Phenanthrenoids from *Juncus acutus* L., New Natural Lipopolysaccharide-Inducible Nitric Oxide Synthase Inhibitors

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The novel natural product juncutol (1), 1,4,7-trimethyl-8,9-dihydro-4*H*-cyclopenta[*def*]phenanthrene-2,6-diol, along with the three related metabolites juncusol (2), dehydrojuncusol (3), and 6-hydroxymethyl-1-methyl-5-vinyl-9,10-dihydrophenanthrene-2-ol (4), were isolated from the rhizomes of *Juncus acutus* L. (Juncaceae) growing in Egypt. The structural identity of 1 was determined on the basis of spectroscopic analyses, including 2D NMR spectroscopy. The inhibitory effect of these natural products on the expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide-stimulated RAW264.7 macrophage cells was determined for the first time. The unprecedented symmetrical compound juncutol (1) was found to be the most potent inhibitor against the induction of the proinflammatory iNOS protein.

Key words *Juncus acutus* L.; Juncaceae; juncutol; phenanthrenoid; iNOS inhibitor; antiinflammatory activity

The wetland *Juncus* plants (*e.g.*, *Juncus acutus*, *Juncus roemerianus* and *Juncus effusus*) are considered a rich source of nitrogen-free alkylated phenanthrenoids.[1—11] Juncusol, 1,6-dimethyl-5-vinyl-9,10-dihydrophenanthrene-2,7-diol (**2**), isolated from *J. roemerianus*[1] is the first example of this class of natural products. A wide range of biological activities such as cytotoxicity,[1,2] antitumor,[3] antialgal,[4—7,9] antimicrobial, and DNA-binding photosentizing antimicrobial[8] have been reported for *Juncus* phenanthrenoids. Although the phenanthrenoid-rich plant *J. effusus* is used traditionally in Japan, China, and Taiwan as an antipyretic and antiphlogistic agent,[8,10] no antiinflammatory studies have been reported of its phenanthrenoids. Our chemical investigation of the rhizomes of *J. acutus* L. (Juncaceae) growing in Egypt has led to the isolation of a novel phenanthrenoid, juncutol (**1**), along with three related compounds (**2**—**4**). The structure of the novel metabolite was mainly determined on the basis of spectroscopic analyses, including 2D NMR (^{1}H–^{1}H COSY, HMQC, and HMBC) spectroscopy and by spectral comparison. The antiinflammatory effects of the isolated phenanthrenoids were evaluated *in vitro* by measuring the percent inhibition of proinflammatory inducible nitric oxide synthase (iNOS) protein expression in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells.

The dried rhizomes of *J. acutus* were powdered and exhaustively extracted with MeOH. The MeOH-free extract was partitioned with H_2O/light petrol and H_2O/EtOAc to afford the petrol and EtOAc fractions, respectively. The resulting fractions were chromatographed on a column of Si gel and RP-18 Si gel to afford compounds **1** and **2** from the petrol fraction and **3** and **4** from the EtOAc fraction (Fig. 1). Compounds **2**, **3**, and **4** were identified by comparison of their physical and spectroscopic (MS, UV, and NMR) data with those of the previously isolated compounds from *Juncus* species as juncusol,[1,8] dehydrojuncusol,[11] and 6-hydroxymethyl-1-methyl-5-vinyl-9,10-dihydrophenanthrene-2-ol,[6] respectively

The novel compound juncutol (**1**) was obtained as a yellow powder, mp 214—216 °C. The molecular formula $C_{18}H_{18}O_2$ was determined using HR-EI-MS (*m*/*z* 266.1305 $[M]^+$), appropriate for 10 degrees of unsaturation. The IR absorption bands at 3229 and 1595 cm^{-1} and the six carbon signals appearing at δ_C 109.9—155.7 in the ^{13}C-NMR spectrum indicated the presence of a phenolic moiety in **1**. The UV spectrum of **1** in MeOH showed an intense λ_{max} (log ε) at 212 (4.34), 285 (4.04), and 296 sh (4.02) nm, which were found to be similar to those of juncusol (**2**).[8] Unlike **2**, the ion peaks appearing in the EI-MS at *m*/*z* 251 $[M-15]^+$, 236 $[M-2\cdot15]^+$, and 221 $[M-3\cdot15]^+$, suggested the presence of three methyls in **1**. This was supported by the proton signals at δ_H 2.16 (6H, s) and 1.42 (3H, d, J=7.2 Hz) attributable to two chemically equivalent aromatic methyls and one secondary methyl, respectively. Moreover, two pairs of chemically equivalent aromatic methine (δ_H 6.74, 2H, s) and benzylic methylene (δ_H 3.02, 4H, s) protons were also deduced from the ^{1}H-NMR spectrum of **1**. The fifth proton signal appearing in the ^{1}H-NMR spectrum at δ_H 3.79 (1H, q, J=7.2 Hz) was found to show HMQC correlation with the single sp^3 me-

