Schisandrene, a Dibenzocyclooctadiene Lignan from *Schisandra chinensis*: Structure−Antioxidant Activity Relationships of Dibenzocyclooctadiene Lignans[⊥]

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Phytochemical investigation of the fruits of *Schisandra chinensis* led to the isolation of 13 lignans including schisandrene (**13**), a new lignan based on a dibenzocyclooctadiene backbone with an exocyclic double bond. Its structure and absolute configuration were established using NMR, MS, and CD data. Antioxidant activity of the lignans was evaluated using a DCFH-DA cellular-based assay. The structure−activity relationships of the dibenzocyclooctadiene lignans showed that the exocyclic methylene functionality was essential for antioxidant activity, with the benzoyloxy group probably enhancing such effects.

Lignans are the most common constituents of *Schisandra chinensis* (Turcz.) Baill. (Magnoliaceae). Dried fruits of *S. chinensis* are extensively used in the traditional medicinal systems of Korea, China, and Japan.[1] Several C_{18} dibenzocyclooctadiene lignans were previously isolated from *S. chinensis* and showed anticancer,[2,3] antihepatocarcinogenesis,[4,5] antihepatotoxic,[6] anti-HIV,[7] antioxidant,[8,9] and anti-inflammatory[10] activities. *S. chinensis* has also been employed in the treatment and prevention of some chronic diseases such as inflammation, hepatitis, and cancer,[11] which may result from damage to biomolecules by free radicals and reactive oxygen species (ROS). Since ROS and oxidative stress play an important role in the etiology and progression of major human degenerative diseases[12] such as cancer, inflammation, atherosclerosis, and aging,[13] considerable attention has been focused on natural products that act as endogenous and exogenous antioxidants. In particular, phenolic compounds in foods have shown an ability to scavenge free radicals and are reportedly linked to anticarcinogenic and antiatherogenic activities.[14] Several studies of antioxidants examined the reduction potential of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) or the radical-scavenging effects of natural products in solution-based or TLC-based DPPH bioautographic chemical assays.[15] However, it is important to evaluate the effects of antioxidants within living cells. Fluorescent technology has recently focused on the antioxidant effects of natural products in living cell systems using specific probes such as 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA).[16,17] This cell-based fluorescent method is useful to directly examine the ability of natural products to penetrate cell membranes and inhibit ROS in living human cells. The antioxidant activity of the phenolic constituents is related to their structures, but little information on antioxidant properties of lignans has been published.[8,18] The present study focuses on the structure elucidation of a new dibenzocyclooctadiene lignan (**13**) and an investigation of lignan structure−activity relationships using the DCFH-DA cellular-based assay for antioxidant activity.

Results and Discussion

The dried fruits of *S. chinensis* were successively extracted with hexane, EtOAc, and then MeOH. The hexane extract led to the isolation of schisandrin A (**1**),[19] gomisin K3 (**2**),[20] schisandrin (**3**),[21] gomisin A (**4**),[22] tigloylgomisin H (**6**),[23] benzoylgomisin H (**7**),[22] schisandrin C (**8**),[23] gomisin N (**9**),[23] and gomisin J (**10**).[23] The EtOAc extract was chromatographed on a silica gel column to afford gomisin B (**11**),[21] tigloylgomisin H (**6**), and schisandrene (**13**). The MeOH extract was chromatographed on a silica gel column to yield gomisin G (**12**),[21] angeloylgomisin H (**5**),[22] and schisandrin (**3**). The structures of these compounds are shown in Table 1.

