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(7*R*,8*S*) AND (7*S*,8*R*) 8–5' LINKED NEOLIGNANS FROM EGYPTIAN HERBAL MEDICINE *ANASTATICA HIEROCHUNTICA* AND INHIBITORY ACTIVITIES OF LIGNANS ON NITRIC OXIDE PRODUCTION

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Abstract — Three new (7*R*,8*S*) and (7*S*,8*R*) 8–5' linked neolignans named hierochins A (**1**), B (**2**), and C (**3**) were isolated from an Egyptian herbal medicine, the whole plants of *Anastatica hierochuntica*. The absolute stereostructures of new compounds were elucidated on the basis of chemical and physicochemical evidence. Both enantiomers, (7*R*,8*S*) and (7*S*,8*R*)-type neolignans, were found to coexist in a state of a little differing functional structure. The effects of isolated lignans on nitric oxide production in lipopolysaccharide-activated macrophages were examined and three known neolignan constituents were found to show inhibitory effects on nitric oxide production and induction of inducible nitric oxide synthase.

The Cruciferae plant, *Anastatica hierochuntica*, is widely distributed in the Sahara-Arabian desert areas and the whole plants of *A. hierochuntica* are prescribed for the treatment of fatigue and uterine haemorrhage in Egyptian folk medicine and are used by women as a charm for child birth.[1,2] During the course of our studies on bioactive constituents of Egyptian herbal medicines,[3–6] we reported the structure elucidation of then new skeletal benzofuranoflavanones, anastains A and B, with hepatoprotective activities from this herbal medicine.[7] As a continuation of the characterization studies on this natural medicine, we additionally isolated optically pure (7*R*,8*S*) and (7*S*,8*R*) 8–5' linked neolignans including three new neolignans termed hierochins A (**1**), B (**2**), and C (**3**) from the whole plants of *A. hierochuntica*. In addition, we examined the inhibitory effect of isolated lignans from the whole plants of *A. hierochuntica* on nitric oxide (NO) production in lipopolysaccharide