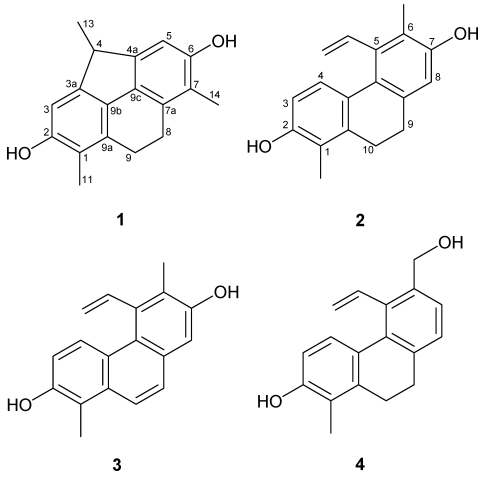

Fig. 1. Juncutol (**1**) and Related Compounds (**2**—**4**) from *J. acutus*

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Table 1. ^{1}H- and ^{13}C-NMR Spectral Data for Compound **1**

C/H	**1**	
	δ_H [a)]	δ_C [b)]
1, 7		121.5 (qC)
2, 6		155.7 (qC)
3, 5	6.74 2H, s	109.9 (CH)[d)]
3a, 4a		144.9 (qC)
4	3.79 1H, q (7.2)[c)]	44.8 (CH)
8, 9	3.02 4H, s	25.3 (CH_2)
11, 14	2.16 6H, s	11.0 (CH_3)
13	1.42 3H, d (7.2)	18.7 (CH_3)
7a, 9a		128.9 (qC)
9b, 9c		132.0 (qC)

Spectra recorded at *a*) 300 MHz and *b*) 75 MHz in CD_3OD. *c*) *J* values (in Hz) parentheses. *d*) Attached protons were deduced by DEPT experiments.

thine carbon at δ_C 44.8. Although **1** possesses the same molecular formula, $C_{18}H_{18}O_2$, as that of **2**, the ^{13}C-NMR spectrum of **1** showed the presence of only 10 carbon signals (Table 1). Therefore **1** was deduced to have a symmetrical structure. Structural differences between **1** and **2** can be assigned on the basis of the comparison of the NMR data (measured in CD_3OD) of **1** with those of **2**. It was found that the signals of the vinyl side chain (δ_H 6.74, 1H, dd, J=18.0, 11.7 Hz; 5.40, 1H, br d, J=11.7 Hz; 5.11, 1H, br d, J=18.0 Hz and δ_C 139.4, CH; 119.2, CH_2) in **2** were replaced by the signals of one secondary methyl and one sp^3 methine in **1** (Table 1). Since the protons of the two latter groups were found to be correlated with each other in the ^{1}H–^{1}H COSY spectrum (Fig. 2), therefore the remaining eight carbon signals in the ^{13}C-NMR spectrum of **1** should correspond to a 9,10-dihydrophenenthrene moiety, symmetrically substituted with two hydroxy and two methyl groups. The two aromatic methyls in **1** were thought to be located at C-1 and C-7 due to their chemical shift (δ_C 11.0), which resembles that at C-1 in **2** (δ_C 11.7). This was further supported by the HMBC correlations (Fig. 2) observed from the protons of aromatic methyls and the benzylic methylenes to the same quaternary carbons at δ_C 121.5 (2C, C-1, and C-7) and 128.9 (2C, C-7a, and C-9a). Moreover, the HMBC correlations found from these methyls to the oxycarbons at δ_C 155.7 indicated the C-2 and C-6 positions of the hydroxy groups in **1**. Furthermore, a $^2J_{CH}$ coupling was found between C-2 and an aromatic proton (δ_H 6.74, s, H-3), which in turn showed HMBC correlation with the single sp^3 methine carbon (δ_C 44.8, C-4) of the molecule. This established the bridging of C-3a and C-4a by the sp^3 methine carbon (C-4) in **1** to form a cyclopenta[*def*]-phenanthrene structure. This was further confirmed by the $^3J_{CH}$ couplings observed between each of C-3a, C-4a (δ_C 144.9, 2C), and C-4 (δ_C 44.8, 1C) with the protons of the secondary methyl (δ_H 1.42, 3H, d, J=7.2 Hz, H_3-13). On the basis of the above findings and other detailed HMBC correlations (Fig. 2), the structure of juncutol (**1**) was fully established as shown in Fig. 1 to be 1,4,7-trimethyl-8,9-dihydro-4*H*-cyclopenta[*def*]phenanthrene-2,6-diol.

