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Phenolic Compounds from *Caesalpinia sappan* Heartwood and Their Anti-inflammatory Activity

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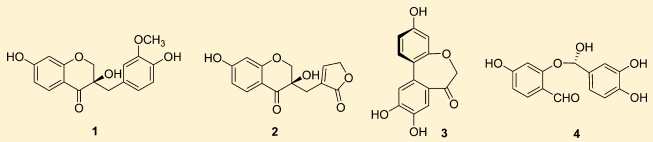
Supporting Information

ABSTRACT: Four new phenolic compounds, caesalpiniaphenols A–D (**1**–**4**), together with eight known compounds were isolated from *Caesalpinia sappan* heartwood. The chemical structures were established mainly by NMR, MS, ECD, and Mosher's method. Compounds **4**, **5**, and **7** showed weak inhibitory activity against the LPS-induced NO production in macrophage RAW264.7 cells with IC_{50} values of 12.2, 3.5, and 5.7 μM, respectively.



Inflammation is a central feature of many pathophysiological conditions in response to tissue injury and host defenses against invading microbes. Chronic inflammation and infections lead to the up-regulation of a series of enzymes and signaling proteins in affected tissues and cells. Proinflammatory cells, mainly activated macrophages, mediate most of the cellular and molecular pathophysiology of inflammation by producing cytokines and other pro-inflammatory molecules, including prostaglandins, enzymes, and free radicals such as NO. These enzymes are known to be involved in the pathogenesis of many chronic diseases including multiple sclerosis, Parkinson's and Alzheimer's diseases, and colon cancer.[1,2] NO is produced by iNOS in macrophages, hepatocytes, and renal cells, under stimulation of lipopolysaccharide (LPS), tumor necrosis factor-alpha (TNF-α), interleukin-1, or interferon-gamma.[3] After exposure to stimulants such as LPS from Gram-negative bacteria or lipoteichoic acid from Gram-positive bacteria, iNOS can be induced to trigger several disadvantageous cellular responses, including inflammation.[4] Therefore, NO production induced by LPS through iNOS can reflect the degree of inflammation, and a change in NO level through inhibition of iNOS enzyme activity or iNOS induction provides a means of assessing the effect of agents on the inflammatory process.

Caesalpinia sappan (Leguminosae) is distributed in southeast Asia, and its heartwood, sappan lignum, is famous as a red dyestuff. Sappan lignum is also used as an herbal medicine for inflammation and to improve blood circulation,[5] as well as for its anti-influenza,[6] anti-allergic,[7] and neuroprotective activities.[8] Many reports have shown that the main compounds in sappan lignum are phenolics, and they are divided into four structural subtypes: brazilin, chalcone, protosappanin, and homoisoflavonoids. Among the protosappanin derivatives, protosappanin B and isoprotosappanin B, 10-*O*-methyprotosappanin B and 10-*O*-methylisoprotosappanin B, and protosappanin E1 and protosappanin E2 occur as pairs of epimers. The homoisoflavonoid epimers sappanol and episappanol, 4-*O*-methylsappanol and 4-*O*-methylepisappanol, and 3′-*O*-methylsappanol and 3′-*O*-methylepisappanol have been successively isolated.[9]

Choi et al. confirmed that an ethanol extract of *C. sappan* ameliorates hypercholesterolemia in C57BL/6 mice and suppresses inflammatory responses in human umbilical vein endothelial cells (HUVECs) through an anti-oxidant mechanism.[10] Other compounds with a sappanchalcone skeleton show anti-inflammatory,[11,12] antibacterial, and anti-influenza activity.[13] In particular, brazilein, one of the main compounds of *C. sappan*, shows hepatoprotective,[14] anti-oxidant,[11] and neuroprotective activities.[15] To further study the inhibitory effect of LPS-induced NO production in macrophage RAW264.7 cells, fractionation of the EtOAc-soluble fraction resulted in the isolation of four new phenolic compounds (**1**–**4**), together with eight known compounds (**5**–**12**). This study describes the isolation and structural elucidation of these compounds, as well as the evaluation of their inhibitory effects on LPS-induced NO production in macrophage RAW264.7 cells.

RESULTS AND DISCUSSION

Repeated column chromatography (silica gel, RP-18, and semi-preparative HPLC) of the EtOAc-soluble fraction from *C. sappan*