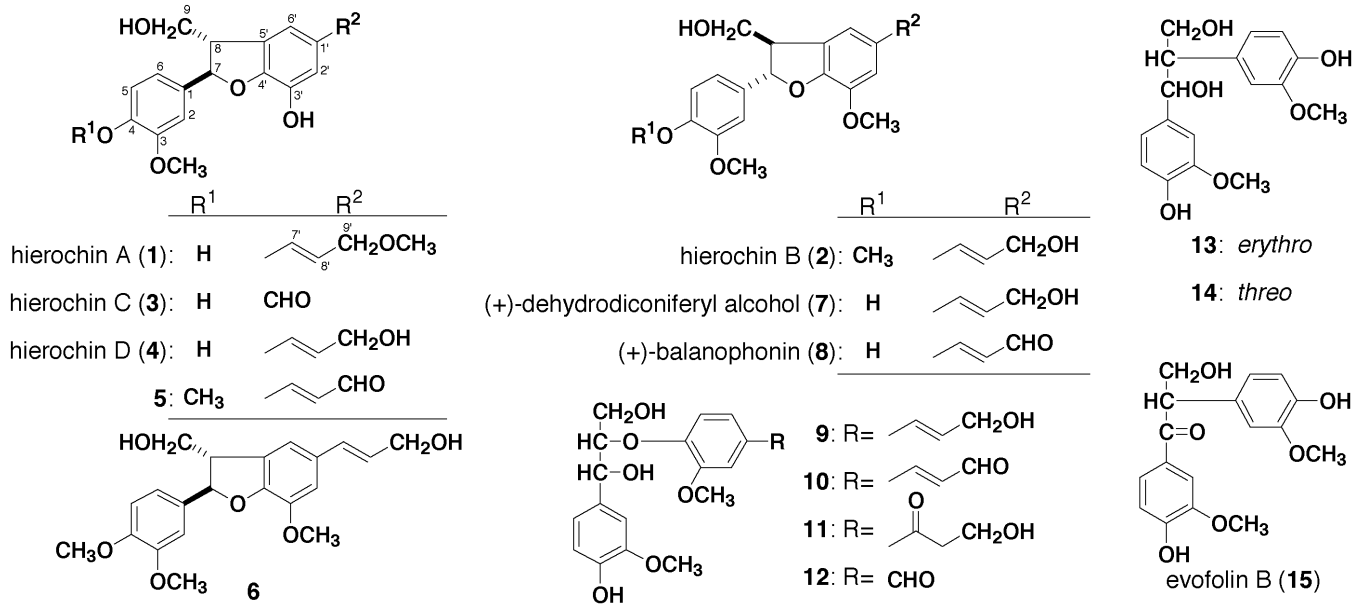

Chart 1

(LPS)-activated macrophages. This paper deals with the isolation of optically pure (7*R*,8*S*) and (7*S*,8*R*) type neolignans and the structure elucidation of three new neolignans as well as the NO production inhibitory activity of lignan constituents from this herbal medicine. Furthermore, we describe the inhibitory effect of several constituents with potent NO production inhibitory effect on induction of inducible NO synthase (iNOS).

The methanolic extract from the dried whole plants of Egyptian *A. hierochuntica* was partitioned into a mixture of ethyl acetate (EtOAc) and water to provide the EtOAc–soluble portion and an aqueous phase as described.[7] The aqueous phase was further extracted with 1-BuOH to give 1-BuOH and H_2O–soluble portions. The 1-BuOH–soluble portion was subjected to silica gel and ODS column chromatographies and finally HPLC to give hierochins A (**1**, 0.0005% from the herbal medicine), B (**2**, 0.0003%), and C (**3**, 0.0007%) together with hierochin D (**4**)[8] (0.0010%), **9**[9] (0.0006%), (+)-lariciresinol[10] (0.0024%), kaempferol[11] (0.0006%), luteolin[11] (0.0008%), rutin[11] (0.0007%), and β-sitosterol 3-*O*-β-D-glucopyranoside[12] (0.0025%). From the EtOAc–soluble portion, **5**[13] (0.0061%), (+)-dehydrodiconiferyl alcohol (**7**,[14] 0.0011%), (+)-balanophonin (**8**,[15] 0.0005%), **10**[16] (0.0002%), **11**[17] (0.0002%), **12**[18] (0.0006%), **13**[16,19] (0.0029%), **14**[16,19] (0.0011%), and evofolin B (**15**,[20] 0.0009%) were isolated by the similar procedure.[21]

Hierochin A (**1**), a pale yellow oil, $[\alpha]_D^{24}$ –32.2° (*c*=0.53, MeOH), $C_{20}H_{22}O_6$,[22] EI-MS *m/z* 358 (M^+, base peak), showed absorption maximum at 279 nm (log ε4.20) in the UV spectrum (in MeOH). The IR spectrum (film) of **1** showed absorption bands at 3432, 3282, 1655, 1630, 1518, 1509, 1275, and 1034 cm^{-1} ascribable to hydroxyl, olefin, aromatic ring, and ether functions. The ^{1}H- and ^{13}C-NMR (acetone-d_6, Table 1) spectra[23] of **1** showed signals assignable to two methoxyl groups [δ 3.29, 3.82 (3H each, both s, OCH_3-9' and 3)], dihydrofuran moiety [δ 3.54 (1H, m, H-8), 5.55 (1H, d, *J*=6.7 Hz, H-7)], two methylenes with an oxygen function [δ 3.83 (2H, m, H_2-9), 4.01 (2H, dd, *J* = 1.2, 6.1 Hz, H_2-9')], *trans*-olefinic protons [δ 6.11 (1H, dt, *J* = 15.8, 6.1 Hz, H-8'), 6.49 (1H, dt, *J* = 15.8, 1.2 Hz, H-7')], and five aromatic protons {*meta*-coupled protons [δ 6.85, 6.91 (1H each, both d, *J* = 1.8 Hz, H-2' and 6')], *ortho*- and *meta*-coupled ABC-type protons [δ6.83 (1H, d, *J* = 8.2 Hz, H-5), 6.89 (1H, dd, *J* = 1.9, 8.2 Hz, H-6), 7.06 (1H, d, *J* = 1.9 Hz, H-2)]. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR data of **1** were superimposable on those of **4**, except for the signals due to the 9'-methoxyl group. The 8–5' linked neolignan structure of **1** was clarified by ^{1}H–^{1}H COSY and HMBC experiments. Thus, the ^{1}H–^{1}H COSY experiments on **1** indicated the presence of four partial structures shown in bold lines in Figure 1. In the HMBC experiment, long-range correlations were observed between the following proton and carbon pairs: H-2 and C-3, 4, 6, 7; H-5 and C-1, 3; H-6 and C-2, 4, 7; H-7 and C-2, 6, 9; H-8 and C-1, 4', 5'; H-9 and C-7; H-2' and C-3', 4', 6', 7'; H-6' and C-8, 2', 7'; H-7' and C-1', 2', 6'; H-8' and C-1'; H-9' and O$\underline{C}H_3$-9'; O$C\underline{H}_3$-3 and C-3; O$C\underline{H}_3$-9' and C-9', so that the connectivities of the quaternary carbons and the positions of methoxyl groups in **1** were clarified.

