Coumarins with Monoamine Oxidase Inhibitory Activity and Antioxidative Coumarino-lignans from *Hibiscus syriacus*

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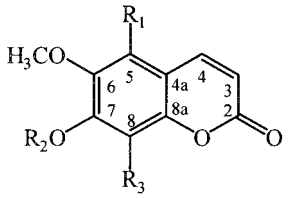
A previously undescribed coumarin and a new coumarino-lignan, together with the known compounds scopoletin and cleomiscosins A, C, and D, have been isolated from the root bark of *Hibiscus syriacus*, and their structures were assigned on the basis of various spectral studies. The coumarin analogue and scopoletin inhibited monoamine oxidase with moderate IC_{50} values. The new coumarino-lignan and cleomiscosin C showed lipid peroxidation inhibitory activity comparable to vitamin E.

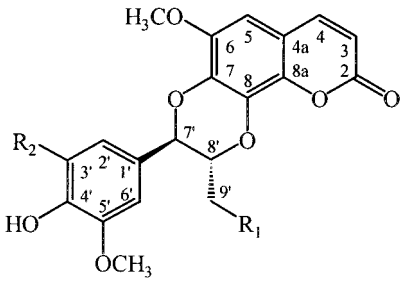
Hibiscus syriacus (Malvaceae) is widely distributed over East Asia. The dried flower of *H. syriacus* is used as material for making herbal teas in Europe, and the root bark has been used as a fungicide, antipyretic, and anthelmintic in Chinese traditional medicine.[1,2] Recently we reported the isolation of a cyclic peptide[3] and triterpene caffeates[4] from the root bark of *H. syriacus*. In our ongoing investigation of biologically active compounds from this plant, we have isolated from the $CHCl_3$ extract of the root bark two coumarins and four coumarino-lignans with monoamine oxidase (MAO) inhibitory activity and antioxidative activity. MAO catalyzes the oxidative deamination of biogenic amines that have neurotransmitter functions in the central nervous system as well as in peripheral tissues.[5] The deficiency of monoamines such as norepinephrine, epinephrine, dopamine, and serotonin at critical synapses relates to depression.[6] In this paper, we describe the isolation, structure elucidation, and biological activity of these compounds.

The methanolic extract of *H. syriacus* was purified by solvent partition and various flash column chromatographies using Si gel, Sephadex LH-20, and ODS resins to afford compounds **1**−**6**. Compounds **2**, **4**, **5**, and **6** were identified as scopoletin and cleomiscosins A, C, and D, respectively, by comparison of their spectra with literature data.[7−10]

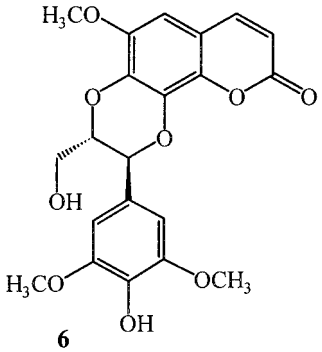
The molecular formula of **1** was established as $C_{12}H_{12}O_6$ by HREIMS (*m/z* 252.0630 M^+ − 0.4 mmu). Its IR spectrum suggested the presence of hydroxyl (3378 cm^{-1}) and carbonyl (1738 cm^{-1}) groups. The ^1H NMR spectrum of **1** in CD_3OD exhibited the signals for two coumarin hydrogens [δ 6.32 and 8.05 (1H each, *d*, *J* = 9.6 Hz)] and three aromatic methoxyl groups at δ 4.01, 3.95, and 3.93. In DMSO-d_6, it showed one additional exchangeable proton signal at δ 9.60, which was quenched by addition of D_2O. The ^{13}C NMR spectrum in DMSO-d_6 revealed signals for an unsaturated ester carbonyl group (δ 159.8), two methines (δ 139.2, 114.0), six sp^2 quaternary carbons (δ 145.2, 142.4, 140.9, 139.3, 134.5, 109.1), and three aromatic methoxyl carbons (δ 62.2, 61.1, 61.0). The above spectral data suggested that **1** is a highly substituted coumarin analogue. The attachment positions of a hydroxyl and three methoxyl groups on the coumarin skeleton were assigned by an HMBC experiment. The hydroxyl proton at δ 9.60 showed a long-range correlation to the quaternary carbon at δ 139.3 (C-8a), which was correlated with the methine proton at δ 8.01 (H-4). Also, the methoxyl protons at δ 3.86, 3.84, and 3.83 were correlated to the oxygenated sp^2 quaternary carbons at δ 145.2, 140.9, and 142.4, respectively, as described in Table 1. Therefore, the structure of **1** was established as 8-hydroxy-5,6,7-trimethoxycoumarin. Although this compound was recently reported,[11] full physical and spectral properties are described here for the first time.