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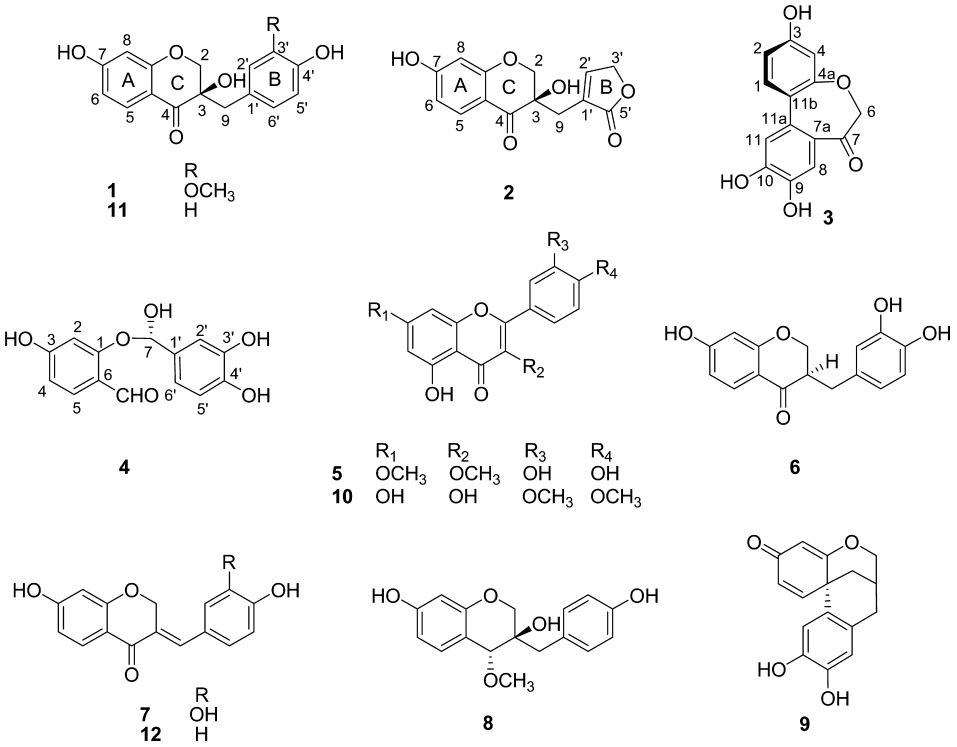

Figure 1. Chemical structures of isolated compounds (**1**–**12**).

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data for Compounds 1–4 (400 MHz and 100 MHz, methanol-d_4, δ values)

	1		2		3		4	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1					7.10, d (8.4)	131.1		147.2
2	4.14, d (11.6)	73.5	4.30, d (11.6)	74.8	6.70, dd (2.4, 8.4)	113.5	7.28, s	115.4
	4.02, d (11.6)		4.19, d (11.6)					
3		74.3		73.3		159.8		153.8
4		195.9		194.2	6.66, d (2.4)	108.9	6.89, d (8.4)	116.3
4a		113.4		113.4		159.2		
5	7.72, d (8.8)	130.5	7.72, d (8.8)	130.7			7.29, d (8.4)	126.5
6	6.55, dd (2.4, 8.8)	112.3	6.55, br d (8.8)	112.5	4.46, s	78.9		130.9
7		166.8		167.0		207.9	5.19, s	104.9
7a						125.0		
8	6.36, d (2.4)	103.7	6.35, br s	103.6	6.72, s	117.5		
8a		165.0		164.9				
9	2.91, d (14.0)	41.3	2.82, d (14.8)	30.9		145.8		
	2.85, d (14.0)		2.71, d (14.8)					
10						145.5		
11					6.72, s	117.6		
11a						131.9		
11b						127.3		
1′		127.8		128.3				131.2
2′	6.81, d (2.0)	115.5	7.51, br s	152.3			6.84, s	114.9
3′		148.6	4.98 (2H, overlap)	72.4				146.7
4′		146.6						146.6
5′	6.71, d (8.0)	115.9		177.1			6.71, d (8.0)	115.9
6′	6.66, dd (2.0, 8.0)	124.5					6.73, d (8.0)	119.6
3′-OCH_3	3.83, s	56.4						
6-CHO							9.67, s	193.2

heartwood resulted in the isolation of 12 phenolic compounds. The chemical structures of the known compounds were identified as quercetin-3,7-di-*O*-methyl ether (**5**), 3-deoxysappanone B (**6**), sappanone A (**7**), 3′-deoxy-4-*O*-methylepisappanol (**8**), 10,11-dihydroxydracaenone C (**9**), quercetin-3′,4′-di-*O*-methyl ether (**10**), 3′-deoxysappanone B (**11**), and 3′-deoxysappanone A (**12**), by comparing the physicochemical and spectroscopic data with those previously reported.[16−22]

