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Pseudoguaiane-type sesquiterpenes and inhibitors on nitric oxide production from *Dichrocephala integrifolia*

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Abstract—Three new pseudoguaiane-type sesquiterpenes, dichrocepholides A–C, and two new pseudoguaiane-type sesquiterpene dimers, dichrocepholides D and E, were isolated from the aerial part of *Dichrocephala integrifolia*. Their stereostructures were determined on the basis of chemical and physicochemical evidence. In addition, the extract and its principal sesquiterpene constituent, parthenin, showed an inhibitory activity on nitric oxide (NO) production and on induction of inducible NO synthase.

1. Introduction

The Asteraceae plant, *Dichrocephala integrifolia* (L. f.) O. Kuntze, is a sparingly branched annual herb, which is widely distributed in African, Middle Eastern, and Asian countries.[1,2] Many genus of *Dichrocephala* have been commonly used as folk medicines for pneumonia, hypertension, fever, and ulcers. The leaves of *D. integrifolia* have also been used for the treatment of malaria and hepatitis.[2] The composition of the essential oil from the leaves and flowers of *D. integrifolia* was reported.[2] However, the studies on the pharmacologic activity and biological constituents of *D. integrifolia* are left uncharacterized. Previously, we have reported on bioactive constituents from several Egyptian and Yemeni natural medicines such as *Cyperus longus*,[3,4] *Anastatica hierochuntica*,[5,6] *Nigella sativa*,[7,8] and *Crinum yemens*.[9] As a continuing study, we found that the aqueous methanolic extract from the aerial parts of *D. integrifolia* showed potent inhibitory effect on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in mouse peritoneal macrophages. From the aqueous methanolic extract, three new pseudoguaiane-type sesquiterpenes, dichrocepholides A (**1**), B (**2**), and C (**3**) and two new pseudoguaiane-type sesquiterpene dimers, dichrocepholides D (**4**) and E (**5**), were isolated together with six known constituents, parthenin (**6**),[10–12] **7**,[13] 6-methoxy-3-*O*-methylkaempferol (**8**),[14] quercetagetin 3,7-dimethyl ether (**9**),[15] 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone (**10**),[16] and kaempferol 3-*O*-β-D-glucopyranoside (**11**).[17] In this paper, we describe the isolation and structure elucidation of five new sesquiterpenes (**1**–**5**) from the aerial parts of *D. integrifolia* as well as inhibitory effects of the isolated constituents on NO production and induction of NO synthase inhibitory activities.

2. Results and discussion

The 80% aqueous methanolic extract of the aerial part of *D. integrifolia* was found to show NO production inhibitory activity on LPS-activated mouse peritoneal macrophages (IC_{50}=15 μg/mL). From the active fraction (the EtOAc-soluble fraction, IC_{50}=4.2 μg/mL), three new pseudoguaiane-type sesquiterpenes, dichrocepholides A (**1**, 0.0013%), B (**2**, 0.0014%), and C (**3**, 0.0007%), and two new pseudoguaiane-type sesquiterpene dimers, dichrocepholides D (**4**, 0.0055%) and E (**5**, 0.0047%), were isolated together with six known constituents, **6** (0.75%), **7** (0.0070%), **8** (0.0007%), **9** (0.0033%), **10** (0.0004%), and **11** (0.0015%) (Table 1).

Dichrocepholide A (**1**) was isolated as colorless fine crystals, mp 151–153 °C, with positive optical rotation ($[\alpha]_D^{24}$ +23.0). The EIMS spectrum of **1** showed a molecular ion peak at *m/z* 280 (M^+) and the molecular formula $C_{15}H_{20}O_5$ of **1** was elucidated by HREIMS measurement. The UV spectrum of **1**

Keywords: Dichrocepholide; Pseudoguaiane-type sesquiterpene; *Dichrocephala integrifolia*; Nitric oxide inhibitor.
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Table 1. Inhibitory effects of the 80% aqueous methanolic extract and EtOAc-, *n*-BuOH-, and H_2O-soluble fractions from *D. integrifolia* on NO production in LPS-activated mouse peritoneal macrophages

	Inhibition (%)						IC_{50} (μg/mL)
	0 μg/mL	1 μg/mL	3 μg/mL	10 μg/mL	30 μg/mL	100 μg/mL	
80% Aqueous methanolic extract	0.0±2.8	0.2±3.5	2.9±2.1	35.3±4.2**	100.0±0.2**	101.0±0.2**,[a]	15
EtOAc-soluble fraction	0.0±5.8	9.2±4.1	24.0±1.8**	91.0±2.5**	100.4±0.1**,[a]		4.2
n-BuOH-soluble fraction	0.0±4.9	−2.1±2.8	1.1±3.6	3.5±3.2	0.0±6.3	31.5±3.2**	
H_2O-soluble fraction	0.0±5.0	1.3±2.9	4.7±1.8	5.0±1.5	3.6±1.3	7.0±1.2	

Each value represents the mean±SEM, (N=4).
Significantly different from the control, *p<0.05, **p<0.01.
[a] Cytotoxic effect was observed.

