}), ester carbonyl (1710 cm^{-1}), and phenyl (1600, 1470 cm^{-1}) groups. Their ^{1}H-NMR spectra were also similar and revealed signals due to a tertiary methyls, three oxymethines, two aliphatic methylenes, two methoxyls, and two methylenes together with those of a *p*-substituted benzoyl group. Their ^{13}C-NMR spectra showed the signals of nine carbons including those of two acetal carbons and two oxygentaed carbons, together with those of a *p*-substituted benzoyl and two methoxyl groups. The ^{1}H–^{1}H shift correlation spectroscopy (COSY) and heteronuclear multiple-quantum coherence (HMQC) spectra revealed the partial connectivities (bold line) of C3–C4–C5–C6–C7 and C5–C9 (Fig. 1). These partial structures were connected based on the long-range correlations observed in the heteronuclear multiple-bond correlation (HMBC) spectrum as shown in Fig. 1, which led to the assignment of planar iridoid framework having methoxyl substituents at C-1 and C-3 in both **1** and **2**. The HMBC data further indicated the presence of a 4-hydroxybenzoyl substituent at C-6, indicating that they should be stereoisomers.

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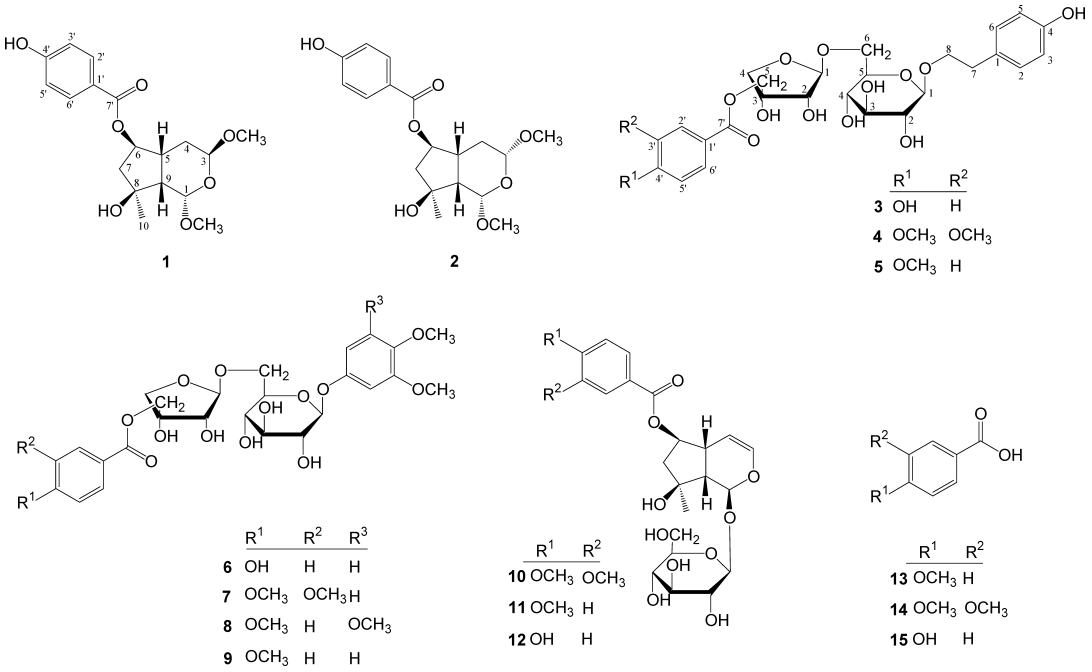