Schisandrene (**13**) was obtained as a yellow oil with $[\alpha]^{25}_{D}$ −13 (*c* 0.51, $CHCl_3$). The positive HRTOFMS indicated a molecular ion at *m*/*z* 520 corresponding to $[M + NH_4]^+$, thus indicating a molecular formula of $C_{30}H_{28}NH_4O_8$. The IR spectrum displayed a band at 1717 cm^{-1}, suggesting the presence of an ester carbonyl functionality. Analysis of 1D- and 2D-NMR data with homo- and heteronuclear direct and long-range correlations permitted assignments of the ^1H and ^{13}C NMR resonances as listed in Table 2. The ^{13}C NMR and DEPT spectra showed 29 signals, including 18 carbons for three aromatic rings, two methylenedioxy groups (*δ* 101.5 and 100.8), two *O*-methyls at *δ* 59.9 and 59.2, and an ester carbonyl carbon at *δ* 165.2. It further showed the presence of a benzylic methylene at *δ* 41.1 and an oxygenated carbon at *δ* 81.2. The presence of a quaternary olefinic carbon at *δ* 149.8 and an olefinic methylene at *δ* 111.9 indicated an exocyclic double bond. The two aromatic protons of the biphenyl moiety resonated at *δ* 6.75 and 6.50, respectively, and two methylenedioxy groups at *δ* 5.97 (2H, d) and 5.75 (1H, s) and 5.83 (1H, s). One-proton resonances at *δ* 5.06 and 4.78 were due to the exocyclic methylene protons. The three-proton doublet at *δ* 1.23 is indicative of the C-8 methyl group. Proton multiplets at *δ* 7.63 (2H), 7.32 (2H), and 7.48 (1H) indicated the presence of a monosubstituted phenyl ring. HMBC correlations observed between H-7′ and C═O and from H-2″ and H-6″ to C═O suggested the presence of a benzoyloxy group at C-7′ of the cyclooctadiene ring. Cross-peaks were also observed between H-8 and C-9 and between H-9 and C-8′, C-8, and C-7. Correlations between H-9′ and C-8′, C-7′, and C-8 established that the terminal methylene of the double bond was at C-8′. Moreover, the HMBC spectrum confirmed the position of the *O*-methyl groups, showing correlations between the OCH_3 protons at *δ* 3.79 and C-3′ and at *δ* 3.59 with C-3, and the methylenedioxy protons showed correlations between resonances at *δ* 5.97 (2 × OCH_2O) and C-4′ and C-5′ and at *δ* 5.83 and 5.75 (OCH_2O, 1H each) with C-4 and C-5.

The CD spectrum showed a negative Cotton effect at 265 nm, indicating *M*-helicity (conformation) and hence an *S*-configured

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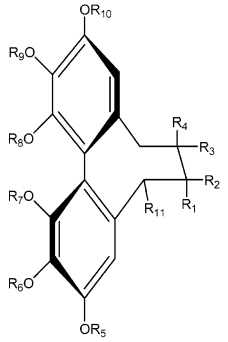
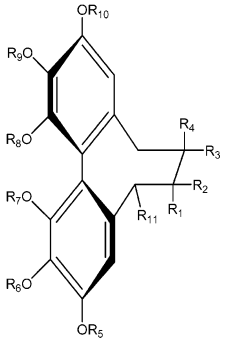
Table 1. Structures of Lignans from Fruits of *S. chinensis*



R-biphenyl configuration



S-biphenyl configuration

No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}
1	H	CH_3	H	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	H
2	H	CH_3	H	CH_3	CH_3	CH_3	CH_3	H	CH_3	CH_3	H
3	OH	CH_3	H	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3	H
4	OH	CH_3	H	CH_3	CH_3	CH_3	CH_3	CH_3	CH_2		H
5	OH	CH_3	CH_3	H	CH_3	CH_3	CH_3	Angeloyl-	CH_3	CH_3	H
6	OH	CH_3	CH_3	H	CH_3	CH_3	CH_3	Tigloyl-	CH_3	CH_3	H
7	OH	CH_3	CH_3	H	CH_3	CH_3	CH_3	Benzoyl-	CH_3	CH_3	H

No.	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}
8	CH_3	H	CH_3	H	CH_2		CH_3	CH_3	CH_2		H
9	CH_3	H	CH_3	H	CH_3	CH_3	CH_3	CH_3	CH_2		H
10	CH_3	H	CH_3	H	H	CH_3	CH_3	CH_3	CH_3	H	H
11	OH	CH_3	CH_3	H	CH_3	CH_3	CH_3	CH_3	CH_2		Angeloyloxy
12	OH	CH_3	CH_3	H	CH_2		CH_3	CH_3	CH_3	CH_3	Benzoyloxy
13	$=CH_2$		H		CH_3	CH_2	CH_3	CH_3	CH_2		Benzoyloxy