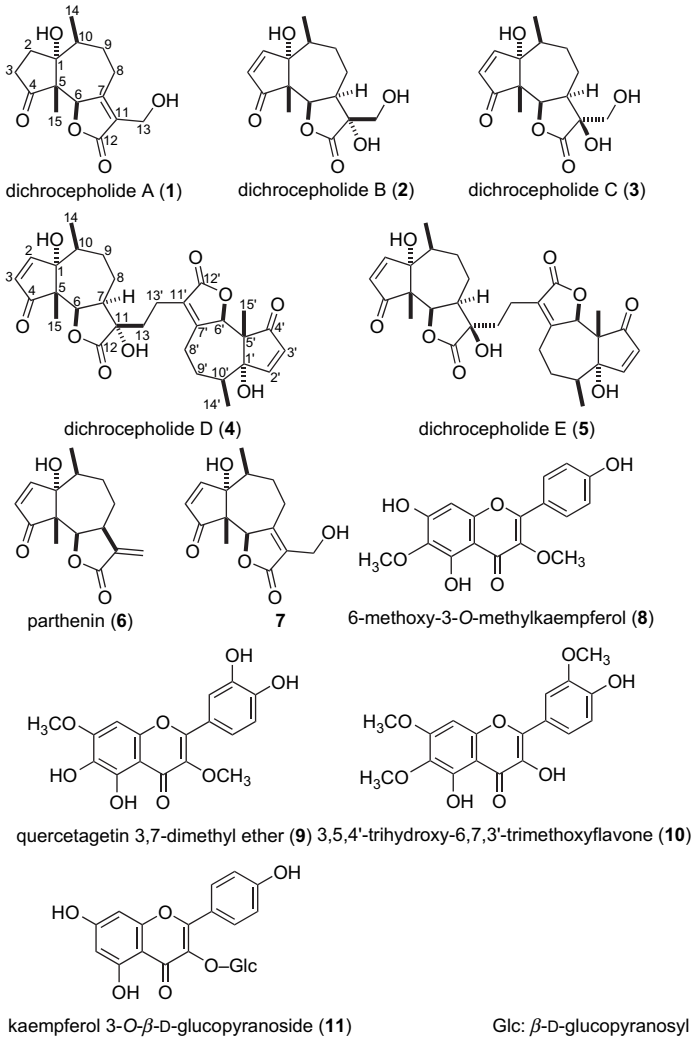


Chart 1.

showed an absorption maximum at 219 (log ε 3.13) and the IR spectrum of **1** showed absorption bands at 3565, 1762, and 1740 cm^{-1} ascribable to hydroxyl, carbonyl, and α,β-unsaturated γ-lactone functions. The ^1H (CD_3OD, Table 2) and ^{13}C NMR (Table 2) spectra of **1** showed signals due to two methyl [δ 0.86 (3H, s, H_3-15), 1.10 (3H, d, J=7.0 Hz, H_3-14)], four methylenes [δ 1.45 (1H, ddd, J=6.9, 10.1, 13.5 Hz, Hβ-9), 2.33 (1H, ddd, J=3.5, 6.9, 13.5 Hz, Hα-9), 1.82, 2.56 (1H each, both m, H_2-2), 2.41 (2H, m, H_2-3), 2.97 (2H, d-like, H_2-8)], a methine [δ 2.16 (1H, m, H-10)] together with a methylene and a methine bearing an oxygen function [δ 4.28 (2H, s, H_2-13), 5.43 (1H, s, H-6)]. The proton and carbon signals in the ^1H and ^{13}C NMR spectra of **1** were found to be similar to those of **7**, except for the 2- and 3-positions. The 1,13-dihydroxy-4-oxo-7(11)-pseudoguaien-12,6-olide structure in **1** was constructed on the basis of homo- and heterocorrelation spectroscopy (^1H–^1H, ^{13}C–^1H COSY), distortionless enhancement by polarization transfer (DEPT), and heteronuclear multiple bond connectivity (HMBC) experiments. Thus, the ^1H–^1H COSY experiment on **1** indicated the presence of partial structures shown by bold lines in Figure 1. In the HMBC experiment of **1**, long-range correlations were observed between the following proton and carbon pairs: H_2-2 and C-1, 4, 5; H_2-3

Table 2. ^{1}H and ^{13}C NMR data of dichrocepholides A–C (**1**–**3**) in CD_3OD

	1		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		84.0		85.0		85.0
2	1.82 m 2.56 m	32.4	7.61 d (6.0)	166.2	7.60 d (6.0)	166.2
3	2.41 m (2H)	34.2	6.13 d (6.0)	131.6	6.12 d (6.0)	131.6
4		218.5		213.7		213.6
5		59.0		60.0		60.4
6	5.43 s	83.7	5.01 d (6.3)	80.6	4.93 d (7.3)	80.2
7		170.3	2.96 ddd (2.3, 6.3, 6.3)	52.9	3.04 ddd (3.9, 7.3, 7.3)	48.6
8	2.97 d-like (2H)	24.0	1.79 m 2.07 m	21.5	1.80 m 1.90 m	20.5
9α	2.33 ddd (3.5, 6.9, 13.5)	31.0	2.21 ddd (3.6, 6.7, 13.2)	32.7	2.17 ddd (4.1, 7.2, 14.1)	32.1
9β	1.45 ddd (6.9, 10.1, 13.5)		1.68 ddd (6.7, 9.8, 13.2)		1.75 ddd (7.2, 10.2, 14.1)	
10	2.16 m	43.3	2.33 m	41.3	2.34 m	41.6
11		127.1		78.7		78.1
12		176.1		177.9		180.0
13	4.28 s (2H)	53.6	3.70 d (12.0) 3.80 d (12.0)	63.2	3.57 d (11.0) 3.70 d (11.0)	67.8
14	1.10 d (7.0)	18.3	1.12 d (6.8)	18.0	1.11 d (7.7)	18.0
15	0.86 s	11.3	1.31 s	20.4	1.31 s	19.8