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Caesalpiniaphenol A (**1**) was obtained as colorless needles following crystallization from MeOH, with the molecular formula $C_{17}H_{16}O_6$, as determined by the HRFABMS at *m/z* 317.1025 $[M + H]^+$. The IR spectrum of **1** suggested the presence of a hydroxy group (3450 cm^{-1}), a carbonyl group at 1720 cm^{-1}, and aromatic absorption (1430 cm^{-1}). The ^1H NMR spectrum showed the typical splitting pattern for a 3-hydroxy-3-benzyl-4-chromanone-type homoisoflavonoid with the H-2 resonances occurring as a pair of doublets at δ_H 4.02 and 4.14 (each 1H, d, J = 11.6 Hz), with a benzylic methylene signal H-9 at δ_H 2.85 and 2.91 (each 1H, d, J = 14.0 Hz). The O-substituted quaternary carbon at δ_C 74.3 was assigned to C-3.[23] The ^1H NMR spectrum showed one methoxy resonance at δ_H 3.83 (3H, s). One aromatic proton singlet at δ_H 6.81 (d, J = 2.0 Hz, H-2′), and a pair of *ortho*-coupled resonances at δ_H 6.71 (d, J = 8.0 Hz, H-5′) and 6.66 (dd, J = 2.0, 8.0 Hz, H-6′), accounting for the three protons of the B-ring. Resonances ascribed to H-5 (δ_H 7.72, d, J = 8.8 Hz), H-6 (δ_H 6.55 dd, J = 2.4, 8.8 Hz), and H-8 (δ_H 6.36, d, J = 2.4 Hz) were located on ring A (Table 1). Correlations in the NOESY spectrum between the H-2′ resonances and the methoxy proton resonances at δ_H 3.83, respectively, enabled placement of the methoxy group at C-3′. The ^{13}C NMR spectrum of **1** contained 17 carbon signals. The signal at δ_C 56.4 belonged to the methoxy group, and the other 16 signals were consistent with the chroman-4-one skeleton. The HMQC and ^{13}C NMR spectra indicated the presence of eight quaternary carbons in **1**. The chemical shifts of these quaternary carbons indicated that of the 12 aromatic carbons, four were oxygen-bearing carbons. The C-4 carbonyl functionality was evident at δ_C 195.9 (Table 1). The complete NMR assignments and connectivity of **1** were further determined by analysis of the HMQC and HMBC spectroscopic data. The HMBC spectrum confirmed the correlations between H-2′ (δ_H 6.81) and the C-3′ methoxy protons (δ_H 3.83) and C-3′ (δ_C 148.6). Diagnostic HMBC correlations were observed for H-2 to C-3 (δ_C 74.3), C-4 (δ_C 195.9), and C-8a (δ_C 165.0) and H-9 to C-3 (δ_C 74.3), C-4 (δ_C 195.9), and C-1′ (δ_C 127.8) (Figure 2).

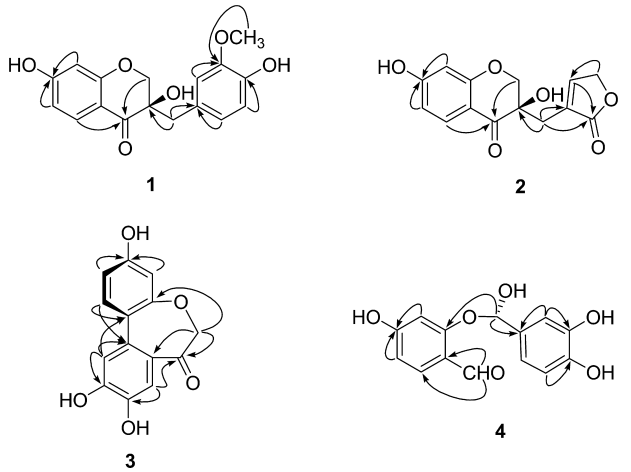

Figure 2. Selected HMBC correlations (H → C) for new compounds **1**–**4**.

The electronic circular dichroism (ECD) spectrum of **1** showed a negative Cotton effect at $\Delta\varepsilon_{249}$ −3.11 and a positive Cotton effect at $\Delta\varepsilon_{281}$ +9.21, suggesting a 3*R* configuration by comparison with (3*R*)-3-(3,4-dihydroxybenzyl)-7-hydroxychroman-4-one.[22,24] On the basis of the above analysis, the structure of compound **1** was elucidated as (3*R*)-3,7-dihydroxy-3-(4′-hydroxy-3′-methoxybenzyl)chroman-4-one.

Caesalpiniaphenol B (**2**) was obtained as a colorless, amorphous powder, with the molecular formula $C_{14}H_{12}O_6$, as determined by the HRFABMS at *m/z* 277.0712 $[M + H]^+$. The IR spectrum of **2** suggested the presence of a hydroxy group (3448 cm^{-1}) and two carbonyl groups (at 1745 and 1740 cm^{-1}). The ^1H NMR spectrum showed the H-2 resonances as a pair of doublets at δ_H 4.19 and 4.30 (each 1H, d, J = 11.6 Hz) of ring C and the methylene signal H-9 at δ_H 2.71 and 2.82 (each 1H, d, J = 14.8 Hz). The three aromatic protons of ring A resonated at δ_H 7.72 (d, J = 8.8 Hz, H-5), 6.55 (br d, J = 8.8 Hz, H-6), and 6.35 (br s, H-8) (Table 1). Diagnostic signals for the unsaturated furanone portion of **2** were the olefinic proton at δ_H 7.51 (br s, H-2′), the methylene protons δ_H 4.98 (2H, overlap), olefinic ^{13}C NMR signals at δ_C 128.3 (C-1′) and 152.3 (C-2′), an oxymethylene carbon signal at δ_C 72.4 (C-3′), and a carbonyl at δ_C 177.1 (C-5′) (Table 1). These conclusions were further supported by the analysis of the HMQC and HMBC spectroscopic data. The HMBC spectrum confirmed the correlations between protons H-9, H-2′, and H-3′ and C-5′ and between H-2′ and C-1′, C-3′, and C-5′. Diagnostic HMBC correlations were observed between H-9, H-2, and H-5 and C-4 (Figure 2). The ECD spectrum of **2** showed a negative Cotton effect at $\Delta\varepsilon_{256}$ −2.64 and a positive Cotton effect at $\Delta\varepsilon_{278}$ +6.84, suggesting a 3*R* configuration by comparison with (3*R*)-3-(3,4-dihydroxybenzyl)-7-hydroxychroman-4-one.[22] On the basis of the above analysis, the structure of compound **2** was elucidated as (3*R*)-3,7-dihydroxy-3-[furan-2′(5*H*)-one]chroman-4-one.