Table 2. ^1H (500 MHz) and ^{13}C NMR (150 MHz) Data of Schisandrene (**13**)

position	δ_C	δ_H	H−C long-range correlations[a]
1	135.4		
2	121.2		
3	141.3		
4	134.6		
5	149.0		
6	102.8	6.50, s	149.0, 134.6, 135.4, 121.2, 41.1
7	41.1	β 2.53, dd, 2.0/2.0	149.8, 135.4, 121.2, 102.8, 39.0,29.9,20.8,
		α 2.25, dd, 6.8/6.8	149.8, 135.4, 121.2, 102.8, 39.0
8	39.0	2.64, m	41.1
9	20.8	1.23, d, 4.4	41.1, 39.0
1′	133.4		
2′	121.6		
3′	141.6		
4′	137.0		
5′	148.8		
6′	104.2	6.75, s	148.8, 137.0, 133.4, 121.6, 81.
7′	81.2	6.46, s	165.2, 149.8, 133.4, 121.6, 111.9, 104.2, 39.0
8′	149.8		
9′	111.9	a 5.06, s	149.8, 81.2, 39.0
		b 4.78, s	81.2, 39.0
OCH_3	59.9	3.79, s	141.6
OCH_3	59.2	3.59, s	141.3
OCH_2O	101.5	5.97, d, 8.0	148.8, 137.0
OCH_2O	100.8	5.83, s	149.0, 134.6
		5.75, s	149.0, 134.6
1″	130.2		
4″	132.9	7.48, t	129.8, 128.1
2″, 6″	129.8×2	7.63, d, 5.2	165.2, 130.2, 128.1
3″, 5″	128.1×2	7.32, t	130.2, 132.9, 129.8
C=O	165.2		

[a] $^1H-^{13}C$ long-range correlations (HMBC) correspond to two- or three-bond connectivities.

biphenyl axis.[21] With the axial chirality defined, a NOESY experiment was used to establish the absolute configuration at the remaining stereocenters. In the NOESY experiment, H-7′ showed correlations with H-9′a, H-9′b, and H-8, while H-8 correlated with H-7α. These observations were consistent only with a cyclooctadiene lignan with a twisted boat/chair conformation having C-7′ (*S*) and C-8 (*R*) absolute configuration. The computer-generated perspective drawing (Figure 1) confirmed the proximity of these protons and hence the feasibility of the observed NOESY interactions.

The lignans from *S. chinensis* were assessed for their antioxidant capacity. To clarify their structure−antioxidant activity relationships, the relevant pure lignans were obtained through separation and purification by column chromatography and confirmed by HPLC. Schisandrin (**3**) and gomisin N (**9**) were the major lignans in the fruits of *S. chinensis*. Both *R*- and *S*-configured biphenyl bonds were found for the dibenzocyclooctadiene functionality (Table 1). Substituents comprised hydroxy, benzoyl, and exocyclic methylene groups on the cyclooctadiene ring, and angeloyl, tigloyl, benzoyl, methyl, and methylenedioxy units on the two aromatic rings.

Several studies of plant-derived antioxidants have examined the reduction potential or radical-scavenging effects of natural products in solution-based chemical assays.[24−27] However, researchers have started to probe the antioxidant effects of natural products in living systems.[16] A cell-based method to directly examine the ability of natural products to penetrate living human cells and inhibit ROS-catalyzed oxidation was used to evaluate the 13 lignans from *S. chinensis* for their ability to scavenge exogenous ROS induced by PMA (phorbol 12-myristate-13-acetate) in HL-60 cells.

The structural differences of the dibenzocyclooctadiene lignans significantly affected the DCFH-DA cellular-based antioxidant activity. Seven of the lignans from *S. chinensis* were found to possess DCFH-DA antioxidant activity (2.8−160.9 μM) (Table 3). Generally, the number and positions of hydroxy, benzoyl, tigloyl, angeloyl, or exocyclic methylene groups in the cyclooctadiene ring and methylenedioxy or methyl groups of the phenolic rings influenced the antioxidant activity of these lignans. An exocyclic methylene on the cyclooctadiene ring resulted in a markedly increased antioxidant activity, but a benzoyl group induced only a small increase. Exocyclic methylene and benzoyl groups of lignans were found only for *S*-biphenyl dibenzocyclooctadiene lignans. The latter group of lignans showed poor activity in the DCFH-DA cellular-based assay. The present study also revealed the importance

