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ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · FEBRUARY 2004

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New Geranyl Stilbenes from *Dalea purpurea* with in Vitro Opioid Receptor Affinity

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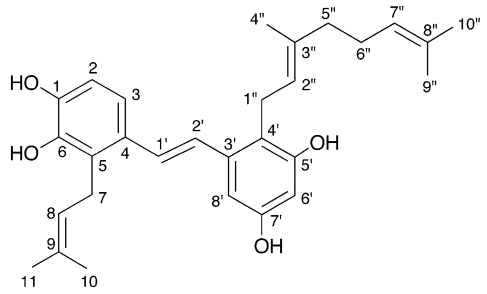
Received June 5, 2003

Three new geranyl stilbenes, pawhuskins A, B, and C (**1**, **2**, and **3**), were isolated from organic extracts of *Dalea purpurea*. The structures of the three compounds were determined by NMR and HRMS methods. The activities of these compounds, along with that of the known compound petalostemumol (**4**), were evaluated in an opioid receptor assay in vitro. Pawhuskin A (**1**) exhibited the strongest activity of the four compounds with a K_i value of 0.29 ± 0.11 μM.

The genus *Dalea* (Fabaceae) includes more than one hundred species that, in newer taxonomies, contain members of the genera *Petalostemon*, *Parosela*, and *Psorothamnus*. A number of *Dalea* spp. have been the subject of detailed chemical study in recent years, with chalcones,[1] flavonoids,[2−4] and a pterocarpan[5] having been reported, among others.[6,7] *Dalea purpurea* (syn.: *Petalostemon purpureum*) was one of a group of plants from the Nature Conservancy's Tallgrass Prairie Preserve, near Pawhuska, Oklahoma, selected for extraction and testing in an in vitro opioid receptor assay.[8] *D. purpurea* was used by Plains Indians to ward off disease and for unspecified ailments.[9] The Pawnee used it in this way as a prophylactic by pulverizing the root, steeping it in hot water, and drinking the liquid above the resulting sediment.[10]

Organic extracts of *D. purpurea* exhibited moderate activity in the opioid assay.[8] Fractionation of these extracts afforded three new geranyl stilbenes, pawhuskins A−C (**1**−**3**), and one known compound, petalostemumol (**4**), previously isolated from this plant.[2] The structures of compounds **1**−**4** were determined by NMR and HRMS techniques. Details of the isolation, structure determination, and biological activities of these metabolites are reported here.



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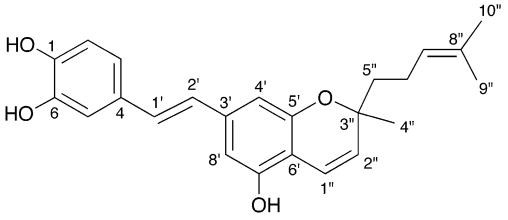

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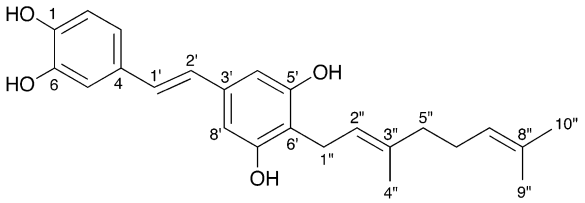

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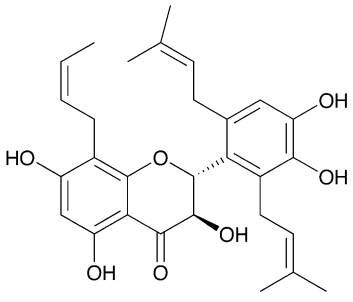

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Results and Discussion

Fractionation of methanolic extracts of *D. purpurea* by silica gel VLC, Sephadex LH-20, and repeated gradient chromatography over silica gel afforded metabolites **1**−**4**. The HRESIMS, ^{13}C NMR (Table 1), and DEPT data for compound **1** indicated a molecular formula of $C_{29}H_{36}O_4$. Signals for four phenolic hydroxyl groups and for two *trans* double-bond protons (J_{HH} = 15.9 Hz) were present in the ^{1}H NMR spectrum. Also present were four aromatic proton signals, two *ortho*-coupled (J_{HH} = 8.2 Hz) and two *meta*-coupled (J_{HH} = 2.4 Hz). Assignments for all of the corresponding ^{1}H and ^{13}C NMR signals in the molecule were made according to DEPT and HMQC data. The five methyl singlets present in the ^{1}H NMR spectrum showed HMBC correlations characteristic of prenyl and geranyl side chains, for example, correlations from H_3-10 to the sp^2 carbons at C-8 and C-9, as well as to the C-11 methyl group. COSY correlations between H-7 and H-8 and between H-5″, H-6″, and H-7″ further confirmed the structures of these portions of the molecule. Weak long-range couplings to the methyl protons were also observed (e.g., H-8 and H_2-7 →