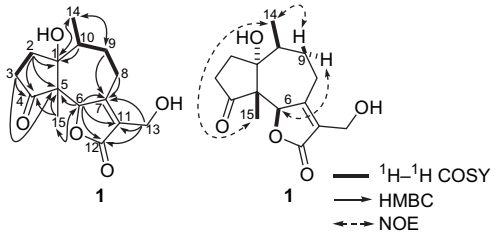
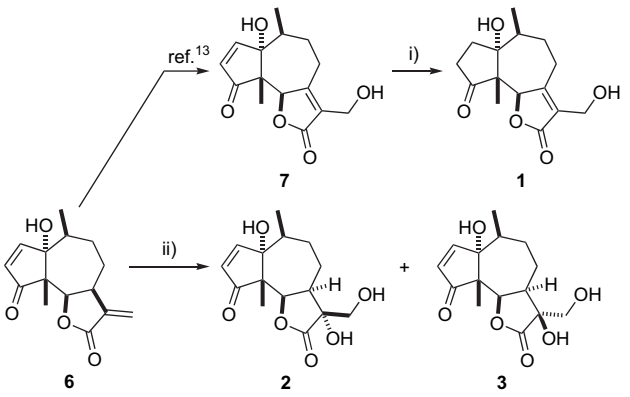


Figure 1.

and C-4, 5; H-6 and C-5, 7, 11, 12, 15; H_2-8 and C-7; H_2-9 and C-7, 14; H-10 and C-1; H_2-13 and C-7, 11, 12; H_3-14 and C-1, 9; H_3-15 and C-4, 5, 6 (Fig. 1). The relative stereostructure of **1** was determined by nuclear Overhauser enhancement spectroscopy (NOESY) experiment in which NOE correlations were observed as shown in Figure 1. To clarify the absolute stereostructure of **1**, we carried out the chemical correlation of **1** to **6**, whose absolute stereostructure was elucidated previously.[18] That is, hydrogenation of **7**, which was derived from **6**,[13] with 10% palladium–carbon (Pd–C) under a hydrogen atmosphere furnished **1** as shown in Scheme 1. This evidence led us to designate the absolute stereostructure of dichrocepholide A (**1**) as shown.



Scheme 1. Reagents and conditions: (i) 10% Pd–C, H_2/MeOH, rt, 30 min, 97%; (ii) MC-OsO_4, NMO/CH_3CN–acetone–H_2O (1:1:1, v/v/v), rt, 48 h, **2** (59%), **3** (6%), recover **6** (26%).

Dichrocepholides B (**2**) and C (**3**) were obtained as colorless fine crystals with positive optical rotation (**2**: $[\alpha]_D^{24}$ +26.0, **3**: $[\alpha]_D^{24}$ +42.5) and the molecular formulas of **2** and **3** were determined by EIMS and HREIMS measurements to be $C_{15}H_{20}O_6$. The UV spectra of **2** and **3** showed an absorption maximum [**2**: 215 nm (log ε 3.78); **3**: 214 (3.70)], while their IR spectra showed absorption bands due to hydroxyl, γ-lactone, and α,β-unsaturated carbonyl functions (**2**: 3550, 1755, and 1742 cm^{-1}; **3**: 3550, 1755, and 1741 cm^{-1}). The proton and carbon signals in the ^{1}H and ^{13}C NMR spectra of **2** and **3** were superimposable on those of **6**, except for the *exo*-methylene part in **6**. Namely, ^{1}H (CD_3OD, Table 2) and ^{13}C NMR (Table 2) spectra of **2** and **3** showed signals assignable to two methyls, two methylenes, two methines, a methylene and a methine bearing an oxygen function, and two olefin protons. As shown in Figure 2, the ^{1}H–^{1}H COSY experiment on **2** and **3** indicated the presence of partial structures drawn in bold lines. In the HMBC experiment, long-range correlations were observed between the following proton and carbon pairs of **2** and **3** (H-2 and C-1, 4, 5; H-3 and C-4, 5; H-6 and C-5, 11, 12, 15; H-7 and C-11, 12; H_2-9 and C-14; H-10 and C-1; H_2-13 and C-7, 11, 12; H_3-14 and C-1, 9; H_3-15 and C-4, 5, 6). Those findings led us to confirm the planar structures of **2** and **3** to be the same. Next, the relative structure of **2** was clarified by the NOESY experiment, which showed NOE correlations between the following protons (H-6 and H-7, Hα-9; Hβ-9 and H_3-14; H_3-15 and H_3-13, H_3-14), while NOE correlations were observed between the following protons of **3** (H-6 and H-7, Hα-9,

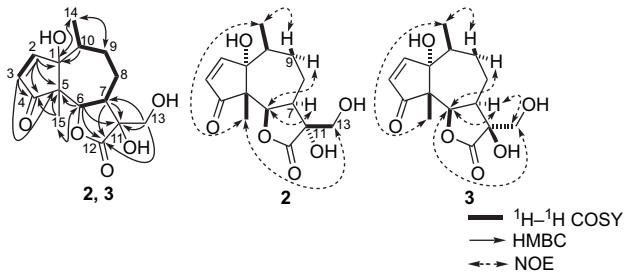


Figure 2.

H_2-13; H-7 and H_2-13; H_3-14 and Hβ-9, H_3-15). Furthermore, osmium oxidation of **6** with microcapsuled osmium tetraoxide (MC-OsO_4) and *N*-methylmorpholine *N*-oxide (NMO) yielded **2** and **3** (ca. 10:1 ratio). Consequently, the absolute stereostructures of **2** and **3** were determined as shown.