Caesalpiniaphenol C (**3**) was obtained as yellow needles following crystallization from MeOH, with the molecular formula $C_{14}H_{10}O_5$ as determined by the HRFABMS at *m/z* 259.0612 $[M + H]^+$. The IR spectrum of **3** suggested the presence of a hydroxy group (3448 cm^{-1}) and a carbonyl group at 1739 cm^{-1}. The ^1H NMR spectrum of **3** showed a singlet methylene signal at δ_H 4.46. The ^{13}C NMR spectrum of **3** (Table 1) exhibited one methylene and one carbonyl carbon signal. The HMBC spectrum indicated a correlation between H-6 and the carbonyl carbon signal (δ_C 207.9), suggesting the relative position of these atoms (Figure 2). On the basis of these results, a partial structure A was proposed for **3**.[25] The ^1H NMR spectrum also showed an aromatic ABC system at δ_H 7.10 (1H, d, J = 8.4 Hz, H-1), 6.70 (1H, dd, J = 2.4, 8.4 Hz, H-2), and 6.66 (1H, d, J = 2.4 Hz, H-4) and a two-proton aromatic singlet at δ_H 6.72 (2H, s) (Table 1). The ^{13}C NMR spectrum of **3** exhibited 12 aromatic carbon signals, and the HMQC revealed these to be methine signals at δ_C 131.1, 117.6, 117.5, 113.5, and 108.9, with the remaining seven signals due to quaternary carbons. The HMBC spectrum revealed correlations of H-1 and H-11 with C11a (δ_C 131.9) and δ_H 6.70 (H-2) and 6.72 (H-11) with a carbon signal at δ_C 127.3 (C-11b). In the NOE spectrum, an association was observed between the protons signals δ_H 6.72 (H-11) and 7.10 (H-1). These observations led to a biphenyl-type partial structure for B.[25] The connection of the two partial A and B structures of **3** was established by HMBC correlations between H-6 and C-4a and C-7a and also between H-8 and the carbonyl carbon (δ_C 207.9) (Figures 2 and 3). The ECD spectrum showed a negative Cotton effect at $\Delta\varepsilon_{256}$ −8.64, indicating *M*-helicity (conformation) and hence an *S*-configured biphenyl axis.[26] Thus, compound **3** was determined to be (a*S*)-3,9,10-trihydroxy-7-hydro-6*H*-dibenz[*b*,*d*]oxocin-7-one.

Caesalpiniaphenol D (**4**) was isolated to give a light brown, amorphous powder with the molecular formula $C_{14}H_{12}O_6$, as determined by HRFABMS at *m/z* 277.0714 $[M + H]^+$. The IR spectrum suggested the presence of a hydroxy group (3440 cm^{-1}), a

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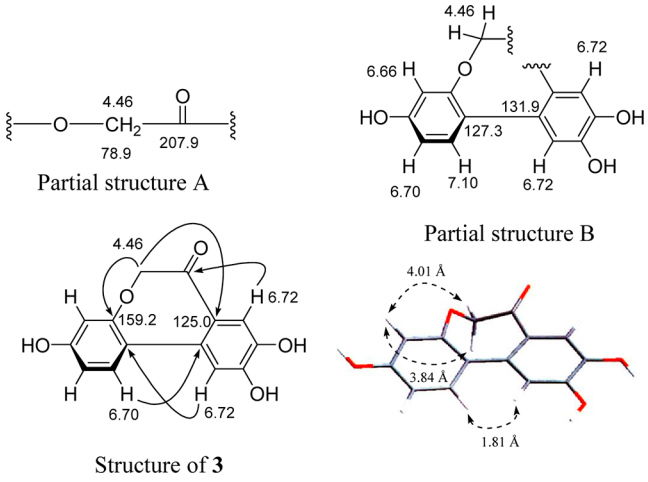

Figure 3. ^{1}H and ^{13}C chemical shifts and NOE observed in the NOE difference spectrum (↔) in methanol-d_4.