1 $R_1 = OCH_3$, $R_2 = CH_3$, $R_3 = OH$
2 $R_1 = R_2 = R_3 = H$



3 $R_1 = H$, $R_2 = OCH_3$
4 $R_1 = OH$, $R_2 = H$
5 $R_1 = OH$, $R_2 = OCH_3$



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The molecular formula of **3** was determined to be $C_{21}H_{20}O_8$ by HRFABMS analysis. The UV maxima at 322

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Table 1. ^{1}H and ^{13}C NMR Spectral Data for Compounds **1** and **3** in DMSO-d_6

	1			**3**		
no.	δ_H	δ_C	HMBC (H→C)	δ_H	δ_C	HMBC (H→C)
2		159.8			160.3	
3	6.35 (1H, d, J = 9.7)[a]	114.0	C-2, 4a	6.33 (1H, d, J = 9.3)	113.4	C-2, 4a
4	8.01 (1H, d. J = 9.7)	139.2	C-2, 5, 8a	7.96 (1H, d, J = 9.3)	145.1	C-2, 5, 8, 8a
4a		109.1			111.3	
5		140.9		6.91 (1H, s)	101.0	C-4, 7, 8a
6		142.4			145.4	
7		145.2			137.8	
8		134.5			131.7	
8a		139.3			138.2	
1′					126.1	
2′,6′				6.74 (2H, s)	105.9	C-2′, 4′, 6′, 7′
3′,5′					148.3	
4′					136.7	
7′				4.73 (1H, d, J = 8.1)	80.7	C-1′, 2′, 6′, 9′
8′				4.47 (1H, dq, J = 8.1, 6.3)	73.2	C-7′, 9′
9′				1.17 (3H, d, J = 6.3)	16.9	C-8, 7′, 8′
5-OMe	3.84 (3H, s)	62.2	C-5			
6-OMe	3.83 (3H, s)	61.1	C-6	3.78 (3H, s)	56.0	C-6
7-OMe	3.86 (3H, s)	61.0	C-7			
8-OH	9.60 (1H, s)		C-7, 8, 8a			
3′,5′-OMe				3.77 (6H, s)	56.3	C-3′, 5′
4′-OH				8.58 (1H, s)		C-3′, 4′, 5′

[a] Proton resonance integral, multiplicity, and coupling constant (J = Hz) are in parentheses.

and 233 nm and IR absorption at 1721 cm^{-1} suggested that **3** had characteristics of a coumarin derivative. This was supported by the appearance of two coumarin methine proton doublets at δ 6.33 (H-3) and 7.96 (H-4) with coupling constants of 9.3 Hz in the ^{1}H NMR spectrum. Additional features of **3** that could be inferred from the chemical shift and splitting pattern of its ^{1}H NMR signals in DMSO-d_6 were the presence of one hydroxyl proton signal at δ 8.58, which was quenched by the addition of D_2O, three aromatic methines at δ 6.91 and 6.74 (2H), three aromatic methoxyl groups at δ 3.78 and 3.77 (×2), and a propanoid moiety [(CH$_3$−CH(−O−)−CH(−O−)−) at δ 4.73 (1H), 4.47 (1H), and 1.17 (3H)]. The chemical equivalence of two aromatic methines at δ 6.74 and two aryl methoxyl methyls at δ 3.77 suggested a symmetrical substitution pattern of an aromatic ring. The above spectral data were closely related to those of the coumarino-lignans represented by cleomiscosins[7,8] and propacins.[9] The structure of **3** was further assigned by an HMBC experiment (Table 1). The presence of a phenylpropanoid moiety was confirmed by the long-range correlations between H-7′ and C-2′/6′ and between C-7′ and H-2′/6′, suggesting the presence of the benzodioxan moiety for a coumarino-lignan, and also the methoxyl group at δ 3.78 was linked to C-6 at δ 145.4. The coumarino-lignans occur naturally as regioisomeric pairs due to the linkage of the benzodioxan moiety to the coumarin core. The structure of **3** was decided by HMBC, which showed the four-bond long-range correlation from the methyl protons at δ 1.17 to the C-8 quaternary carbon at δ 131.7. The *trans* diaxial arrangement of H-7′ and H-8′ in the benzodioxan is in conformity with the large coupling constant of 8.1 Hz between the two vicinal oxymethine protons.[7,8]