Dichrocepholide D (**4**) was obtained as colorless needles, mp 174–176 °C, with positive optical rotation ($[\alpha]_D^{24}$ +49.0) and its UV spectrum showed an absorption maximum at 216 nm (log ε 4.58). The IR spectrum of **4** showed absorption bands at 3575, 1765, and 1742 cm^{-1}. The EIMS spectrum of **4** showed a molecular ion peak at *m/z* 540 (M^+) and the molecular formula $C_{30}H_{36}O_9$ of **4** was elucidated by HREIMS measurement. The proton and carbon signals due to the sesquiterpene part (C-1–C-15) having a saturated lactone moiety in the ^1H and ^{13}C NMR data of **4** were found to be very similar to those of **2**, whereas the proton and carbon signals of the sesquiterpene part (C-1′–C-15′) having an unsaturated lactone moiety were superimposable on those of **7**, except for the signals due to the 13- and 13′-positions. That is, the ^1H (CD_3OD, Table 3) and ^{13}C NMR (Table 3) spectra of **4** showed signals assignable to four methyls [δ 0.94 (3H, s, H_3-15′), 1.07 (3H, d, *J*=7.2 Hz, H_3-14′), 1.13 (3H, d, *J*=7.0 Hz, H_3-14), 1.31 (3H, s, H_3-15)], six methylenes {δ 1.41 (1H, ddd, *J*=6.8, 9.5, 13.5 Hz, Hβ-9′), 2.34 (1H, ddd, *J*=3.7, 6.8, 13.5 Hz, Hα-9′), 1.68 (2H, m, H_2-13), 1.72 (1H, ddd, *J*=6.8, 10.0, 13.4 Hz, Hβ-9), 2.18 (1H, ddd, *J*=3.4, 6.8, 13.4 Hz, Hα-9), 1.90 (2H, m, H_2-8), 2.30, 2.70 (1H each, both m, H_2-13′), [2.85 (1H, m), 3.05 (1H, dd-like), H_2-8′]}, three methines [δ 2.30 (1H, m, H-10), 2.34 (1H, m, H-10′), 2.98 (1H, m, H-7)], two methines bearing an oxygen function [δ 5.12 (1H, d, *J*=5.2 Hz, H-6), 5.43 (1H, s, H-6′)], and four olefinic protons [δ 6.09, 7.70 (1H each, both d, *J*=5.8 Hz, H-3′ and H-2′), 6.14, 7.63 (1H each, both d, *J*=5.8 Hz, H-3 and H-2)]. The ^1H–^1H COSY experiment on **4** indicated the presence of five partial structures shown by bold lines in Figure 3 (C-2–C-3, C-6–C-10–C-14, C-13–C-13′, C-2′–C-3′, and C-8′–C-10′–C-14′). In the HMBC experiment, long-range correlations were observed

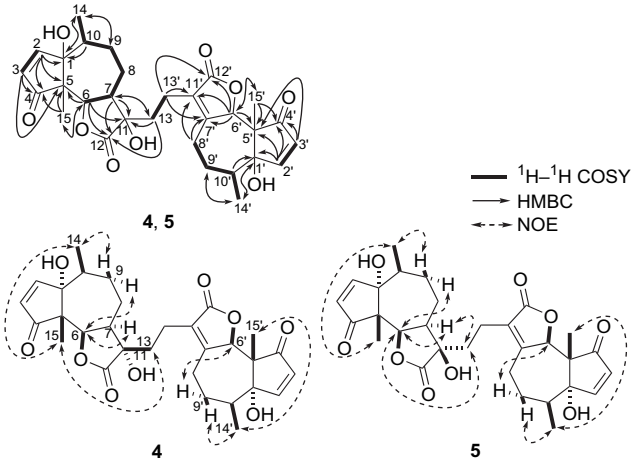


Figure 3.

Table 3. ^1H and ^{13}C NMR data of dichrocepholides D (**4**) and E (**5**) in CD_3OD

	4		**5**	
	δ_H	δ_C	δ_H	δ_C
1		85.6		85.6
2	7.63 d (5.8)	166.3	7.63 d (5.8)	166.3
3	6.14 d (5.8)	131.5	6.14 d (5.8)	131.5
4		213.5		213.5
5		60.0		60.3
6	5.12 d (5.2)	80.7	4.85 d (6.6)	79.4
7	2.98 m	53.0	2.93 ddd (2.7, 5.5, 6.6)	51.9
8	1.90 m (2H)	31.9	1.44 m (2H)	38.0
9α	2.18 ddd (3.4, 6.8, 13.4)	33.0	2.18 ddd (3.0, 6.6, 13.3)	32.3
9β	1.72 ddd (6.8, 10.0, 13.4)		1.78 ddd (6.6, 10.2, 13.3)	
10	2.30 m	41.3	2.34 m	41.4
11		78.5		77.0
12		178.9		179.8
13	1.68 m (2H)	21.3	1.87 m (2H)	20.3
14	1.13 d (7.0)	18.2	1.13 d (7.0)	18.2
15	1.31 s	20.9	1.32 s	21.0
1′		84.5		84.5
2′	7.70 d (5.8)	163.9	7.70 d (5.8)	163.9
3′	6.09 d (5.8)	130.8	6.09 d (5.8)	130.8
4′		211.4		211.4
5′		57.4		57.4
6′	5.43 s	83.6	5.43 s	83.6
7′		166.5		166.5
8′	2.85 m	25.1	2.85 m	25.1
	3.05 dd-like		3.05 dd-like	
9′α	2.34 ddd (3.7, 6.8, 13.5)	33.1	2.34 ddd (4.0, 7.1, 14.2)	33.1
9′β	1.41 ddd (6.8, 9.5, 13.5)		1.41 ddd (7.1, 10.3, 14.2)	
10′	2.34 m	41.2	2.34 m	41.2
11′		128.7		127.9
12′		176.8		176.8
13′	2.30 m	17.8	2.50 m (2H)	18.4
	2.70 m			
14′	1.07 d (7.2)	18.7	1.07 d (7.2)	18.7
15′	0.94 s	15.2	0.94 s	15.2