formyl carbonyl group at 1730 cm^{-1}, and aromatic absorption (1421 cm^{-1}). ^{1}H NMR signals at δ_H 9.67 (1H, s) and δ_H 5.19 (1H, s) revealed the presence of formyl and hemiketal methine groups (Table 1). The spectrum also showed protons at δ_H 6.71−7.29 ppm (6H), indicating the presence of 1,2,4-trisubstituted and 1,3,4-trisubstituted benzene rings. The ^{13}C NMR spectrum showed 14 carbon signals. Those at δ_C 193.2 and 104.9 belonged to formyl and hemiketal methine groups, and the remaining were aromatic carbon signals. The full NMR assignments and connectivities of **4** were determined by analysis of HMQC and HMBC spectroscopic data. The HMBC spectrum confirmed the correlations between H-7 (δ_H 5.19, s) and C-1, C-6′, and C-2′, in agreement with the structure shown. Diagnostic HMBC correlations were observed between the formyl proton (δ_H 9.67, s) and C-1, C-6, and C-5, suggesting that the formyl group was located at C-6. The modified Mosher's method was used to produce (*R*)- and (*S*)-MTPA esters (**4a**, **4b**), and signals corresponding to H-4, H-5, and 6-CHO were relatively shielded in **4a** compared to **4b**. In contrast, signals for H-2′ and H-6′ were relatively deshielded in **4a** compared to **4b** (Figure 4),

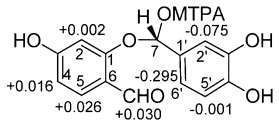

Figure 4. Results with the modified Mosher's method ($\Delta = \delta_S - \delta_R$) for compound **4**.

indicating the absolute configuration of **4** as 7*S*. On the basis of the above data analyses, compound **4** was established to be 1-[(7*S*)-7-hydroxy-1′-(3′,4′-dihydroxyphenyl)methanol]-3-hydroxybenzaldehyde.

The cytotoxic effects of the isolated compounds (**1**−**12**) were evaluated in the presence or absence of LPS by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. These compounds did not affect the cell viabilities of RAW 264.7 cells in either the presence or absence of LPS, even at a dose of 50 μM after a period of 24 h (data not shown). As shown in Table 2, compounds **4**, **5**, and **7** showed inhibitory effects with IC_{50} values of 12.2, 3.5, and 5.7 μM. Compounds **6**, **8**, and **10** displayed moderate effects with IC_{50} values of 42.4, 33.4, and 35.0 μM, respectively, but the others were inactive. Because compounds **4**, **5**, and **7** exhibited an inhibitory effect on the LPS-induced production of NO in macrophage RAW264.7 cells, the effects of these compounds on LPS-induced iNOS expression were investigated. RAW264.7 cells were stimulated with 1 μg/mL of LPS for 18 h in the presence of increasing concentrations of **4**, **5**, and **7**, and the expression levels of iNOS protein were determined by immunoblot analyses (Figure 5). Compounds **4**, **5**, and **7** (0−10 μM) showed a dose-dependent reduction in LPS-induced iNOS expression, but did not change the alpha-tubulin expression. The results showed that **4**, **5**, and **7** inhibited iNOS activities in LPS-stimulated RAW264.7 cells, suggesting that these compounds could suppress LPS-induced iNOS expressions at the transcription level. In addition, these compounds were further investigated on the LPS-induced TNF-α release. Except for compound **4**, pretreatment of cells with compounds **5** and **7** in several concentrations (5−50 μM) decreased the TNF-α production (Table 2). Since degradation of IκB-α protein is an essential step for NF-κB activation, we examined the effect of compounds **5** and **7** on the induced degradation and phosphorylation of IκB-α protetin by LPS (Figure 6). Cystoplasmic extracts were analyzed in the presence of IκB-α; it was almost completely degraded in 15 min after stimulation with LPS and resynthesized in 30 min. However, preincubation with **5** significantly prevented the induced degradation of IκB-α protein at 10 and 15 min; meanwhile, preincubation with **7** prevented the induced degradation of IκB-α protein at 5 and 15 min. The resynthesis of IκB-α, which is under control of NF-κB, was also significantly suppressed by both compounds (Figure 6). In the NO-scavenging activity assay, all the isolated compounds showed potential scavenging activity with IC_{50} values ranging from 65.3 to 156.5 μM.

Table 2. Inhibition of LPS-Induced NO, TNF-α Production in Macrophage RAW264.7 Cells, and NO Scavenging Activity of Compounds 1−12

	IC_{50} value (μM)[a]		
compound	NO	TNF-α	NO-scavenging
1	>50	N.D.[d]	102.56 ± 25.3
2	>50	N.D.	95.62 ± 18.6
3	>50	N.D.	86.25 ± 18.8
4	12.2 ± 1.5	>50	112.52 ± 27.4
5	3.5 ± 0.4	5.8 ± 0.6	102.95 ± 19.5
6	42.4 ± 5.2	N.D.	65.34 ± 11.2
7	5.7 ± 0.6	8.3 ± 0.9	96.58 ± 23.5
8	33.4 ± 0.5	N.D.	114.56 ± 28.2
9	>50	N.D.	156.54 ± 30.4
10	35.0 ± 4.1	N.D.	85.34 ± 12.1
11	>50	N.D.	116.82 ± 18.9
12	>50	N.D.	95.45 ± 9.5
celastrol[b]	1.0 ± 0.1	0.8 ± 0.2	–[e]
ascorbic acid[c]	–	–	0.71 ± 0.1