Compounds **1** and **2** inhibited monoamine oxidase in a dose-dependent fashion with IC$_{50}$ values of 44.5 and 19.4 μg/mL, respectively. Compound **3** and cleomiscosins did not show activity up to 70 μg/mL. The antioxidative activity of these compounds was also investigated by means of a lipid peroxidation inhibition test using rat liver microsomes. Cleomiscosin C (**5**) and compound **3** inhibited lipid peroxidation with IC$_{50}$ values of 0.7 and 1.4 μg/mL, respectively. These activities were comparable to vitamin E (0.8 μg/mL), which was used as a control. Compound **6** was about 8 times less active (IC$_{50}$ 5.5 μg/mL) than compound **5**, its regioisomer with the alternative linkage orientation of the benzodioxan moiety. Compound **4** was about 13 times less active (IC$_{50}$ 9.0 μg/mL) than compound **5**, from which it differs only in the lack of a methoxyl group at C-3′. In addition, compounds **1** and **2** did not show significant antioxidative activity. These results imply that in the microsomal assay the linkage orientation of the benzodioxan on the coumarin core and the substituents on the phenyl group are important determinants for lipid peroxidation inhibitory activity. Among the samples tested, compounds **3** and **5**, with a galloyl dimethyl ether group and the same regioisomeric structure of the benzodioxan, exhibited relatively strong antioxidative activity.

Experimental Section

General Experimental Procedures. Melting points were determined on a Electrothermal 9100 and were uncorrected. Specific rotation was determined using a Polartronic polarimeter. UV spectra were recorded on a Shimadzu UV-260 spectrophotometer in MeOH, and IR spectra were determined on a FT-IR Equinox 55 spectrophotometer using a NaCl plate for **1** and a KBr pellet for **3**. NMR spectra were obtained using Varian UNITY 300 and UNITY 500 NMR spectrometers with samples dissolved in CD$_3$OD or DMSO-d_6 with TMS as an internal standard. The chemical shifts are given in ppm (δ) values. EIMS and HREIMS spectra were taken on a JEOL JMS-SX 102A mass spectrometer, operating at 70 eV. FABMS and HRFABMS spectra were obtained on a JEOL JMS-HX 110A/HX110A mass spectrometer. The fluorescence intensities for MAO inhibitory activity were determined on a Hitachi F-300 fluorophotometer.

Plant Material. The root bark of *H. syriacus* was collected at Yusong, Chungnam Province, Korea, in September 1997, and identified by staff at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Fresh root bark was dried in a dark, well-ventilated place. The voucher specimen is deposited in the Antibiotics Research Laboratory of the KRIBB.

Extraction and Isolation. The dried root bark of *H. syriacus* (1.8 kg) was cut into small pieces and extracted with 70% aqueous MeOH at room temperature for 2 days. The MeOH extract was filtered and concentrated under reduced pressure. The liquid residue was washed with *n*-hexane and

then partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ layer was concentrated in vacuo, and the residue was chromatographed on a column of Si gel eluted stepwise with $CHCl_3$−MeOH mixtures. A fraction eluted with $CHCl_3$−MeOH (50:1) was concentrated and then rechromatographed on a Si gel column using $CHCl_3$ with increasing amounts of MeOH. The coumarin eluate was purified by Sephadex-LH20 column chromatography with MeOH, followed by an ODS gel column with 50% aqueous MeOH to give compounds **1** (15 mg) and **2** (37 mg). Another fraction, also eluted with $CHCl_3$−MeOH (50:1), was concentrated in vacuo and then chromatographed twice on Sephadex-LH20 columns eluting with MeOH and 50% aqueous MeOH, respectively. The crude coumarino-lignan eluate was applied to an ODS gel column eluting with 50% aqueous MeOH, followed by preparative HPLC to afford small amounts of compounds **3** (46 mg), **4** (2 mg), **5** (9 mg), and **6** (7 mg). HPLC was performed on a YMC ODS-H80 column (ϕ 4.6 × 250 mm) with a flow rate of 0.5 mL/min using 30% aqueous MeOH as a mobile phase and by monitoring with a photodiode array detector (190−650 nm).