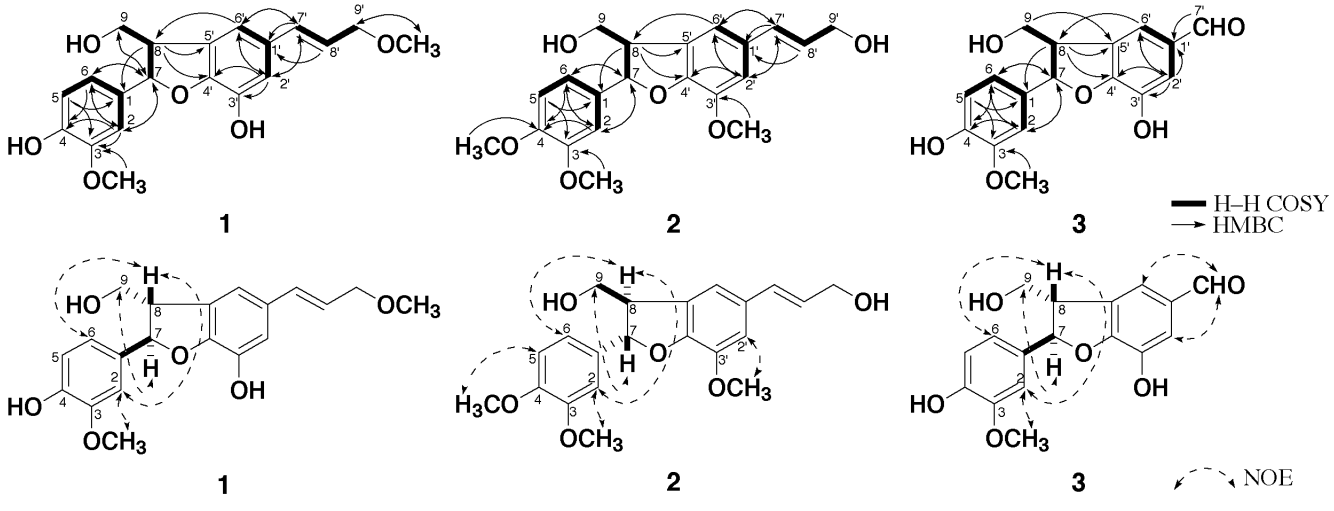

Figure 1

The relative stereostructure of the dihydrofuran moiety in **1** was elucidated by nuclear Overhauser enhancement spectroscopy (NOESY) experiment, which showed the NOE correlations between the 2, 6-protons and 8-proton and between the 7-proton and 9-protons as shown in Figure 1. The absolute stereostructure of **1** was elucidated by the circular dichroic (CD) spectroscopic analysis. Thus, the CD spectrum (in MeOH) of **1** showed positive [234 nm ($\Delta\varepsilon$ +1.62)] and negative Cotton effects [269 nm (–3.18), 287 nm (–3.46)], which indicated the absolute configurations of the 7 and 8-positions to be 7*R* and 8*S* orientations.[14] On the basis of this evidence, the structure of **1** was elucidated as shown.