between the following protons and carbons of **4** (H-2 and C-1, 4, 5; H-3 and C-4, 5; H-6 and C-5, 11, 12, 15; H-7 and C-11, 12, 13; H_2-9 and C-14; H-10 and C-1; H_2-13 and C-7, 11, 12; H_3-14 and C-1, 9; H_3-15 and C-4, 5, 6; H-2′ and C-1′, 4′, 5′; H-3′ and C-4′, 5′; H-6′ and C-5′, 7′, 11′, 12′, 15′; H_2-8′ and C-7′, 11′; H_2-9′ and C-14′; H-10′ and C-1′; H_2-13′ and C-7′, 11′, 12′; H_3-14′ and C-1′, 9′; H_3-15′ and C-4′, 5′, 6′). The stereostructure of **4** was determined by the NOESY experiment, which showed NOE correlations between the following proton pairs (H-6 and H-7, Hα-9; H_2-13 and H_3-15; H_3-14 and Hβ-9, H_3-15; H-6′ and Hα-9′; H_3-14′ and Hβ-9′, H_3-15′). Those findings led us to indicate the dimeric sesquiterpene structure composed of **2** and **7**. Finally, the stereostructure of **4** was confirmed by the X-ray crystallographic analysis as shown in Figure 4.

Dichrocepholide E (**5**) was also obtained as colorless needles, mp 169–171 °C, with negative optical rotation ($[\alpha]_D^{24}$ −3.0) and its molecular formula $C_{30}H_{36}O_9$, which was the same as that of **4**, was determined from the EIMS and HREIMS measurements. The UV spectrum of **5** showed an absorption maximum at 217 nm (log ε 4.26), whereas the IR spectrum showed absorption bands at 3560, 1760, and 1740 cm^{-1}, which was similar to those of **4**. The proton and carbon signals in the ^1H (CD_3OD, Table 3) and ^{13}C NMR (Table 3) spectra of **5** were found to be superimposable on those of **4** and indicated the presence of the same functional groups. In the ^1H–^1H COSY and the HBMC experiments on **5**, the planar structure was constructed to be the same dimeric sesquiterpene skeleton as that of **4**. The stereostructure of **5** was elucidated by the NOESY experiments, which showed NOE correlations were observed between the following proton pairs (H-6 and H-7, Hα-9, H_2-13; H-7 and H_2-13; H_3-14 and Hβ-9, H_3-15; H-6′ and Hα-9′; H_3-14′ and Hβ-9′, H_3-15′). Consequently, the stereostructure of **5** was elucidated as shown.

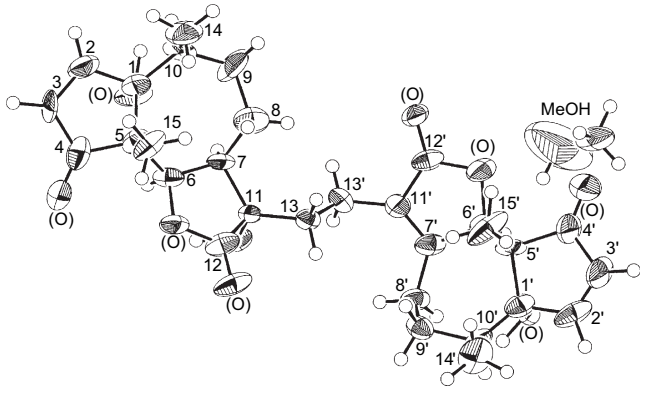


Figure 4. X-ray crystallographic analysis of dichrocepholide D (**4**).

The inorganic free radical NO has been implicated in physiologic and pathologic processes, such as vasodilation, nonspecific host defense, ischemia-reperfusion injury, and chronic or acute inflammation. NO is produced by the oxidation of L-arginine by NO synthase (NOS). In the family of NOS, inducible NOS (iNOS) is specifically involved in pathologic aspects with the overproduction of NO and can be expressed in response to proinflammatory 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 investigated various NO production inhibitors, i.e., higher unsaturated fatty acids,[19] polyacetylenes,[20–22] coumarins,[20,22,23] flavonoids,[21,24] stilbenes,[25,26] lignans,[6,27,28] sesquiterpenes,[29–35] diterpenes,[36,37] triterpenes,[38–41] diarylheptanoids,[42–44] cyclic peptides,[40] alkaloids,[9,45] and phenylpropanoids.[28,46,47] Continuing of these studies, the effects of the sesquiterpene constituents (**1**–**7**) from the aerial parts of *D. integrifolia* on NO production from LPS-activated macrophages were examined, and the results were summarized in Table 4. Among them, parthenin (**6**, IC_{50}=1.4 μM) and **7** (29 μM) exhibited inhibitory activity. The inhibitory activity of **6** was more potent than that of guanidinoethyldisulfide (GED), a selective inducible nitric oxide synthase (iNOS) inhibitor (IC_{50}=7.4 μM).[9]

Next, the effects of the two sesquiterpenes (**6**, **7**) 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 [reference compound, caffeic acid phenethyl ester (CAPE, an inhibitor of NF-κB activation)].[9,47] As shown in Figure 5, iNOS inductions of LPS-activated macrophages were suppressed by **6** and **7** closely related to their

Table 4. Inhibitory effects of constituents from *D. integrifolia* on NO production in LPS-activated mouse peritoneal macrophages