Chart 1. Structures of the Compounds Isolated from *Tabebuia avellanedae*

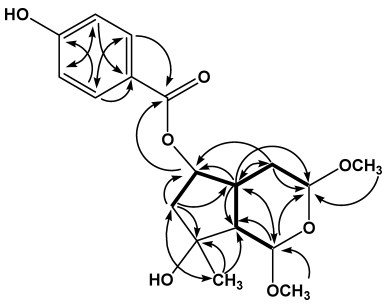
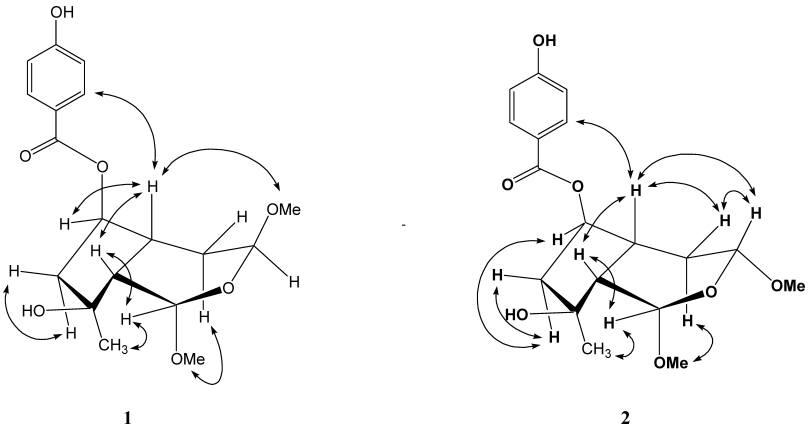


Fig. 1. Connectivities (Bold Line) Deduced by the COSY and HMQC Spectra and Significant HMBC Correlations (Arrow) Observed for **1**

The relative stereochemistry of **1** and **2** were both assigned on the basis of the rotating-frame Overhauser enhancement spectroscopy (ROESY) spectral analysis and coupling constant data. In **1**, the ROESY correlations H-1/H-9, H-1/H_3-10, H-5/H-9, H-5/H-2′,6′, and H-5/MeO-3) were observed, which indicated rings A and B to be *cis*-fused, H-5 and H-9 and 3-OMe and 6-(4-hydroxybenzoyl) substituents to be β-oriented, and the C-1 methoxyl substituent to be α-*axial*-oriented (Fig. 2). Contrary to **1**, the ROESY spectrum of **2** showed correlations between H-3 and H-5, suggesting the α-orientation of the C-3 methoxyl substutient. Thus, the structures of **1** and **2** were established as shown.

Compound **3** was obtained as a colorless amorphous solid with $[\alpha]_D^{25}$ −26.2° (MeOH). It showed the quasimolecular ion at *m/z* 553.1959 $(M+H)^+$ in HR-FAB-MS, which corresponds to the molecular formula $C_{26}H_{33}O_{13}$. The IR spectrum of **3** showed absorptions of hydroxyl (3400 cm^{-1}), ester carbonyl (1710 cm^{-1}), and phenyl (1600, 1510, 1470 cm^{-1}) groups. The ^{1}H-NMR spectrum of **3** exhibited signals due to four methylene protons (δ_H 3.92, 3.65; 2.78), four sets of *ortho*-coupled aromatic protons (δ_H 7.90, 6.82, 7.01, 6.65), a glucosyl anomeric proton (δ_H 4.26), and apiosyl anomeric proton (δ_H 5.05), together with signals ascribable to sugar moieties. The ^{13}C-NMR spectrum of **3**, on the other hand, showed 26 signals including those for an ester carbonyl, 12 aromatic carbons ascribable to two phenyl rings, and two anomeric carbons (δ_C 110.7, 104.4). These data were similar to those of 2-(4-hydroxyphenyl)ethyl-1-*O*-β-D-[5-*O*-(4-methoxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside[15] (**5**), isolated from the same extract, but they differs due to the lack of a methoxyl signal in **5**. This suggests that the structure of **3** should be 2-(4-hydroxyphenyl)ethyl-1-*O*-β-D-[5-*O*-(4-hydroxybenzoyl)]-apiofuranosyl-(1→6)-β-D-glucopyranoside. Moreover, GC analysis[21] of a chiral derivative of an acid hydrolysate of **3** showed its sugar moiety to be D-glucose and D-apiose, respectively. Thus, the structure of **3** was concluded as shown.