[a]The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean ± SD). [b]Positive control for NO and TNF-α production. [c]Positive control for NO-scavenging. [d]N.D.: not determined. [e](−): no test.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured with a JASCO DIP 370 digital polarimeter. UV spectra were obtained in MeOH using a Thermo 9423AQA2200E UV spectrometer,

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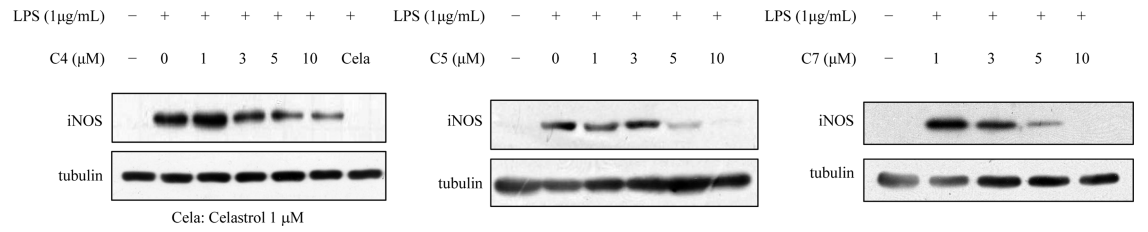

Figure 5. Inhibition of LPS-induced iNOS expression in RAW264.7 cells by compounds **4**, **5**, and **7** (C4, C5, and C7). RAW264.7 cells were pretreated for 30 min with the indicated concentrations of **4**, **5**, and **7**, followed by stimulation with LPS (1 μg/mL) for 18 h. Total lysates were prepared, and the expression levels of iNOS were determined by analysis of immunoblot. Histograms show densitometry analyses of relative iNOS expression levels normalized against α-tubulin.

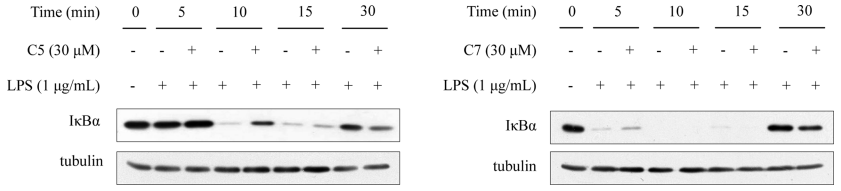

Figure 6. Inhibition of LPS-induced IκB-α degradation expression in RAW264.7 cells by compounds **5** and **7** (C5 and C7). RAW264.7 cells were pretreated for 30 min with the indicated concentrations of **5** and **7**, followed by stimulation with LPS (1 μg/mL) for 18 h. Total lysates were prepared, and the expression levels of IκB-α were determined by analysis of immunoblot. Histograms show densitometry analyses of relative IκB-α expression levels normalized against α-tubulin.

and IR spectra were obtained on a Bruker Equinox 55 FT-IR spectrometer. The nuclear magnetic resonance (NMR) spectra were obtained on a Varian Unity Inova 400 MHz spectrometer. ECD spectra were recorded on a JASCO J-810 spectropolarimeter. Silica gel (Merck, 63−200 μm particle size) and RP-18 silica gel (Merck, 75 μm particle size) were used for column chromatography. TLC was carried out using Merck silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was performed using a Waters 600 Controller system with a UV detector and a YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size, YMC Co., Ltd., Japan), and HPLC solvents were from Burdick & Jackson, USA.

Plant Material. The *C. sappan* heartwood was purchased from a folk medicine market "Yak-ryoung-si" in Daegu, Korea, in May 2010. One of the authors (B.S.M.) performed botanical identification, and a voucher specimen (CUD-3174) was deposited at the Herbarium of the College of Pharmacy, Catholic University of Daegu, Korea.

