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Sesquiterpenes from the Rhizomes of *Cyperus rotundus* and Their Potential to Inhibit LPS-induced Nitric Oxide Production

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The rhizomes of *Cyperus rotundus* L. (Cyperaceae) have been used in traditional Chinese medicine as an estrogenic and *anti*-inflammatory agent for the treatment of women's diseases and also used for treatment of stomach ache, bowel disorders, and menstrual disorders.[1] The extract of the rhizomes of *C. rotundus* has been showed a broad range of biological activities, such as *anti*-diabetic activity,[2] acetylcholinesterase inhibitory activity,[3] and inhibitory activities on nitric oxide and superoxide production.[4] Previous phytochemical investigations on *C. rotundus* have resulted in the isolation of a series of sesquiterpenes possessing diverse skeletons[5-7] as well as sesquiterpene alkaloids, triterpenes, sterols, and flavonoids.[7-9] Recently, new patchoulane-type sesquiterpenes were isolated from the rhizomes of *C. rotundus* by our group.[10] We also investigated the inhibitory effect of the *n*-hexane fraction of the 80% EtOH extract on LPS-induced nitric oxide (NO), a pro-inflammatory mediator, in RAW 264.7 cells.[11] In the present study, further investigation of the *n*-hexane-soluble fraction from the rhizomes of *C. rotundus* led to the isolation and characterization of a new natural (**1**) and a new (**2**) patchoulane-type sesquiterpenes, along with seven known eudesman-type sesquiterpenes (**3**-**9**). The structures of **1** and **2** were determined by spectroscopic data interpretation, particularly by extensive 1D and 2D NMR studies. To our knowledge, this is the first report on the ^{13}C NMR assignment of **1**. The structures of other known compounds were identified to be α-rotunol (**3**),[5] β-rotunol (**4**),[5] (–)-eudesma-3,11-diene-5-ol (**5**),[12] ligucyperonol (**6**),[13] 14-hydroxy-α-cyperone (**7**),[14] britanlin E (**8**),[15] and 1β,4β-dihydroxyeudesma-11-ene (**9**)[16] by physical (mp, $[\alpha]_D$) and spectroscopic data (^{1}H NMR, ^{13}C NMR, 2D NMR, and MS) measurement and by comparison with published values.

Compound **1** was obtained as a colorless solid. Its HR-DART-MS gave a pseudo-molecular ion peak at *m/z* = 319.1910 $[MH–H_2O]^+$ (calcd for $C_{19}H_{27}O_4$: 319.1909), indicated a molecular formula of $C_{19}H_{28}O_5$. The ^{1}H-NMR spectrum of **1** (Table 1) showed the presence of three oxymethine protons [δ 5.57 (1H, d, *J* = 6.0 Hz), δ 4.96 (1H, br t, *J* = 7.0 Hz), and δ 4.66 (1H, td, *J* = 10.5 and 7.0 Hz)] and two acetoxyl methyl groups [δ 2.17 (3H, s) and δ 2.03 (3H, s)]. The ^{13}C-NMR and DEPT spectra of **1** (Table 2) allowed the identification of 19 carbon atoms: six methyl groups, two methylene groups, five methine groups, and six quaternary carbon atoms. The chemical shifts of the latter indicated two ester carbonyl (δ 171.2 and 170.9), two aliphatic (δ 61.6 and 41.5), and two sp^2 carbons (δ 140.9 and 139.1). The presence of two acetoxyl groups in **1** was supported by HR-DART-MS spectrum of **1** which showed two fragment ion peaks at *m/z* 277.1794 $[MH–Ac]^+$ and 217.1623 $[MH–2Ac]^+$. Further examination of the ^{1}H-NMR spectrum of **1** revealed some signals common to patchoulane-type sesquiterpenes, namely the two geminal methyl groups H-12 and H-13 (δ 0.88 and 1.07, respectively; each 3H, s), the methyl group H-15 at δ 0.81, appearing as a doublet (3H, *J* = 6.5 Hz) and coupling with the methine proton H-10 (δ 2.12, 1H, dq, *J* = 10.5 and 6.5 Hz), and the vinyl methyl group H-14 at δ 1.74, appearing as a singlet. The ^{1}H- and ^{13}C-NMR spectra of **1** exhibited strong similarities with those of sugetriol triacetate[10,17] (Tables 1 and 2), which was isolated from the same plant in the previous study.[10] The inspection of the ^{1}H- and ^{13}C-NMR spectra of **1** readily indicated the absent of an acetyl group (δ_H 2.05, δ_C 21.1 and δ_C 170.7) of sugetriol triacetate. It was also observed that both the oxymethine proton at δ_H 5.90 and the oxymethine carbon at δ_C 84.8 in the ^{1}H- and ^{13}C-NMR spectra of sugetriol triacetate were shifted to upfield in the those of **1** (δ_H 4.96 and δ_C 83.2) due to deacetylation. The COSY and HMBC correlations (Figure 2) confirmed the