Compound 1: pale yellow solid; mp 153−156 °C; UV λ_{max} (MeOH) (log ϵ) 212 (4.25), 260 (3.79), 311 (3.80) nm; IR (NaCl) ν_{max} 3378, 2928, 2855, 1738, 1615, 1468, 1399, 1059, 1040 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 252 M^+; HREIMS *m*/*z* 252.0630 M^+ ($C_{12}H_{12}O_6$ requires 252.0634).

Compound 3: pale yellow solid; mp 240−244 °C; $[\alpha]_D$ −6.4° (*c* 0.7, $CHCl_3$/MeOH, 1:1); UV λ_{max} (MeOH) (log ϵ) 212 (4.67), 233 (sh 4.29), 322 (3.91) nm; IR (KBr) ν_{max} 3433, 2931, 1721, 1615, 1574, 1444, 1416, 1304, 1218, 1135, 837 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; FABMS *m*/*z* 401 $(M + H)^+$; HRFABMS *m*/*z* 401.1238 $(M + H)^+$ ($C_{21}H_{21}O_8$ requires 401.1236).

Monoamine Oxidase Inhibitory Activity. The crude MAO was prepared by Naoi's method.[12] Mouse brains were quickly removed after death by decapitation and homogenized with 4 volumes of 10 mM potassium phosphate buffer containing 0.25 M sucrose solution (pH 7.4) under ice cooling. The homogenates were centrifuged at 1200*g* for 10 min, and the supernatants were centrifuged at 16,000*g* for 20 min. The mitochondrial pellet was suspended in 10 mM sodium phosphate buffer (pH 7.4) to 100−300 μg/mL and then was divided into aliquots and kept at −20 °C until used. Monoamine oxidase activity with kynuramine as the substrate was assayed fluorometrically by the Krajl method.[13] Samples (5 μL) dissolved in methanol were added to 0.2 M potassium phosphate buffer (70 μL, pH 7.4) which contained 5 μL of mitochondrial suspension and 20 μL of 500 μM kynuramine. After incubation at 37 °C for 30 min, the reaction was stopped by addition of 25 μL of 10% $ZnSO_4$ and 5 μL of 1 N NaOH, and the assay mixture centrifuged at 3000*g* for 5 min. Seventy microliters of the supernatant was transferred into a fluoro 96-well plate, and 140 μL of 1 N NaOH was added. The fluorescence intensity of the reaction product, 4-hydroxyquinoline, was measured at 380 nm (emission) with excitation at 315 nm in a fluorophotometer. As a blank test, the reaction was carried out omitting the substrate.

Antioxidative Activity. Antioxidative activities were evaluated as the inhibitory activities of compounds against lipid peroxidation in rat liver microsomes according to a previously described thiobarbituric acid (TBA) method.[14] In brief account for TBA assay, microsomes were isolated from rat livers and suspended in 100 mM Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of 100 μM $FeSO_4 \cdot 7H_2O$ into a mixture of Tris-HCl buffer (0.7 mL), ascorbic acid (0.2 mM), microsomal suspension (0.5 μg protein/mL), and 10 μL of sample solution. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of a 1:1 mixture of 0.25 mL of TCA (3 M) and HCl (2 N) and then centrifuged at 3500*g* for 10 min. The reaction supernatant (1 mL) was mixed with 0.67% (w/v) thiobarbituric acid (0.25 mL) and then heated in boiling water for 10 min. Lipid peroxidation was assessed by measuring the thiobarbituric acid reactive products at 532 nm. Lipid peroxidation inhibitory activity was calculated as follows: $[1 - (T-B)/(C-B)] \times 100$ (%), in which *T*, *C*, and *B* are absorbance values of the sample treatment, the control without sample, and the zero time control, respectively.

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