Hierochin B (**2**), a colorless oil, $[\alpha]_D^{24}$ +29.7°(*c*=0.40, MeOH), $[\alpha]_D^{26}$ +40.9° (*c*=0.30, $CHCl_3$), $C_{21}H_{24}O_6$,[24] showed absorption maximum at 279 (log ε 4.20) nm in the UV spectrum (in MeOH). The IR spectrum (film) of **2** showed absorption bands at 3567, 3420, 1653, 1636, 1507, 1264, and 1026 cm^{-1} assignable to hydroxyl, aromatic ring, olefin, and ether functions. The ^{1}H- and ^{13}C-NMR (acetone-d_6, Table 1) spectra[23] of **2** showed signals assignable to a dihydrofuran moiety [δ 3.53 (1H, m, H-8), 5.60 (1H, d, *J*=6.4 Hz, H-7)], three methoxyl groups [δ 3.79, 3.80, 3.87 (3H each, all s, OCH_3-3, 4, and 3')], two methylenes with an oxygen function [δ 3.83 (2H, m, H_2-9), 4.18 (2H, dd, *J*=1.2, 5.8 Hz, H_2-9')], *trans*-olefinic protons [δ 6.24 (1H, dt, *J*=15.9, 5.8 Hz, H-8'), 6.53 (1H, dt, *J*=15.9, 1.2 Hz, H-7')], and five aromatic protons: *meta*-coupled protons [δ 6.95, 6.98 (1H each, both d, *J*=1.8 Hz, H-2' and 6')], *ortho*- and *meta*-coupled ABC-type protons [δ 6.92 (1H, d, *J*=8.3 Hz, H-5), 6.96 (1H, dd, *J*=1.9, 8.3 Hz, H-6), 7.04 (1H, d, *J*=1.9 Hz, H-2)]. The ^{1}H- and ^{13}C-NMR signals of **2** were very similar to those of **7**, except for the signals due to additional methyl group. The planer structure of **2** was determined by HMBC experiment, in which long-range correlations were observed as shown in Figure 1. Furthermore, the relative stereostructure of the dihydrofuran moiety in **2** was characterized on the basis of the NOESY experiment, in which the NOE correlations were observed between the following proton pairs of **2** (H-2, 6 and H-8; H-7 and H-9). Next, the CD spectrum of **2** [MeOH, 233 nm ($\Delta\varepsilon$ –4.94), 269 nm (+3.89), 286 nm (+4.16)] were observed and thus the absolute configurations of the 7 and 8-positions were elucidated as 7*S* and 8*R* orientations.[14] Furthermore, diazomethane methylation of (+)-dehydrodiconiferyl alcohol (**7**),[14] which was previously reported to be 7*S* and 8*R* configurations, yielded **2**. In order to confirm the optical purity of **2**, the (7*R*,8*S*) enantiomer (**6**)[25] of **2** was obtained by diazomethane methylation of **4**. Comparison of the physical data [**6**: $[\alpha]_D^{25}$ –32.0° (*c*=0.20, MeOH), $[\alpha]_D^{24}$ –47.8° (*c*=0.20, $CHCl_3$), CD spectra (MeOH): 232 nm ($\Delta\varepsilon$ +4.64), 269 nm (–4.59), 285 nm (–5.11)] and HPLC [detection: UV (279 nm), column: Ceremospher Chiral RU-2, mobile phase: CH_3CN–H_2O (50:50, v/v), flow rate: 0.5 ml/min, t_R: **2** (11.25 min), **6** (9.42 min)] for **2** with those for **6** led us to determine the absolute stereostructure of **2** as shown.

Table 1. ^{13}C-NMR Spectral Data for Hierochins A—C (**1**—**3**) and **6**

	1[a]	**2**[a]	**2**[b]	**3**[c]	**6**[b]		**1**[a]	**2**[a]	**2**[b]	**3**[c]	**6**[b]
C-1	134.4	135.6	133.5	133.9	133.5	C-1'	131.6	132.0	130.9	132.8	130.9
C-2	110.5	110.8	109.3	110.6	109.4	C-2'	115.1	111.7	110.5	118.0	110.6
C-3	148.4	150.2	149.1	149.2	149.1	C-3'	142.0	145.2	144.5	143.3	144.5
C-4	147.3	150.5	149.2	147.8	149.2	C-4'	148.0	149.0	148.4	154.8	148.4
C-5	115.7	112.7	111.0	116.3	111.1	C-5'	130.4	130.3	128.0	131.2	128.1
C-6	119.7	119.0	118.7	119.9	118.7	C-6'	115.0	116.1	114.8	120.7	114.8
C-7	88.6	88.4	88.2	90.3	88.2	C-7'	133.1	130.5	131.3	192.9	131.3
C-8	55.0	54.9	53.5	54.6	53.5	C-8'	124.3	128.5	126.5		126.5
C-9	64.6	64.7	64.0	64.6	64.0	C-9'	73.7	63.4	63.9		63.9
3-OCH_3	56.3	56.1	56.0	56.4	56.0	3'-OCH_3		56.4	56.1		56.1
4-OCH_3		56.2	56.0		56.0	9'-OCH_3	57.7				

Measured in [a]acetone-d_6 , [b]$CDCl_3$, and [c]CD_3OD at 125 MHz.