LPS is an endotoxin, which induces septic shock syndrome and stimulates the production of inflammatory mediators such as NO and prostaglandins.[12,13] The production of these two inflammatory mediators is associated with the expression of the proinflammatory iNOS and cyclooxygenase

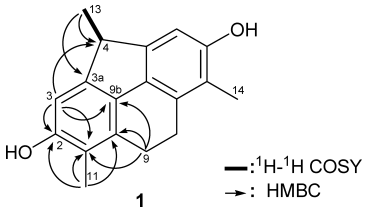

Fig. 2. ^{1}H–^{1}H COSY and HMBC Correlations for Juncutol (**1**)

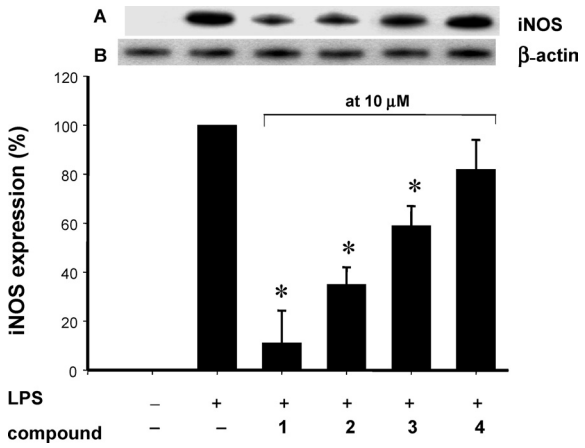

Fig. 3. Effect of Compound **1**—**4** on iNOS Protein Expression of LPS-Stimulated RAW264.7 Macrophage Cells by Immunoblot Analysis[a)]

(A) Immunoblot of iNOS. (B) Immunoblot of β-actin. Equal loading of proteins was verified by β-actin immunoblot. The values are mean±S.E.M. (n=6). Relative intensity of the LPS-stimulated control group was taken as 100%. Significantly different from LPS-stimulated group (*p<0.05). *a*) CAPE (caffeic acid phenylethyl ester) inhibited LPS-iNOS expression, under the same experimental condition, with IC_{50} value 0.86±0.14 μM.

(COX)-2 proteins. Therefore agents able to block or reduce LPS-induced expression of iNOS and/or COX-2 proteins might be beneficial in the treatment of inflammatory responses. We utilized an *in vitro* assay system of LPS-stimulated RAW264.7 macrophage cells linked with immunoblot analysis[14,15] with the aim of identifying a new class of iNOS and/or COX-2 inhibitors. The inhibitory effects of the isolated phenanthrenoids **1**—**4** from *J. acutus* L. were thus estimated. It was found that all compounds showed selective inhibition of iNOS protein expression in LPS-stimulated RAW264.7 macrophage cells with different degrees of potency, while they were inactive against COX-2 protein expression. The symmetrical compound juncutol (**1**) has been shown to be the most potent phenanthrenoid and strongly reduced the level of iNOS protein to 11.2±13.1% at 10 μM relative to that (100%) of the control LPS-stimulated RAW264.7 macrophage cells. Related compounds **2** and **3** also exhibited significant activities at 10 μM, reducing the iNOS protein expression to 35.0±7.0% and 59.0±8.0%, respectively, relative to the controls (Fig. 3). Finally, compound **4**, which has only one phenolic hydroxy group in the molecule, was found to be weakly active and only inhibited iNOS protein expression to 82.0±12.0% at 10 μM. It appears that the presence of the two phenolic hydroxyl groups at C-2 and C-7 in the molecule is essential for inhibiting iNOS expression.

From the above observations, the nonnitrogenous phenanthrene-2,7-diol derivatives may be regarded as a new class of natural antiinflammtory agents. A correlation of the antiin-

flammatory activity of these natural products to the traditional use of the *Juncus* herbs as antipyretic and antiphlogistic agents in the Far East[8,10] has thus been experimentally established for the first time.

Experimental

Melting points were determined using a Fisher John melting point apparatus. IR spectra were recorded on a Hitachi I-2001 infrared spectrophotometer. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. The NMR spectra were recorded on a Bruker Avance-DPX 300 FT-NMR at 300 MHz for ^{1}H-NMR and at 75 MHz for ^{13}C-NMR in CD_3OD using TMS as an internal standard. Mass spectral data were obtained by EI and FAB with a VG Quattro GC/MS spectrometer. HR-EI-MS spectra were recorded on a Finnigan MAT-95XL mass spectrometer. Silica gel 60 (Merck, 230—400 mesh) and RP-18 Silica gel (Merck, 230—400 mesh) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.2 mm) were used for analytical TLC.

Plant Material The rhizomes of *J. acutus* L. were collected near New Damietta (north coast of Egypt) during late spring (May 2002) and identified by Prof. Ibrahim A. Mashaly, Department of Botany, Faculty of Science, Mansoura University. A voucher sample was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University.