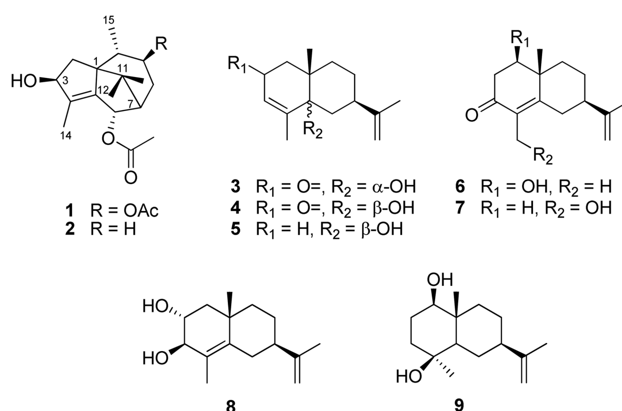

Figure 1. Structures of **1**-**9** isolated from the rhizomes of *C. rotundus*.

[a]These authors contributed equally to this work.

Table 1. ^{1}H-NMR Spectral Data for **1** and **2** (in $CDCl_3$)[a]

Position	δ_H (*J* in Hz)		
	1	**2**	sugetriol triacetate[10,17]
2	2.12 dd (13.0, 6.5)	2.07 dd (13.0, 6.5)	2.17 dd (13.5, 6.5)
	1.38 dd (13.5, 6.5)	1.35 dd (13.5, 7.0)	1.49 dd (13.5, 7.0)
3	4.96 br t (7.0)		
		4.94 br t	5.90 dd (7.0, 6.5)
6	5.57d (6.0)	5.63 d (6.0)	5.57 d (6.0)
7	2.32 dt (6.0, 3.0)	2.18 td (6.0, 3.0)	2.35 dt (6.0, 3.5)
8	1.86 ddd (13.5, 7.0, 4.0)	1.76 m	1.87 ddd (13.5, 7.0, 3.5)
	1.65 ddd (13.5, 10.5, 3.0)	1.29 m	1.65 ddd (13.5, 10.5, 3.5)
9	4.66 td (10.5, 7.0)	1.48 m	4.65 td (10.5, 7.0)
		1.21 m	-
10	2.17 overlap[b]	2.05 overlap[b]	2.17 dq (10.5, 6.5)
12	0.88 s	0.86 s	1.06 s
13	1.07 s	0.99 s	0.88 s
14	1.74 s	1.74 s	1.65 s
15	0.81 d (6.5)	0.73 d (6.5)	0.87 d (6.5)
3-COO*CH*$_3$	-	-	2.05 s
6-COO*CH*$_3$	2.03 s	2.09 s	2.03 s
9-COO*CH*$_3$	2.17 s	-	2.17 s

[a]The assignments were based on COSY, HMQC, and HMBC experiments. [b]Overlapping with other signals.