Hierochin C (**3**),[26] a pale yellow oil, $[\alpha]_D^{27}$ –60.1°(*c*=1.00, MeOH), $C_{17}H_{16}O_6$, showed absorption bands due to hydroxyl, aromatic ring, olefin, and ether functions (3652, 3569, 1684, 1670, 1655, 1509, 1277, and 1032 cm^{-1}) in the IR spectrum (film). The proton and carbon signals in the ^{1}H- and ^{13}C-NMR (CD_3OD, Table 1) spectra[23] of **3** showed signals assignable to a dihydrofuran moiety [δ 3.58 (1H, m, H-8), 5.65 (1H, d, *J*=6.4 Hz, H-7)], a methoxyl group [δ 3.82 (3H, s, OCH_3-3)], a methylene with an oxygen function [δ 3.85 (2H, m, H_2-9)], five aromatic protons: *meta*-coupled protons [δ 7.28, 7.39 (1H each, both d, *J*=1.6 Hz, H-2' and 6')], *ortho*- and *meta*-coupled ABC-type protons [δ 6.78 (1H, d, *J*=8.3 Hz, H-5), 6.85 (1H, dd, *J*=2.1,

8.3 Hz, H-6), 6.97 (1H, d, *J*=2.1 Hz, H-2)], and an aldehyde [δ 9.72 (1H, s, H-7')]. The planar structure of **3** was constructed on the basis of ^{1}H–^{1}H COSY and HMBC experiments (Figure 1). In the NOESY experiment of **3**, NOE correlations were observed between the 2, 6-protons and 8-proton, and between the 7-proton and 9-proton, so that the relative structure of **3** was elucidated. Furthermore, the absolute stereostructure of **3** was determined by CD spectrum. Thus, the CD spectra of **3** showed positive [MeOH, 233 nm ($\Delta\varepsilon$ +5.17)] and negative Cotton effects [246 nm (–4.63), 295 nm (–4.02)], which suggested the absolute configurations of 7 and 8-positions were 7*R* and 8*S* orientations.[14] On the basis of above-mentioned evidence, the absolute stereostructure of **3** was elucidated as shown.

In this study, optically pure (7*R*,8*S*) and (7*S*,8*R*) 8–5' linked neolignans were found to coexist in the whole plants of *A. hierochuntica*. It is noteworthy that all (7*R*,8*S*)-type neolignans (**1**, **3**—**5**) have a hydroxyl group at the 3'-position, while the (7*S*,8*R*)-type neolignans (**2**, **7**, **8**) were found to have a methoxyl group at the same position. Although a regiospecific *O*-demethylation reaction with *Pinus taeda* cell suspension culture was reported to occur at the 3'-position of 8–5' linked racemic neolignans,[27] this evidence led us to presume that enantiomer selective 3'-*O*-demethylation, 3'-*O*-methylation or phenylpropanoid coupling reaction may be related to the biosynthesis of those optically pure neolignans in this plant.

Table 2. Inhibitory Effects of Constituents from *A. hierochuntica* on NO Production in LPS–activated Mouse Peritoneal Macrophages