In this paper, we have reported two new iridoids (**1**, **2**) and a new phenylethanoid glycoside (**3**), together with twelve known compounds (**4**—**15**). Compounds **1** and **2** have two methyl acetals in the molecule, which have possibility to be produced during the isolation. However, TLC analysis of the water extract with **1** and **2** as standard suggested both **1** and **2** were present in an original water extract. Thus, the possibility of **1** and **2** to be an artifact may be excluded. All the isolated compounds were tested for their inhibitory activities against NO production in LPS-activated macrophage-like J774.1 cells. All of them displayed significant dose-dependent inhibition, and the activities of **1**, **3**, **10**, **11**, and **12** were more potent than the positive control, N^G-monomethyl-L-arginine (L-NMMA). Among the isolated compounds, 6-*O*-(*p*-methoxybenzoyl)-ajugol (**11**) displayed the most potent activity with an IC_{50} value of 13.8 μg/ml (Table 1). Although structure–activity relationship could not be deduced from the observed activity data, it is evident that iridoids are active to NO production inhibition, while simple phenolic compounds are inactive. NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological



1 2

Fig. 2. ROESY Correlations (⟷) Observed for **1** and **2**

Table 1. Inhibitory Effects on NO Production in LPS-Activated Macrophage-like J774.1 Cells

Compound	IC_{50} (μg/ml)[a)]
1	17.4
2	34.6
3	22.0
4	31.1
5	55.1
6	44.1
7	75.9
8	35.6
9	38.2
10	15.1
11	13.8
12	26.1
13	141
14	129
15	150
L-NMMA	27.4

a) IC_{50} values were calculated from the mean of data of four determinations.

processes. When certain cells are activated by specific proinflammatory agents such as endotoxin, tumor necrosis factor (TNF), interferon-gamma (IFN-γ) and interleukin-1 (IL-1), NO is produced by inducible nitric oxide synthase (iNOS) and acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities.[22)] However, excessive production has detrimental effects on many organ systems of the body leading to tissue damage, even leading to a fatal development (septic shock).[23)] The NO production inhibitory activity of the constituents of *T. avellanedae* further indicated the traditional utility of this plant as an anti-inflammatory medicine.

Experimental

General Methods Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solutions. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in δ values. HR-FAB-MS measurements were carried out on a JEOL JMS-700T spectrometer and glycerol was used as matrix. Column chromatography was performed with BW-820MH silica gel (Fuji Silysia, Aichi, Japan). Analytical and preparative TLC were carried out on precoated silica gel plates (Merck, 0.25 or 0.50 mm thickness).

Plant Material Commercial dried inner bark of *T. avellanedae* Lorenz was provided by IJ INC. (Tokyo, Japan) for present investigation.

Extraction and Isolation Dried small pieces of inner bark of *T. avellanedae* (2.6 kg) were extracted with water (2 l, 90 °C, 2 h×3). The water extract (450 g) was suspended in H_2O (1 l) and partitioned with EtOAc (1 l×3) to yield an EtOAc-soluble (25 g) and H_2O-soluble (415 g) fractions, respectively. The EtOAc-soluble fraction (25 g) was chromatographed (9×45 cm) with an MeOH–$CHCl_3$ solvent system to give six fractions [Fr. 1: 1% MeOH eluate, 2.08 g; Fr. 2: 5% MeOH eluate, 2.27 g; Fr. 3: 10% MeOH eluate, 1.92 g; Fr. 4: 15% MeOH eluate, 6.52 g; Fr. 5: 20% MeOH eluate, 1.58 g; Fr. 6: 30% MeOH eluate, 3.90 g].

Fraction 1 (1 g) was rechromatographed (5×45 cm) with MeOH–$CHCl_3$ (1%→5%) to give **13** (700 mg) and **14** (20 mg).

Fraction 2—4 (each 100 mg) were subjected to preparative TLC with 10% MeOH–$CHCl_3$ to give **13** (18 mg); **10** (7.0 mg) and **11** (5.6 mg); and **15** (1.6 mg), **1** (3.1 mg) and **2** (2.5 mg), respectively.

Fraction 5 (100 mg) was subjected to preparative TLC with MeOH–CH_3CN–H_2O (1 : 1 : 2) to give **11** (9.0 mg) and **12** (23 mg).