Table 2. ^{13}C-NMR Spectral Data for **1** and **2** (in $CDCl_3$)[a]

Position	δ_C		
	1	**2**	sugetriol triacetate[10,17]
1	61.6	62.2	62.1
2	39.7	39.9	36.5
3	83.2	83.4	84.8
4	139.1	138.1	142.8
5	140.9	142.1	135.9
6	70.9	71.3	70.7
7	50.7	50.9	50.8
8	28.3	22.6	28.2
9	75.1	28.9	74.7
10	40.0	35.0	39.6
11	41.5	41.6	41.7
12	20.4	26.2	20.3
13	25.7	20.3	25.6
14	10.5	10.5	10.8
15	14.3	18.3	14.2
3-COO*C*H$_3$	-	-	21.1
6-COO*C*H$_3$	21.2	21.0	21.2
9-COO*C*H$_3$	20.9	-	20.8
3-*C*OOCH$_3$	-	-	170.7
6-*C*OOCH$_3$	170.9	171.0	170.8
9-*C*OOCH$_3$	171.2	-	171.2

[a]The assignments were based on COSY, HMQC, and HMBC experiments.

assignments of all proton and carbon resonances and the location of the double bond (C-4) and two acetoxyl groups (C-6 and C-9). The absolute configuration at C-3, C-6 and C-9 was proposed as 3*S*, 6*S*, and 9*S* on the basis of NOESY correlations (Figure 3) from δ_H 5.57 (H-6) to δ_H 0.88 (H-12) and δ_H 4.66 (H-9) to δ_H 0.81 (H-15) and on the basis of

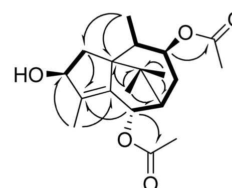

Figure 2. Selected correlations observed in the COSY (▬) and HMBC (⟶) spectra of **1**.

sugetriol triacetate of known absolute configuration.[17] Thus, **1** was determined to be sugetriol 6,9-diacetate. Although **1** was synthesized from sugetriol triacetate,[18] it is the first report on the isolation of **1** from natural source, and of its full NMR assignment.

Compound **2** was obtained as a colorless oil. Its HR-DART-MS gave a pseudo-molecular ion peak at *m/z* 261.1837 ($[MH–H_2O]^+$, Calcd for $C_{17}H_{25}O_2$: 261.1855), indicated a molecular formula of $C_{17}H_{26}O_3$. The proton and carbon signals in the ^{1}H- and ^{13}C-NMR spectra of **2** exhibited strong similarities with those of **1** except for the absence of one of two acetoxyl groups in **1**. In detail, the ^{1}H- and ^{13}C-NMR spectra of **2** revealed the absence of an oxymethine and acetoxyl group (δ_H 4.66 and 2.17/δ_C 75.1, 171.2, and 20.9) and the presence of a methylene group (δ_H 1.48 and 1.21/δ_C 28.9). Thus, the structure of **2** was suggested as a 9-deacetoxyl analogue of **1**. The positions of the acetoxyl (C-6) and hydroxyl groups (C-3) in **2** were confirmed using the HMBC NMR technique. Therefore the new compound **2** was elucidated as sugebiol 6-acetate (cyperene-3,6-diol 6-acetate). The absolute conformation at C-3 and C-6 in **2** was proposed as 3*S* and 6*S* in a similar manner to that of **1** (Figure 3).

We evaluated the abilities of **1-9** isolated from the rhizomes of *C. rotundus* to inhibit LPS-induced nitric oxide

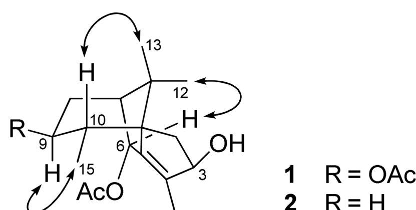

Figure 3. Selected correlations observed in the NOESY (↔) NMR spectra of **1** and **2**.