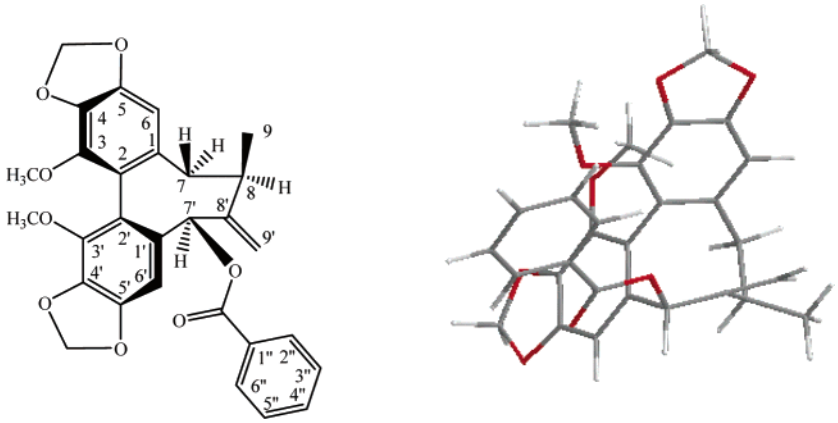


Figure 1. Structure and computer-generated perspective drawing of schisandrene (**13**).

Table 3. Inhibition on DCFH-DA Oxidation by TPA-Stimulated Hydrogen Peroxide and Cytotoxic Activity of Lignans in HL-60 Cells

compound	DCF [IC_{50} (μM)][a]	XTT [IC_{50} (μM)][b]
schisandrin A (**1**)	NA[c]	NA
gomisin K3 (**2**)	>155.3	56.2 ± 4.0
schisandrin (**3**)	NA	NA
gomisin A (**4**)	NA	NA
angeloylgomisin (**5**)	81.5 ± 4.8[d]	45.3 ± 2.2
tigloylgomisin H (**6**)	>124.9	44.3 ± 0.6
benzoylgomisin H (**7**)	NA	41.2 ± 1.0
schisandrin C (**8**)	NA	41.2 ± 2.4
gomisin N (**9**)	NA	52.4 ± 3.2
gomisin J (**10**)	>160.9	54.8 ± 2.8
gomisin B (**11**)	>116.5	NA
gomisin G (**12**)	38.2 ± 1.9	48.3 ± 1.7
schisandrene (**13**)	2.8 ± 0.8	55.8 ± 8.2
vitamin C	10.2 ± 1.1	NA
Trolox	3.6 ± 0.4	NA
NDGA[e]	2.3 ± 0.6	12.2 ± 1.3

[a] DCFH-DA: 2′,7′-dichlorofluorescin diacetate. [b] XTT: 3′-1[(phenylamino)carbonyl]-3,4-tetrazoliumbis(4-methoxy-6-nitro)benzenesulfonic acid hydrate. [c] NA = not active. [d] The data are presented as means ± SD of IC_{50} based on triplicate measurements of fluorescence at 530 nm. [e] NDGA: nordihydroguaiaretic acid.

and influence of the benzoyl group on the cyclooctadiene and angeloyl groups on the aromatic rings of lignans on their DCFH-DA cellular-based activity. Gomisin G (**12**) and angeloylgomisin H (**5**) had DCFH-DA cellular-based activity, with IC_{50} values of 38.2 and 81.5 μM, respectively. Aromatic methylenedioxy groups in gomisin A (**4**), gomisin N (**9**), and schisandrin (**3**) did not increase antioxidant activity. It is interesting to note that the antioxidant activities of schisandrene (**13**) are comparable to the commercial antioxidant vitamin C and Trolox at the concentration tested.

The lignans (**1**−**13**) did not exhibit any anti-inflammatory activity in a cell-based assay using mouse macrophages and also did not inhibit the activity of COX-2 enzyme (data not shown). The compounds were also tested for their in vitro cytotoxicity toward mammalian kidney fibroblasts (Vero) and kidney epithelial (LLC-PK_1) cells. They were also tested for their in vitro anticancer potential in a panel of human solid tumor cells (SK-MEL malignant melanoma; KB epidermal carcinoma, oral; BT-549 ductal carcinoma, breast; and SK-OV-3 ovary carcinoma), as described previously.[30] However, the compounds did not show any activity up to a highest concentration of 48 μM in any of the cell lines tested (data not shown).