Fraction 6 (3 g) was rechromatographed (5×45 cm) with MeOH–$CHCl_3$ (1→30%) to afford four subfractions (fr. 5-1, 77.5 mg; fr. 5-2, 1.2 g; fr. 5-3, 467 mg; fr. 5-4, 156 mg). Subfraction 5-2 was rechromatographed with MeOH–CH_3CN–H_2O (1 : 1 : 2), followed by reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (1 : 1 : 2), to give **3** (6.5 mg), **5** (4.0 mg), **10** (7.0 mg), and **11** (2.0 mg). Subfraction 5-3 (467 mg) was rechromatographed and then subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (1 : 1 : 2), to give **4** (5.6 mg), **5** (8.2 mg), **9** (4.6 mg), **10** (13 mg), **11** (23 mg), and **12** (9.0 mg). Subfraction 5-4 was subjected to reversed-phase preparative TLC with MeOH–CH_3CN–H_2O (1 : 1 : 2), to give **6** (15.3 mg), **7** (44.5 mg), **8** (3.6 mg), and **11** (9.0 mg).

Compound **1**: Colorless amorphous solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.31 (3H, s, H-10), 1.70 (1H, ddd, J=14.7, 3.1, 1.4 Hz, H-7), 1.88 (1H, dd, J=14.1, 7.1 Hz, H-4), 1.98 (1H, ddd, J=14.1, 5.3, 2.9 Hz, H-4), 2.27 (1H, ddd, J=7.8, 3.9, 1.4 Hz, H-9), 2.45 (1H, dd, J=14.7, 9.1 Hz, H-7), 2.96 (1H, dddd, J=14.1, 7.8, 7.5, 5.3 Hz, H-5), 4.76 (1H, d, J=3.9 Hz, H-1), 4.80 (1H, dd, J=7.1, 2.9 Hz, H-3), 5.22 (1H, ddd, J=9.1, 7.5, 3.1 Hz, H-6), 6.71 (2H, d, J=8.8 Hz, H-3′,5′), 7.79 (2H, d, J=8.8 Hz, H-2′,6′). ^{13}C-NMR (100 MHz, CD_3OD) δ: 23.8 (C-10), 31.1 (C-4), 40.7 (C-5), 48.3 (C-7), 49.0 (C-8), 53.3 (C-9), 55.7 (OMe-3), 56.0 (OMe-1), 81.7 (C-6), 96.5 (C-3), 100.1 (C-1), 116.1 (C-3′,5′), 122.5 (C-1′), 132.8 (C-2′,6′), 163.5 4′, 168.1 (C-7′). IR (KBr) cm^{-1}: 3550, 3400, 1708, 1656, 1607, 1511, 1260, 960. HR-FAB-MS: 353.1605 [Calcd for $C_{18}H_{25}O_7$ $(M+H)^+$ 353.1600]. $[\alpha]_D^{25}$ −110.2° (c=0.033, MeOH).

Compound **2**: Colorless amorphous solid. ^{1}H-NMR (400 MHz, CD_3OD) δ: 1.35 (3H, s, H-10), 1.60 (1H, ddd, J=14.7, 7.9, 5.6 Hz, H-4), 1.90 (1H, m, H-7), 1.94 (1H, m, H-4), 2.14 (1H, dd, J=7.7, 5.5 Hz, H-9), 2.34 (1H, dd, J=14.9, 7.5 Hz, H-7), 2.79 (1H, ddd, J=14.7, 7.7, 4.5 Hz, H-5), 4.70 (1H, d, J=5.5 Hz, H-1), 4.78 (1H, dd, J=5.6, 3.7 Hz, H-3), 5.22 (1H, dd, J=7.5, 4.5 Hz, H-6), 6.80 (2H, d, J=8.8 Hz, H-3′,5′), 7.88 (2H, d, J=8.8 Hz, H-2′,6′). ^{13}C-NMR (100 MHz, CD_3OD) δ: 26.9 (C-10), 30.7 (C-4), 41.9 (C-5), 47.7 (C-7), 49.0 (C-8), 52.9 (C-9), 53.3 (OMe-3), 56.0 (OMe-1), 80.3 (C-6), 98.9 (C-1), 99.3 (C-3), 116.1 (C-3′,5′), 122.6 (C-1′), 132.6 (C-2′,5′), 163.5 (C-4′), 168.5 (C-7′). IR (KBr) cm^{-1}: 3550, 3400, 1708, 1656, 1607, 1511, 1260, 960. HR-FAB-MS: 353.1615 [Calcd for $C_{18}H_{25}O_7$ $(M+H)^+$ 353.1600]. $[\alpha]_D^{25}$ −56.4° (c=0.035, MeOH).