Table 3. Inhibitory effect of **1-9** isolated from on nitric oxide production in LPS-induced RAW 264.7 cells

Compound	IC_{50} value (μM)[a]
1	> 200
2	100 ± 1
3	> 200
4	44 ± 2
5	> 200
6	> 200
7	64 ± 1
8	76 ± 3
9	91 ± 2
L-NIL[b]	15 ± 1

[a]IC_{50} value is defined as the concentration that results in a 50% decrease production of nitric oxide. The values represent the means of the results from three independent experiments with similar patterns. [b]L-N^6-(1-iminoethyl)lysine (L-NIL) was used as assay positive control for NO production.

(NO) production in RAW 264.7 cells at non-toxic concentrations. The effects of the nine compounds were assessed using IC_{50} values (Table 3). Interestingly, sugebiol 6-acetate (**2**), β-rotunol (**4**), 14-hydroxy-α-cyperone (**7**), britanlin E (**8**), and 1β,4β-dihydroxyeudesma-11-ene (**9**) were found to have significant inhibitory effects on NO production (IC_{50} values were 100 μM). Therefore, we suggest that **2**, **4**, **7**, **8**, and **9** may be potently active compounds that have *anti*-inflammatory properties *via* inhibition of NO production.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns melting point apparatus without correction. Optical rotations were measured on a Jasco P-2000 polarimeter, using a 10-cm microcell. HR-Mass spectra were obtained using an AccuTOF-single-reflectron time-off light mass spectrometer (Jeol Ltd, Tokyo, Japan) equipped with a DART ion source (IonSense, Saugus, MA, USA). NMR spectra were obtained using a Varian 500 MHz NMR spectrometer using TMS as an internal standard and chemical shifts are expressed as δ values. IR spectra were obtained using a Varian 640-IR. TLC analyses was performed on Kieselgel 60 F_{254} (Merck) plates (silica gel, 0.25 mm layer thickness); compounds were visualized by dipping plates into 20% (v/v) H_2SO_4 reagent (Aldrich) and then heated at 110 °C for 5-10 min. Silica gel (Merck 60A, 70-230 or 230-400 mesh ASTM), Sephadex LH-20 (Amersham Pharmacia Biotech), and reversed-phase silica gel (YMC Co., ODS-A 12 nm S-150 μm) were used for column chromatography. All solvents used for the chromatographic separations were distilled before use. Dulbecco's modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY). Escherichia coli LPS, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant Material. The rhizomes of *Cyperus rotundus* L. were obtained from a domestic Korean market (Kyungdong Crude Drugs Market, Seoul, South Korea), in June 2011. The origin of the herbal material was identified by one of the authors (D.S.J.) and a voucher specimen (CYRO1-2011) was deposited in the Lab. of Natural Product Medicine, College of Pharmacy, Kyung Hee University.