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-370 digital polarimeter. UV spectra were obtained using a Hewlett-Packard 8453 spectrophotometer. CD spectra were recorded in MeOH on a JASCO J-715 spectrometer. IR spectra were recorded on an AATI Mattson Genesis Series FTIR. NMR spectra (^{1}H, ^{13}C, COSY, HMQC, and HMBC) were recorded in $CDCl_3$ on a Bruker DRX 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C, running gradients and using residual solvent peaks as internal references. High-resolution mass spectra were recorded on a Bruker BioApex FT mass spectrometer.

Plant Material. Fruits of *S. chinensis* were collected in September 2002 from Mujoo, Korea. A voucher specimen (accession number SC-NCNPR-1) has been deposited in the Herbarium of The University of Mississippi. The plant was identified by one of the authors (Y.-W.C.).

Extraction and Isolation. The dried fruits of *S. chinensis* (2.5 kg) were ground to a fine powder and were successively extracted at room temperature with *n*-hexane, EtOAc, and MeOH. The hexane extract (15.2 g) was evaporated in vacuo and chromatographed on a silica gel column with *n*-hexane−EtOAc (0, 5%, 10%, 20%, 30% EtOAc in hexane and 5%, 10%, 15% acetone in $CHCl_3$) to give schisandrin A (**1**) (219 mg), gomisin K3 (**2**) (17.5 mg), schisandrin (**3**) (1326 mg), gomisin A (**4**) (308 mg), tigloylgomisin H (**6**) (21.7 mg), benzoylgomisin H (**7**) (9.3 mg), schisandrin C (**8**) (254.9 mg), gomisin N (**9**) (1126 mg), and gomisin J (**10**) (30 mg). The EtOAc extract was evaporated in vacuo, and the residue (20 g) was chromatographed on a silica gel column with *n*-hexane−EtOAc (0, 5%, 10%, 20%, 30% EtOAc in hexane; 5%, 10%, 15% acetone in $CHCl_3$; and 10% MeOH in $CHCl_3$) to give gomisin B (**11**) (7 mg), tigloylgomisin H (**6**) (16 mg), and schisandrene (**13**). The MeOH layer was dissolved in $CHCl_3$−MeOH (1:1) to give a $CHCl_3$−MeOH (MC) layer. This material (8.8 g) was chromatographed on a silica gel column with *n*-hexane−EtOAc (10%, 20%, 30%, 40% EtOAc in hexane and 5%, 10%, 30, 50, 50, 75% acetone in $CHCl_3$) to give gomisin G (**12**) (11.4 mg), angeloylgomisin H (**5**) (77.5 mg), and schisandrin (**3**) (298 mg).

Schisandrene (13): oily gum; $[\alpha]_D^{25}$ −13 (*c* 0.51, $CHCl_3$); UV (MeOH) λ_{max} 244 nm (log ϵ 1.8919); IR ν_{max}^{KBr} 1717 cm^{-1}; CD (*c* 0.1, $CDCl_3$) $[\theta]_{265}$ −130.828; ^{1}H and ^{13}C NMR data, see Table 2; HRTOFMS *m*/*z* 520.1953 (calcd for $C_{29}H_{26}NH_4O_8$, 520.5504).

Assay for Antioxidant Activity. Antioxidant activity was determined by the detection of the oxidative products with the 2′,7′-dichlorofluorescin diacetate (DCFH) method reported previously.[28]

Assay for in Vitro Cytotoxicity. The XTT (3′-1[(phenylamino)-carbonyl]-3,4-tetrazoliumbis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) assay was performed as reported earlier.[29]

The compounds were also tested for their in vitro cytotoxicity against a panel of human solid tumor cells (SK-MEL malignant, melanoma; KB epidermal carcinoma, oral; BT-549 ductal carcinoma, breast; and SK-OV-3 ovary carcinoma) as well as noncancerous kidney epithelial cells (LLC-PK_1) according to a modification of the procedure of Borenfreund et al.[30] Briefly, the cells were washed with saline followed by incubation for 3 h with a solution of neutral red. The cells were washed again to remove extracellular dye. A solution of acidified EtOH was added to liberate the incorporated dye from viable cells, and the absorbance was read at 450 nm.

Assay for Anti-inflammatory Activity. The effect of lignans on COX-2-mediated production of prostaglandin E2 in LPS-induced mouse macrophages was examined up to a highest concentration of 25 μg/mL.

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