	Inhibition (%)[a]						IC_{50}
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	(μM)
hierochin A (**1**)	0.0±3.4	-0.1±7.9	2.2±2.1	-2.5±7.4	2.0±3.6	36.3±2.0[c]	
hierochin B (**2**)	0.0±1.2	7.6±8.7	-1.0±5.9	12.8±3.6	26.2±7.2[b]	73.4±1.8[c]	59
hierochin C (**3**)	0.0±3.4	17.2±2.6	22.6±7.1	8.0±9.5	15.6±5.6	43.7±3.5[c]	
4	0.0±1.3	-4.4±2.9	-7.1±2.7	-2.2±7.5	-2.0±4.1	49.5±6.1[c]	
5	0.0±4.4	3.3±6.2	5.6±6.4	-0.7±7.9	54.9±1.8[c]	98.5±0.5[c]	31
7	0.0±2.5	-15.6±3.1	-5.1±8.6	21.1±2.7[b]	47.6±3.3[c]	97.4±0.9[c]	26
(+)-balanophonin (**8**)	0.0±3.7	-1.2±4.7	5.6±5.2	28.4±1.9[c]	58.4±5.7[c]	100.2±0.8[c]	23
9	0.0±0.8	-12.7±4.2	-14.2±3.6	-6.1±3.7	-10.5±3.1	-5.8±4.0	
10	0.0±1.0	-5.2±6.6	-11.7±3.8	13.0±5.6	12.1±4.1	19.4±1.8[b]	
11	0.0±7.4	-5.4±10.1	-20.8±5.2	-16.7±4.0	-0.8±7.2	97.4±2.2[c]	*ca.* 51
12	0.0±2.8	0.7±2.3	17.6±5.4	15.6±6.9	21.6±5.8[b]	24.3±3.0[c]	
13	0.0±1.0	6.4±9.8	-6.1±3.9	4.9±3.7	11.6±3.7	-1.7±2.8	
14	0.0±3.3	-3.1±1.7	7.4±5.1	2.0±6.0	-5.2±2.0	-8.2±5.0	
evofolin B (**15**)	0.0±8.1	7.2±3.6	4.0±2.1	3.5±2.7	8.8±2.2	13.4±1.4	
(+)-lariciresinol	0.0±2.4	-10.9±3.1	-6.8±6.9	-7.6±4.2	5.1±2.5	6.3±3.1	
(+)-pinoresinol	0.0±8.2	0.7±1.4	4.3±2.8	3.3±2.7	12.5±2.6	64.3±4.2[c]	80
(+)-isolariciresinol	0.0±6.4	-1.2±7.2	2.2±4.5	1.8±7.4	-10.6±6.4	-12.1±3.1	
L-NMMA	0.0±4.0	5.9±0.9	10.3±3.7	15.0±1.6[c]	34.1±3.2[c]	63.1±1.2[c]	57

[a]Each value represents the mean±S.E.M. (*N*=4). Significantly different from the control, [b]$p<0.05$, [c]$p<0.01$.

The inorganic free radical NO has been implicated in physiological and pathological processes such as vasodilation, nonspecific host defense, ischemia reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS). In the NOS family, inducible NOS in particular is involved in pathological overproduction of NO, and can be expressed in response to pro-inflammatory agents such as interleukin-1β, tumor necrosis factor-α, and LPS in various cell types including macrophages, endothelial cells, and smooth muscle cells.

As a part of our studies to characterize the bioactive components of natural medicines, we have reported various NO production inhibitors; i.e. higher unsaturated fatty acids,[3] polyacetylenes,[28,29] coumarins,[28] flavonoids,[11,29] stilbenes,[30,31] sesquiterpenes,[32-37] diterpenes,[38,39] triterpenes,[10,40-43] diarylheptanoids,[41,42] cyclic peptides,[43] and lignans.[44] In the continuing study of antiinflammatory principles from natural medicines, 17 lignan constituents from *A. hierochuntica* were examined. As shown in Table 2, three neolignans, **5** (IC_{50}=31 μM), (+)-dehydrodiconiferyl alcohol (**7**, 26 μM), and (+)-balanophonin (**8**, 23 μM) showed NO production inhibitory activity without cytotoxic effects in the MTT assay (data not

shown). The inhibitory activities of **5**, **7**, **8** were stronger than that of N^G-monomethyl-L-arginine (L-NMMA), a non-selective NOS inhibitor (IC_{50}= 57 μM).

Next, the effects of three neolignans (**5**, **7**, **8**) on iNOS induction were examined. iNOS was detected at 130 kDa after a 20 h incubation with LPS by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)-Western blotting analysis as shown in Figure 2.[43] iNOS inductions of LPS-activated macrophages were shown to be suppressed by three neolignans (**5**, **7**, **8**) in closely related to their inhibitions of NO. These results suggested that **5**, **7**, and **8** inhibited NO production mainly due to their inhibitory activities against iNOS induction in LPS-activated macrophages.