Compound **3**: Colorless amorphous solid. ^{1}H-NMR (400 MHz, CD_3OD)

δ: 2.78 (1H, t, J=5.2 Hz, H-7), 3.65 (1H, m, H-8), 3.92 (1H, m, H-8), 6.65 (2H, J=8.5 Hz, H-3, H-5), 6.82 (2H, d, J=8.8 Hz, H-3′,5′), 7.01 (2H, d, J=8.5 Hz, H-2, 6), 7.90 (2H, d, J=8.8 Hz, H-2′,6′); glucose: 3.17 (1H, t, J=7.9 Hz, H-2), 3.27 (1H, m, H-4), 3.33 (1H, m, H-3), 3.40 (1H, td, J=6.2, 2.3 Hz, H-5), 3.61 (1H, dd, J=11.3, 6.2 Hz, H-6), 4.00 (1H, dd, J=11.3, 2.3 Hz, H-6), 4.26 (1H, d, J=7.9 Hz, H-1); apiose: 3.87 (1H, d, J=9.6 Hz, H-4), 3.98 (1H, d, J=2.2 Hz, H-2), 4.07 (1H, d, J=9.6 Hz, H-4), 4.31 (1H, d, J=12.4 Hz, H-5), 4.34 (1H, d, J=12.4 Hz, H-5), 5.05 (1H, d, J=2.2 Hz, H-1). ^{13}C-NMR (100 MHz, CD_3OD) δ: 36.4 (C-7), 72.2 (C-8), 116.1 (C-3, C-5), 116.2 (C-3′,5′), 122 (C-1′), 130.8 (C-1), 130.9 (C-2, 6), 133.0 (C-2′,6′), 156.8 (C-4), 163.6 (C-4′), 167.9 (C-7′); glucose: 68.6 (C-6), 71.8 (C-4), 75.0 (C-2), 76.8 (C-5), 78.1 (C-3), 104.4 (C-1); apiose: 67.5 (C-5), 75.1 (C-4), 78.6 (C-2), 79.0 (C-3), 110.7 (C-1). IR (KBr) cm^{-1}: 3400, 1710, 1600, 1510, 1470, 1420, 1345, 1270. HR-FAB-MS: 553.1959 [Calcd for $C_{26}H_{33}O_{13}$ $(M+H)^+$ 553.1921]. $[\alpha]_D^{25}$ −26.2° (c=0.029, MeOH).

Sugar Analysis A solution of **3** (1.0 mg) in 1 N HCl (dioxane–H_2O, 1 : 1; 0.5 ml) was heated at 80 °C for 4 h. The reaction mixture was neutralized with Amberlite IRA67 (OH^- form) and the filtrate was concentrated to dryness *in vacuo*. The residue was dissolved in pyridine (0.1 ml) and 0.1 M L-cysteine methyl ester hydrochloride in pyridine (0.1 ml) was added. After heated at 60 °C for 2 h, trimethylsilylimidazole (0.1 ml) was added and the mixture was heated at 60 °C for 1 h. The reaction mixture was partitioned between hexane and water (each 0.15 ml) and the organic layer was analyzed on GC-MS; column, DB-1 30 m×0.25 mm i.d. (J&W Scientific Inc.); initial temperature, 50 °C (0 min); rate, 15 °C/min (0—18 min); final temperature, 230 °C; carrier gas, He (1 ml/min); injection temperature, 240 °C. Standard sugars gave peaks at t_R (min) 24.97 and 25.98 for D- and L-glucose, respectively. As we could not obtain standard D- and L-apiose samples commercially, a known compound **7** (1 mg) was treated in a same manner as in **3**, and the D-apiose in hydrolysate of **7** gave peak at t_R (min) 18.00.

Nitric Oxide Inhibitory Assay NO production inhibition was assayed as reported in the previous papers.[17—20]

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