Extraction and Isolation. The dried and milled plant material (2.8 kg) was extracted with 10 L of 80% EtOH three times by maceration. The extracts were combined and concentrated in vacuo at 40 °C to give a 80% EtOH extract (399 g). A portion of the 80% EtOH extract (392 g) was suspended in H_2O (2 L) and successively extracted with *n*-hexane (3 × 2 L), EtOAc (3 × 2 L), and BuOH (3 × 2 L) to give *n*-hexane- (45.8 g), EtOAc- (23.5 g), BuOH- (52.4 g), and water-soluble extracts (270.3 g), respectively. The *n*-hexane-soluble extract (44 g) was chromatographed over silica gel (70-230 mesh, ϕ 6.0 × 44 cm) as stationary phase with a *n*-hexane-EtOAc gradient (from 1:0 to 1:1 v/v; final stage, MeOH 100%) as mobile phase to afford 18 pooled fractions (H01-H18). Fraction H7 (2.08 g) was further fractionated using a Sephadex column (ϕ 3.6 × 72 cm) with CH_2Cl_2-MeOH mixture (1:1 v/v), yielding compound **5** (20 mg). Fraction H11 (2.09 g) was subjected to a Sephadex column (ϕ 3.6 × 72 cm) with CH_2Cl_2-MeOH mixture (1:1 v/v) to produce 4 subfractions (H11-01~H11-4). Compound **2** (6.9 mg) was purified from the subfraction H11-3 (880 mg) using a flash chromatography system with Redi Sep-Silica cartridges (40 g, *n*-hexane-EtOAc = 19:1 → 9:1 v/v). Compound **3** (18.2 mg) was also obtained from subfraction H11-3-2 (580 mg) by using a flash chromatographic system with Redi Sep-Silica cartridges (40 g, CH_2Cl_2-EtOAc, 19:1 to 4:1 v/v). Fraction H15 (1.83 g) was chromatographed over Sephadex LH-20 (ϕ 3.6 × 72 cm) as stationary phase with a CH_2Cl_2-MeOH mixture (1:1 v/v) as mobile phase to afford 7 subfractions (H15-1~H15-7). Compound **1** (7.8 mg) was purified from the subfraction H15-3 (110 mg) using a flash chromatographic system with Redi Sep-Silica (24 g, CH_2Cl_2-EtOAc = 9:1 v/v) and Redi Sep-C18 (4.3 g, ACN-H_2O = 3:7 v/v) cartridges. Subfraction H15-5 (1.2 g) was further chromatographed over silica gel (230-400 mesh, ϕ 3.5 × 34 cm) as stationary phase with a *n*-hexane-acetone gradient (*n*-hexane-acetone = 4:1 → 7:3 v/v; final stage, MeOH 100%) as mobile phase to afford **4** (24.8 mg), **6** (10.4 mg), and **7** (15.2 mg). Fraction H16 (2.08 g) was subjected to a Sephadex column (ϕ 3.6 × 72 cm) with CH_2Cl_2-MeOH mixture (1:1 v/v) to obtain 6 subfractions (H16-1~H16-6). Compounds **8** (36.5 mg) and **9** (11.7 mg) were purified by silica gel CC

(230-400 mesh, ϕ 3.7×18 cm) with CH_2Cl_2-EtOAc mixture (4:1 → 2:3 v/v) from fraction H16-6.

Sugetriol 6,9-diacetate (1): Colorless solid. $[\alpha]_D^{25}$: −2.6° (*c* 0.041, $CHCl_3$); IR (ATR) ν_{max} cm^{-1}: 2927, 1735, 1462, 1373, 1241, 758; ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) data, see Table 1 and 2; HR-DART-MS *m/z* = 319.1910 $[MH–H_2O]^+$ (calcd for $C_{19}H_{27}O_4$: 319.1909), 277.1794 $[MH–Ac]^+$, 217.1623 $[MH–2Ac]^+$.

Sugebiol 6-acetate (2): Colorless oil. $[\alpha]_D^{25}$: +19.8° (*c* 0.071, $CHCl_3$); IR (ATR) ν_{max} cm^{-1}: 2935, 1730, 1519, 1215, 1027, 754; ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 125 MHz) data, see Table 1 and 2; HR-DART-MS *m/z* = 261.1837 ($[MH–H_2O]^+$, Calcd for $C_{17}H_{25}O_2$: 261.1855), 219.1754 $[MH–Ac]^+$.

Measurment of Nitric Oxide. RAW264.7 cells were plated at 2×10^5 cells/well in 60-mm dishes and incubated with or without LPS (1 μg/mL) in the absence or presence of indicate concentration of the samples for 24 h. The nitrite which accumulated in culture medium was measured as an indicator of NO production according to the Griess reagent. The culture supernatant (100 μL) was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthyl ethylenediamine-HCl] for 10 min, and then the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was determined with reference to a sodium nitrite standard curve.

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Supporting Information. The spectral data of compounds **1** and **2** are available on request from the correspondence author.

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