Extraction and Isolation. The *C. sappan* heartwood (10 kg) was extracted with MeOH three times (3 h × 3 L) under reflux. After the solvent was removed under reduced pressure, the residue was suspended in H_2O and partitioned with *n*-hexane, EtOAc, and *n*-BuOH, successively. The EtOAc-soluble fraction (960 g) was separated on a silica gel column using a stepwise gradient of $CHCl_3$/MeOH (from 50 mL/1 mL to 100% MeOH, each 10 L) to yield 14 fractions (Fr.1−Fr.14) according to their TLC profiles. Fraction 5 (4.4 g) was subjected to reversed-phase (ODS-A) column chromatography and eluted with MeOH/H_2O (from 2 mL/1 mL to 100% MeOH, 2 L for each step) to afford seven subfractions (Fr.5-1 to Fr.5-7). Further purification of Fr.5-2 (630 mg) by semipreparative HPLC [using an isocratic solvent system of 30% MeOH in 0.1% TFA (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of **1** (14.1 mg; t_R = 38.5 min), **5** (5.8 mg; t_R = 44.8 min), and **6** (74.2 mg; t_R = 48.2 min). Fraction 5-3 (728 mg) was purified by semipreparative HPLC [using an isocratic solvent system of 30% MeOH in 0.1% TFA (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size], resulting in the isolation of **3** (28.5 mg; t_R = 40.8 min), **7** (42.0 mg; t_R = 44.9 min), and **10** (15.2 mg; t_R = 46.3 min), respectively. Further purification of Fr.5-5 (510 mg) by semipreparative HPLC [using an isocratic solvent system of 35% MeOH in 0.1% TFA (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size] resulted in the isolation of **4** (22.4 mg, t_R = 39.2 min), **8** (17.1 mg; t_R = 43.8 min), and **12** (16.3 mg, t_R = 46.2 min). Subfraction F.5-7 (354 mg) was further purified by semipreparative HPLC [using an isocratic solvent system of 40% MeOH in 0.1% TFA (flow rate 5 mL/min) over 90 min; UV detection at 210 nm; YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size], resulting in the isolation of **2** (4.8 mg, t_R = 42.9 min), **9** (12.7 mg, t_R = 46.2 min), and **11** (25.6 mg, t_R = 50.2 min).

Caesalpiniaphenol A (1): colorless needles (MeOH); mp 181−183 °C; $[\alpha]_D^{25}$ 49.2 (*c* 0.13, MeOH); IR ν_{max} (KBr) 3450, 2942, 1720, 1430, 1260, 1166 cm^{-1}; UV (MeOH) λ_{max} (log ε) 232 (4.12), 278 (3.87) nm; ECD (*c* 0.032 mM, MeOH) $\Delta\varepsilon_{249}$ −3.11, $\Delta\varepsilon_{281}$ +9.21; ^{1}H NMR (400 MHz, methanol-d_4) and ^{13}C NMR (100 MHz, methanol-d_4) spectroscopic data, see Table 1; HRFABMS *m*/*z* 317.1025 $[M + H]^+$ (calcd for $C_{17}H_{17}O_6$, 317.1025).

Caesalpiniaphenol B (2): colorless, amorphous powder; $[\alpha]_D^{25}$ 56.2 (*c* 0.10, MeOH); IR ν_{max} (KBr) 3448, 2941, 1745, 1740, 1427, 1257, 1169 cm^{-1}; UV (MeOH) λ_{max} (log ε) 226 (4.16), 270 (3.81), 284 (3.52) nm; ECD (*c* 0.036 mM, MeOH) $\Delta\varepsilon_{256}$ −2.64, $\Delta\varepsilon_{278}$ +6.84; ^{1}H NMR (400 MHz, methanol-d_4) and ^{13}C NMR (100 MHz, methanol-d_4) spectroscopic data, see Table 1; HRFABMS *m*/*z* 277.0712 $[M + H]^+$ (calcd for $C_{14}H_{13}O_6$, 277.0712).

Caesalpiniaphenol C (3): yellow needles (MeOH); mp 262−264 °C; $[\alpha]_D^{25}$ −22.7 (*c* 0.40, MeOH); IR ν_{max} (KBr) 3448, 2926, 1739, 1260, 1158, 1031 cm^{-1}; UV (MeOH) λ_{max} (log ε) 252 (4.46), 285 (4.13) nm; ECD (*c* 0.046 mM, MeOH) $\Delta\varepsilon_{256}$ −8.64; HRFABMS *m*/*z* 259.0612 $[M + H]^+$ (calcd for $C_{14}H_{11}O_5$, 259.0606); ^{1}H NMR (400 MHz, methanol-d_4) and ^{13}C NMR (100 MHz, methanol-d_4) spectroscopic data, see Table 1; ^{1}H NMR (400 MHz, pyridine-d_5) δ_H 7.43 (1H, d, *J* = 8.4 Hz, H-1), 7.13 (1H, dd, *J* = 2.4, 8.4 Hz, H-2), 7.21 (1H, d, *J* = 2.4 Hz, H-4), 4.73 (2H, s, H-6), 7.38 (1H, s, H-8), 7.40 (1H, s, H-11); ^{13}C NMR (100 MHz, pyridine-d_5) δ_C 131.2 (C-1), 113.9 (C-2), 160.7 (C-3), 109.7 (C-4), 159.2 (C-4a), 78.8 (C-6), 206.4 (C-7), 124.7 (C-7a), 118.5 (C-8), 147.4 (C-9), 147.1 (C-10), 118.6 (C-11), 131.5 (C-11a), 126.9 (C-11b).