	Inhibition (%)						IC_{50} (μM)
	0 μM	1 μM	3 μM	10 μM	30 μM	100 μM	
Dichrocepholide A (**1**)	0.0±3.6	−6.7±3.4	−0.1±1.4	−0.1±3.6	−0.3±4.1	10.2±5.7	
Dichrocepholide B (**2**)	0.0±2.0	−2.2±1.3	−3.3±2.1	3.8±4.0	4.2±6.1	65.6±1.6**	
Dichrocepholide C (**3**)	0.0±1.9	−2.0±4.1	1.9±3.2	3.2±2.8	10.2±2.2	11.3±1.8*	
Dichrocepholide D (**4**)	0.0±3.2	0.3±2.1	−0.6±9.7	−6.0±7.7	18.5±1.5	36.1±10.6**	
Dichrocepholide E (**5**)	0.0±5.5	−5.2±5.2	−17.2±14.5	−9.5±9.1	−0.1±2.5	−3.1±10.4	
7	0.0±2.2	−2.5±3.4	−7.1±3.7	−1.9±2.5	51.0±1.6**	95.9±1.1**	29
L-NMMA	0.0±4.0	5.9±0.9	10.3±3.7	15.0±1.6**	34.1±3.2**	63.1±1.2**	57[9]
CAPE	0.0±0.7	3.8±0.1	1.4±0.1	68.2±0.0**	93.7±0.2**	99.6±0.0**,[a]	15[9]
GED	0.0±0.0	6.2±0.1	24.4±0.1**	57.9±0.1**	89.7±0.2**	97.9±0.0**	7.4[9]
	0 μM	0.03 μM	0.1 μM	0.3 μM	1 μM	3 μM	
Parthenin (**6**)	0.0±1.4	1.8±3.7	−5.9±5.3	9.9±5.8	34.8±0.5**	92.7±2.0**	1.4

Each value represents the mean±SEM, (N=4).
Significantly different from the control, *p<0.05, **p<0.01.
[a] Cytotoxic effect was observed.

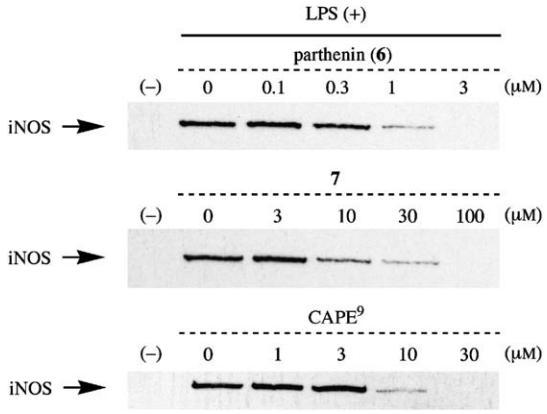


Figure 5. Effects of **6**, **7**, and CAPE on iNOS induction in LPS-activated mouse macrophages.

inhibitions of NO. These results suggested that the two sesquiterpenes (**6**, **7**) inhibited NO production due to their inhibitory activities against iNOS induction in LPS-activated macrophages.

3. Experimental

3.1. General

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); UV spectra, Shimadzu UV-1600 spectrometer; IR spectra, Shimadzu FTIR-8100 spectrometer; EIMS and HREIMS, JEOL JMS-GCMATE mass spectrometer, JEOL JMS-SX 102A mass spectrometer; ^{1}H NMR spectra, JNM-LA500 (500 MHz); ^{13}C NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard; Combustion analysis was done on a Perkin–Elmer Series II CHNS/O Analyzer 2400; HPLC detector, Shimadzu RID-6A refractive index detector; HPLC column, YMC-Pack ODS-A (250×4.6 mm i.d.) and (250×20 mm i.d.) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: ordinary-phase column chromatography; Silica gel BW-200 (Fuji Silysia Chemical, Ltd, 150–350 mesh), reversed-phase column chromatography; Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh); TLC, pre-coated TLC plates with Silica gel 60F_{254} (Merck, normal-phase) and Silica gel RP-18 F_{254S} (Merck, reversed-phase); HPTLC, pre-coated TLC plates with Silica gel 60F_{254} (Merck, normal-phase), Silica gel RP-18 WF_{254S} (Merck, reversed-phase) and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

3.2. Plant material

The aerial parts of *D. integrifolia* was collected in Ibb province, Yemen in July 2000, and was identified by one of the authors, Dr. Osama B. Abdel-Halim (Professor of Pharmacognosy of Mansura University, Egypt). A voucher specimen (No. Y-02) of this natural medicine is on file in our laboratory.