Extraction and Isolation The air-dried rhizomes of *J. acutus* L. (4.5 kg) were powdered and exhaustively extracted with MeOH. The MeOH extract was filtered and concentrated under a vacuum to afford a dark brown viscous residue (674 g). The solvent-free residue was partitioned with H_2O/light petrol (boiling point 60—80 °C) and then with H_2O/EtOAc. The two organic partitions were separately evaporated under a vacuum to give the petrol fraction and the EtOAc fraction, respectively. The petrol fraction (37 g) was chromatographed on a column of Si gel and eluted with EtOAc in light petrol (0—100%, gradient) to yield 15 subfractions. Subfractions 9 to 13, eluted with EtOAc/light petrol (1 : 3 to 1 : 0), were combined (showing two major UV active spots at *Rf* values 0.3 and 0.2 in EtOAc/light petrol, 1 : 4) and rechromatographed on a column of Si gel using EtOAc/light petrol (3 : 17) to afford subfractions P-1 (76 mg) and P-2 (21 mg). Subfractions P-1 and P-2 were separately purified by successive washing with 25% CH_2Cl_2 in light petrol to yield **2** (12 mg) from P-1 and **1** (3.5 mg) from P-2. The EtOAc fraction (85 g) was also chromatographed on a column of Si gel and eluted with EtOAc/light petrol (0 : 100 to 100 : 0, gradient) and EtOAc–MeOH–H_2O (100 : 0 : 0 to 100 : 16.5 : 13.5) to give 16 subfractions (E-1 to E-16). Subfraction E-4, eluted with EtOAc/light petrol (1 : 1), was subjected to Si gel column chromatography using EtOAc/light petrol (0 : 100 to 100 : 0, gradient) to afford **3** (103 mg) and a mixture. The latter mixture was purified by successive Si gel column chromatography, using EOAc/light petrol (0 : 100 to 100 : 0, gradient) and RP-18 using MeOH–H_2O (1 : 1 to 1 : 0, gradient) to yield **4** (2.8 mg).

Juncutol (**1**): Yellow powder, mp 214—216 °C. IR (neat) ν_{max} 3229, 2953, 2922, 1595, 1456, 1279, 1049, 851, 760 cm^{-1}. UV λ_{max} (MeOH) nm (log ε) 408 (3.96), 386 sh (3.81), 296 sh (4.02), 285 (4.04), 212 (4.34). ^{1}H- and ^{13}C-NMR data (CD_3OD), see Table 1. EI-MS *m/z* 266 (100, $[M]^+$), 251 (46.3), 236 (16.4), 221 (6.5), 218 (8.2), 208 (4.3), 205 (13.2), 202 (12.1), 200 (3.4), 190 (9.0), 189 (24.9), 165 (17.2), 152 (12.6). HR-EI-MS *m/z* 266.1305 (Calcd for $C_{18}H_{18}O_2$, 266.1307).

In Vitro* Antiinflammatory Assay** The *in vitro* antiinflammatory assay[14] was modified from the methods of Ho *et al.*[15] and Park *et al.*[16] Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC, no. TIB-71) and cultured in Dulbecco's modified essential medium (DMEM) containing 10% heat-inactivated fetal bovine serum at 37 °C in a humidified 5% CO_2–95% air incubator under standard conditions. Macrophages were activated by incubation in medium containing *Escherichia coli* LPS (0.01 μg/ml; Sigma, St. Louis, MO, U.S.A.) for 16 h in the presence or absence of various compounds. Then cells were washed with ice-cold PBS, lysed in ice-cold lysis buffer, and centrifuged at 20000 ***g for 30 min at 4 °C. The supernatant was decanted from the pellet and retained for Western blot analysis. Protein concentrations were determined using a DC protein assay kit (Bio-Rad, Hercules, CA, U.S.A.) using the modified method of Lowry *et al.*[17] Samples containing equal quantities of protein were subjected to SDS-PAGE and the separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PVDF; Immobilon-P, Millipore, 0.45 μM pore size). The resulting PVDF membranes were incubated with blocking solution and incubated for 180 min at room temperature with antibody against iNOS (1 : 1000 dilution; Transduction Laboratories, U.S.A.) and COX-2 (1 : 1000 dilution; Cayman Chemical, U.S.A.) protein. The blots were detected using ECL detection reagents (Perkin-Elmer, Western Blot Chemiluminescence Reagent Plus) according to the manufacturer instructions and finally exposed to X-ray film (Kodak X-OMAT LS, Kodak, U.S.A.). The membranes were reprobed with a monoclonal mouse anti-β-actin antibody (1 : 2500, Sigma) as the loading control. After X-ray film scanning, the integrated optical density of the bands was estimated (Image-Pro plus 4.5 software) and normalized to the background values. Relative variations between the bands of the drug-treated samples and the LPS-treated samples were calculated in the same image.

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