Caesalpiniaphenol D (4): light brown, amorphous powder; $[\alpha]_D^{25}$ −24.4 (*c* 0.17, MeOH); IR ν_{max} (KBr) 3440, 2924, 1730, 1421, 1264 1165, 1031 cm^{-1}; UV (MeOH) λ_{max} (log ε) 233 (4.30), 279 (4.16), 312 (4.09) nm; ^{1}H NMR (400 MHz, methanol-d_4) and ^{13}C NMR (100 MHz, methanol-d_4) spectroscopic data, see Table 1; HRFABMS *m*/*z* 277.0714 $[M + H]^+$ (calcd for $C_{14}H_{13}O_6$, 277.0712).

Preparation of (*R*)- and (*S*)-MTPA Esters 4a and 4b from 4. A solution of **4** (1.0 mg) in dry pyridine (50 μL) was reacted with 5 μL of (*S*)-(+)-MTPA-Cl and 2 mg of 4-DMAP at room temperature for 30 min. The reaction mixture was dried by N_2 gas. The dried product

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was partitioned with CH_2Cl_2 and H_2O. The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by preparative TLC silica gel (0.25 mm thickness) developed with CH_2Cl_2–MeOH (5 mL/1 mL), and the product was eluted with CH_2Cl_2–MeOH (100 mL/10 mL) to furnish the ester, **4a** (1.3 mg). In a similar manner, **4b** (1.7 mg) was prepared from **4** (1.0 mg) using (*R*)-MTPA-Cl (5 μL) and 4-DMAP (2.0 mg).[27]

*(1-[(7S)-7-Hydroxy-1′-(3′,4′-dihydroxyphenyl)methanol]-3-hydroxybenzaldehyde (R)-MTPA ester (**4a**):* colorless oil; ^{1}H NMR (pyridine-d_5, 400 MHz) δ 9.984 (1H, s, 6-CHO), 8.111 (1H, d, J = 8.4 Hz, H-5′), 7.985 (1H, d, J = 2.0 Hz, H-2′), 7.818 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 7.520 (1H, s, H-2), 7.291 (1H, d, J = 8.0 Hz, H-5), 8.100 (1H, d, J = 7.6 Hz, H-4), 7.46–7.53 (5H, m, aromatic protons).

*(1-[(7S)-7-Hydroxy-1′-(3′,4′-dihydroxyphenyl)methanol]-3-hydroxybenzaldehyde (S)-MTPA ester (**4b**):* colorless oil; ^{1}H NMR (pyridine-d_5, 400 MHz) δ 10.014 (1H, s, 6-CHO), 8.110 (1H, d, J = 8.4 Hz, H-5′), 7.910 (1H, d, J = 2.0 Hz, H-2′), 7.523 (1H, dd, J = 2.0, 8.4 Hz, H-6′), 7.522 (1H, s, H-2), 7.317 (1H, d, J = 8.0 Hz, H-5), 8.260 (1H, d, J = 7.6 Hz, H-4), 7.46–7.53 (5H, m, aromatic protons).

Determination of NO Production and the Cell Viability Assay. The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants as described previously.[28] Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO, USA) for 24 h in the presence or absence of the test compounds (0.5–25 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled H_2O). The absorbance at 540 nm was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, USA), and the absorption coefficient was calibrated using a $NaNO_2$ solution standard. The amount of TNF-α in the culture supernatant was measured using the ELISA kit (R&D systems, Minneapolis, MN, USA). Cell viability was measured with the MTT-based colorimetric assay. For this experiment, celastrol was used as a positive control.

Immunoblot Analysis. Proteins were extracted from cells in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, 1 μg/mL leupeptin, 1 mM sodium vanadate, 150 mM NaCl). A 50 μg amount of protein (for iNOS) per lane was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk and then incubated with the corresponding antibody. The antibody for iNOS was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for α-tubulin was obtained from Sigma. Antibody for IκB-α was obtained from Cell Signaling Technology (Danvers, MA, USA). After binding of an appropriate secondary antibody coupled to horseradish peroxidase, proteins were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Pharmacia Biotec, Buckinghamshire, UK).[29]

Nitric Oxide Free Radical Scavenging Activity. A 50 μL amount of each of the concentrations of compounds previously dissolved in DMSO, as well as ascorbic acid (standard compound), was taken in separate tubes, and the volume was uniformly made up to 150 μL with MeOH. To each tube was added 2.0 mL of sodium nitroprusside (10 mM) in phosphate buffer saline. The solutions were incubated at room temperature for 150 min. A similar procedure was repeated with MeOH as blank, which served as a control. After the incubation, 5 mL of Griess reagent was added to each tube including the control. The absorbance of chromophore formed was measured at 546 nm on an Infinite F200Pro UV–visible spectrometer, Tecan, Switzerland. Ascorbic acid was used as a positive control. The IC_{50} value for each test compound as well as standard preparation was calculated.[30]

$$\%\text{scavenging/reduction} = [\text{absorbance of control} - \text{absorbance of test sample/absorbance of control}] \times 100$$

■ ASSOCIATED CONTENT

Supporting Information

Copies of ^{1}H, ^{13}C NMR and 2D spectra for **1**–**4** can be accessed free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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