3.3. Extraction and isolation

The dried aerial parts of *D. integrifolia* (1.6 kg) was finely cut and extracted three times with 80% aqueous methanol under reflux for 3 h. Evaporation of the solvent under reduced pressure gave the aqueous methanolic extract (213 g, 13.3% from dried aerial parts). The aqueous methanolic extract (213 g) was partitioned in an EtOAc–H_2O (1:1, v/v) mixture. The aqueous layer was extracted with *n*-BuOH and removal of the solvent in vacuo from the EtOAc-, *n*-BuOH-, and H_2O-soluble portions yielded 60 g (3.7%), 99 g (6.2%), and 54 g (3.4%) of the residue, respectively. The EtOAc-soluble fraction (50.0 g) was subjected to ordinary-phase silica gel column chromatography [1.5 kg, *n*-hexane–EtOAc (10:1–5:1–1:1–1:10, v/v)–$CHCl_3$–MeOH–H_2O (10:3:1–7:3:1, lower layer)–MeOH] to afford 10 fractions [Fr. 1 (4.1 g), Fr. 2 (2.7 g), Fr. 3 (7.8 g), Fr. 4 (4.4 g), Fr. 5 (4.1 g), Fr. 6 (9.5 g), Fr. 7 (5.7 g), Fr. 8 (2.2 g), Fr. 9 (3.5 g), Fr. 10 (6.0 g)]. Fraction 4 (4.4 g) was separated by reversed-phase silica gel column chromatography [125 g, MeOH–H_2O (20:80–50:50–70:30, v/v)–MeOH] to give four fractions {Fr. 4-1 (650 mg), 4-2 [=parthenin (**6**, 1200 mg, 0.75%)], 4-3 (851 mg), 4-4 (1685 mg)}. Fraction 6 (9.5 g) was separated by reversed-phase silica gel column chromatography [280 g, MeOH–H_2O (30:70–40:60–60:40–70:30, v/v)–MeOH] to furnish five fractions [Fr. 6-1 (82 mg), 6-2 (3500 mg), 6-3 (1500 mg), 6-4 (750 mg), 6-5 (2800 mg)]. Fraction 6-2 (1.0 g) was further subjected to HPLC [MeOH–H_2O (25:75, v/v)] to give **7** (110 mg, 0.0070%), **1** (21 mg, 0.0013%), **2** (23 mg, 0.0014%), and **3** (11 mg, 0.0007%). Fraction 6-3 (1.0 g) was further purified by HPLC [MeOH–H_2O (55:45, v/v)] to give **4** (88 mg, 0.0055%), **5** (76 mg, 0.0047%), and **9** (53 mg, 0.0033%). Fraction 6-4 (750 mg) was further separated by HPLC [MeOH–H_2O (55:45, v/v)] to give **8** (11 mg, 0.0007%) and **10** (7 mg, 0.0004%). Fraction 8 (2.2 g) was separated by reversed-phase silica gel column chromatography [65 g, MeOH–H_2O (30:70–55:45–70:30, v/v)–MeOH] to give four fractions [Fr. 8-1 (296 mg), 8-2 (422 mg), 8-3 (536 mg), 8-4 (946 mg)]. Fraction 8-2 (422 mg) was subjected to HPLC [MeOH–H_2O (45:55, v/v)] to give **11** (24 mg, 0.0015%).

3.3.1. Dichrocephalide A (1). Colorless fine crystals (from MeOH), mp 151–153 °C, $[\alpha]_D^{24}$ +23.0 (c 1.10, MeOH). HREIMS, calcd for $C_{15}H_{20}O_5$ (M^+): 280.1311. Found: 280.1314. UV [MeOH, nm (log ε)]: 219 (3.13). IR (KBr): 3565, 1762, 1740 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ_H: given in Table 2. ^{13}C NMR (125 MHz, CD_3OD) δ_C: given in Table 2. EIMS: m/z 280 (M^+, 11), 262 (M^+-H_2O, 61), 244 (22), 189 (100), 161 (88), 91 (58), 55 (95).

3.3.2. Dichrocephalide B (2). Colorless fine crystals (from MeOH), mp 177–179 °C, $[\alpha]_D^{24}$ +26.0 (c 1.40, MeOH). HREIMS, calcd for $C_{15}H_{20}O_6$ (M^+): 296.1260. Found: 296.1264. UV [MeOH, nm (log ε)]: 215 (3.78). IR (KBr): 3550, 1755, 1742 cm^{-1}. ^{1}H NMR (500 MHz, CD_3OD) δ_H: given in Table 2. ^{13}C NMR (125 MHz, CD_3OD) δ_C: given in Table 2. EIMS: m/z 296 (M^+, 11), 278 (M^+-H_2O, 16), 260 (16), 91 (67), 55 (100). Anal. requires C, 60.80; H, 6.80%. Found C, 60.65; H, 6.86%.

3.3.3. Dichrocephalide C (3). Colorless fine crystals (from MeOH), mp 166–168 °C, $[\alpha]_D^{24}$ +42.5 (c 0.60, MeOH).

HREIMS, calcd for $C_{15}H_{20}O_6$ (M^+): 296.1260. Found: 296.1265. UV [MeOH, nm (log ε)]: 214 (3.70). IR (KBr): 3550, 1755, 1741 cm^{-1}. ^1H NMR (500 MHz, CD_3OD) δ_H: given in Table 2. ^{13}C NMR (125 MHz, CD_3OD) δ_C: given in Table 2. EIMS: *m/z* 296 (M^+, 13), 278 (M^+-H_2O, 21), 260 (11), 91 (61), 55 (100). Anal. requires C, 60.80; H, 6.80%. Found C, 60.96; H, 6.77%.

3.3.4. Dichrocepholide D (4). Colorless needles (from MeOH), mp 174–176 °C, $[\alpha]_D^{24}$ +49.0 (*c* 2.70, MeOH). HREIMS, calcd for $C_{30}H_{36}O_9$ (M^+): 540.2359. Found: 540.2355. UV [MeOH, nm (log ε)]: 216 (4.58). IR (KBr): 3575, 1765, 1742 cm^{-1}. ^1H NMR (500 MHz, CD_3OD) δ_H: given in Table 3. ^{13}C NMR (125 MHz, CD_3OD) δ_C: given in Table 3. EIMS: *m/z* 540 (M^+, 8), 496 (10), 478 (15), 303 (18), 231 (55), 79 (100).

3.3.5. Dichrocepholide E (5). Colorless needles (from MeOH), mp 169–171 °C, $[\alpha]_D^{24}$ −3.0 (*c* 2.30, MeOH). HREIMS, calcd for $C_{30}H_{36}O_9$ (M^+): 540.2359. Found: 540.2357. UV [MeOH, nm (log ε)]: 217 (4.26). IR (KBr): 3560, 1760, 1740 cm^{-1}. ^1H NMR (500 MHz, CD_3OD) δ_H: given in Table 3. ^{13}C NMR (125 MHz, CD_3OD) δ_C: given in Table 3. EIMS: *m/z* 540 (M^+, 7), 496 (12), 478 (11), 303 (15), 231 (62), 79 (100).