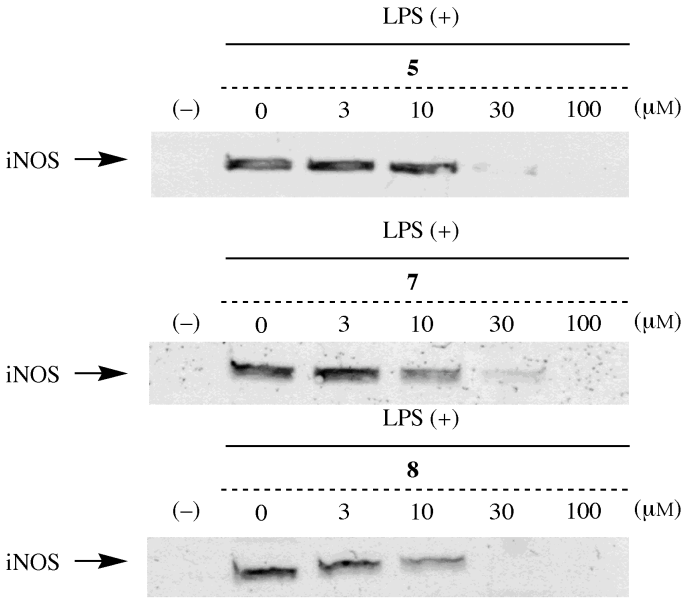

Figure 2. Effects of **5**, **7**, **8** on iNOS Induction in LPS-activated Mouse Macrophages

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21 The optical purities of 8–5' linked neolignans were confirmed by comparison of their CD spectral data, $[\alpha]_D$ values, and HPLC with chiral columns [Ceremospher Chiral RU-1, (MeOH) or Ceremospher Chiral RU-2, (CH_3CN–H_2O)].

22 **1**: High-resolution EI-MS: Calcd for $C_{20}H_{22}O_6$ (M^+): 358.1416. Found: 358.1408.

23 The ^{1}H- and ^{13}C-NMR spectra of **1**—**3** were assigned with the aid of homo- and hetero-correlation spectroscopy (^{1}H–^{1}H, ^{13}C–^{1}H COSY), distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple bond connectivity (HMBC) experiments.

24 **2**: High-resolution EI-MS: Calcd for $C_{21}H_{24}O_6$ (M^+): 372.1573. Found: 372.1606. EI-MS (%): *m/z* 372 (M^+, 47), 354 (M^+–H_2O, 100).

25 **6:** A colorless oil, UV (MeOH, nm, log ε) 278 (4.10). IR (film): 3565, 3424, 1650, 1599, 1516, 1497, 1264, 1142, 1026 cm^{-1}. High-resolution EI-MS: Calcd for $C_{21}H_{24}O_6$ (M^+): 372.1573. Found: 372.1570. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ 3.86, 3.87, 3.91 (3H each, all s, OCH_3-3, 4, and 3'), 3.63 (1H, m, H-8), [3.93 (1H, dd, *J* = 4.9, 11.0 Hz), 3.99 (dd, *J* = 6.1, 11.0 Hz), H_2-9], 4.31 (2H, dd, *J* = 1.5, 5.8 Hz, H_2-9'), 5.60 (1H, d, *J*=6.4 Hz, H-7), 6.24 (1H, dt, *J* = 15.9, 5.8 Hz, H-8'), 6.56 (1H, dt, *J* = 15.9, 1.5 Hz, H-7'), 6.83 (1H, d, *J* = 8.3 Hz, H-5), 6.89, 6.90 (1H each, both d, *J* = 1.8 Hz, H-2' and 6'), 6.93 (1H, d, *J* = 2.2 Hz, H-2), 6.96 (1H, dd, *J* = 2.2, 8.3 Hz, H-6). ^{13}C-NMR spectral data, see Table 1. EI-MS (%): *m/z* 372 (M^+, 38), 354 (M^+–H_2O, 100).

26 **3**: UV (MeOH, nm, log ε) 235 (4.28), 289 (4.10). High-resolution EI-MS: Calcd for $C_{17}H_{16}O_6$ (M^+): 316.0947. Found: 316.0945. EI-MS (%): *m/z* 316 (M^+, 41), 286 (100).

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