3.3.6. Hydrogenation of 7. A solution of **7** (10.0 mg) in MeOH (1.0 mL) was treated with 10% palladium–carbon (Pd–C, 10.0 mg) and the whole mixture was stirred at room temperature under an H_2 atmosphere for 30 min. The catalyst was filtered off, and the solvent from the filtrate was evaporated under reduced pressure to give a residue, which was purified by ordinary-phase silica gel column chromatography [0.5 g, *n*-hexane–acetone (3:2, v/v)] to give **1** (9.8 mg, 97%).

3.3.7. Osmium tetraoxide oxidation of 6. A solution of **6** (9.8 mg) in CH_3CN–acetone–H_2O (1:1:1, v/v/v, 1.0 mL) was treated with microcapsuled osmium tetraoxide (MC-OsO_4, ca. 10 mg) in the presence of *N*-methylmorpholine *N*-oxide (NMO, 6.6 mg) and the whole mixture was stirred at room temperature for 48 h. The MC-OsO_4 was filtered off, and the solvent from the filtrate was evaporated under reduced pressure to give a residue, which was purified by HPLC [MeOH–H_2O (25:75, v/v)] to give **2** (6.5 mg, 59%), **3** (0.7 mg, 6%), and **6** (2.5 mg, 26%).

3.4. Crystal data for 4

Colorless needles, mp 174–176 °C (from MeOH); $C_{30}H_{36}O_9 \cdot CH_3OH$; M=572.65; crystal dimensions: 0.06×0.03×0.25 mm; crystal system: orthorhombic; lattice type: primitive; lattice parameters: a=13.189(3), b=29.814(2), c=7.432(2) Å, V=2922(1) $Å^3$; space group: $P2_12_12_1$ (#19), Z=4, D_{calc}=1.301 g/cm^3, F_{000}=1224.00, μ (Cu Kα)=8.03 cm^{-1}; temperature: 23.0 °C; structure solution: Direct methods (SHELEXS-86); residuals: R=0.257, R_w=0.251, R_1=0.096; goodness of fit indicator: 1.42. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu Kα (λ=1.54178 Å) radiation and a rotating anode generator. The data have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC265768.

3.5. Bioassay methods

3.5.1. Reagents. Lipopolysaccharide (LPS, from *Salmonella enteritidis*), N^G-monomethyl-L-arginine (L-NMMA), and RPMI 1640 medium were purchased from Sigma; 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide (MTT) was from Dojindo Laboratories; protease inhibitor cocktail (Complete Mini) was from Roche Diagnostics GmbM; fetal calf serum (FCS) was from Gibco; thioglycolate (TGC) medium was from Nissui Seiyaku; other reagents was from Wako Pure Chemical.

3.5.2. Effects on production of NO in LPS-stimulated macrophages. Screening test for NO production using TGC-induced mouse peritoneal macrophages was performed as described previously.[9,28,46,47] Briefly, peritoneal exudate cells (5×10^5 cells/well) were collected from the peritoneal cavities of male ddY mice and were suspended in 100 μL of RPMI 1640 supplemented with 5% fetal calf serum (FCS), penicillin (100 units/mL), and streptomycin (100 μg/mL), and pre-cultured in 96-well microplates at 37 °C in 5% CO_2 in air for 1 h. Nonadherent cells were removed by washing with PBS, and the adherent cells were cultured in 200 μL of fresh medium containing 10 μg/mL LPS and various concentrations of test compound for 20 h. NO production in each well was assessed by measuring the accumulation of nitrite (NO_2^-) in the culture medium using Griess reagent. Cytotoxicity was determined by the MTT colorimetric assay, after 20 h incubation with test compounds. Each test compound was dissolved in DMSO and then the solution was added to the medium (final DMSO concentration was 0.5%). Inhibition (%) was calculated using the following formula and IC_{50} was determined graphically (N=4).

$$\text{Inhibition}\,(\%) = [(A-B)/(A-C)] \times 100 \quad A-C:\ NO_2^- \text{ concentration}\,(\mu M)$$

[A: LPS (+), sample (−); B: LPS (+), sample (+); C: LPS (−), sample (−)]

3.5.3. Detection of iNOS. In this experiment, TGC-induced peritoneal exudate cells (7.5×10^6 cells/3 mL/dish) from male ddY mice were pre-cultured in culture dishes (6 cm i.d.) for 1 h, and the adherent cells were obtained as described previously.[9,47] After washing, the culture medium was then exchanged for fresh medium containing 5% FCS, 20 μg/ml LPS, and test compound for 20 h. Cells were collected in lysis buffer [100 mM NaCl, 10 mM Tris, protease inhibitor cocktail (1 tab/10 mL), 0.1% Triton X-100, 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), pH 7.4] and sonicated. After determination of the protein concentration of each suspension by the BCA method (BCA™ Protein Assay Kit, Pierce), the suspension was boiled in Laemmli buffer. For SDS-PAGE, aliquots of 40 μg of protein from each sample were subjected to electrophoresis in 7.5% polyacrylamide gels. Following electrophoresis, the proteins were transferred electrophoretically onto nitrocellulose membranes. The membranes were incubated with 5% nonfat dried milk in Tris-buffered saline (T-TBS, 100 mM NaCl, 10 mM Tris, 0.1% Tween 20, pH 7.4) and probed with mouse monoclonal IgG (dilution of 1:1000) against iNOS. The blots were washed in T-TBS

and probed with the secondary antibody, anti-mouse IgG antibody conjugated with horseradish peroxidase (dilution of 1:5000). Detection was performed using an ECL™ and X-ray film (Hyperfilm-ECL™, Amersham).

3.5.4. Statistics. Values are expressed as means±SEM. One-way analysis of variance followed by Dunnett's test was used for statistical analysis.

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