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10.1021/np030258d CCC: $27.50
Published on Web 12/18/2003

Table 1. NMR Data for Compounds **1**−**3** in Acetone-d_6

	1		**2**		**3**	
position	δ_C [a]	δ_H [b] (mult.; J_{HH})	δ_C [a]	δ_H [b] (mult.; J_{HH})	δ_C [a]	δ_H [b] (mult.; J_{HH})
1	145.1		146.3[e]		146.1	
1-OH		7.06(s)		8.00(s)		7.90[f]
2	113.9	6.73(d; 8.2)	116.4	6.80(d; 8.2)	116.4	6.79(d; 7.9)
3	118.1	7.01(d; 8.4)	120.2	6.91(dd; 8.2, 2.0)	120.0	6.85(dd; 8.2, 2.0)
4	130.6		130.9		131.1	
5	127.7		114.1	7.07(d; 2.0)	114.0	7.03(d; 2.0)
6	144.1		146.3[e]		146.3	
6-OH		8.40(s)		7.87(s)		8.04[f]
7	26.0[c]	3.53(d; 6.8)				
8	124.5	5.17(m)				
9	131.5					
10	18.3	1.81(br s)				
11	25.9[c]	1.66(br s)				
1′	128.8	7.13(d; 15.9)	129.7	6.97(d; 16.3)	128.6	6.84(d; 16.7)
2′	127.4	7.07(d; 15.9)	126.8	6.79(d; 16.5)	127.2	6.76(d; 16.0)
3′	140.0		140.0		137.5	
4′	118.7		106.5	6.51(br s)	106.1	6.57(s)
5′	156.9[d]		155.5		157.2	
5′-OH		8.07(s)				8.09(br s)
6′	102.9	6.36(d; 2.4)	109.8		115.5	
7′	157.0[d]		154.1		157.2	
7′-OH		7.93(s)		8.41(s)		8.09(br s)
8′	104.9	6.65(d; 2.4)	106.8	6.55(d; 1.3)	106.1	6.57(s)
1″	25.0	3.43(d; 6.8)	118.4	6.71(d; 10.1)	23.2	3.36(d; 7.1)
2″	125.6	5.17(m)	128.1	5.59(d; 9.9)	124.4	5.32(m)
3″	134.4		78.9		134.8	
4″	16.6	1.78(br s)	26.7	1.36(s)	16.4	1.77(s)
5″	40.7	1.96(m)	42.1	1.69(m)	40.8	1.95(m)
6″	27.7	2.05(m)	23.6	2.13(m)	27.7	2.05(m)
7″	125.3	5.06(m)	125.4	5.12(m)	125.4	5.09(m)
8″	131.8		132.0		131.7	
9″	17.8	1.52(s)	17.8	1.57(s)	17.8	1.56(s)
10″	25.9[c]	1.58(s)	25.9	1.64(s)	25.9	1.61(s)

[a] 50 MHz. [b] 600 MHz. [c−f] Assignments may be interchanged.

H_3-11). HMBC correlations were critical in determining the substitution pattern around the stilbene core of the molecule. Key correlations were observed from H_2-7 of the prenyl group and H_2-1″ of the geranyl group to the carbons of the rings to which these groups are attached (see Experimental Section). Similarly, HMBC correlations between H-3 and C-1′, H-1′ and C-3, H-8′ and C-2′, and H-2′ and C-4′ helped deduce connectivity in the central part of the molecule. Assignments of the *E* stereochemistry at C-2″−C-3″ and differentiation of the terminal methyl groups were made using ^{1}H and ^{13}C NMR chemical shift and coupling constant values,[11] as well as by comparison with the closest related known compounds, schweinfurthins A−C.[12] The remaining HMBC correlations were sufficient to support the assigned structure of pawhuskin A (**1**) as shown.

The molecular formula of pawhuskin B (**2**) was found to be $C_{24}H_{26}O_4$, as deduced from HRESIMS and NMR data. The stilbene core of the molecule was present in **2**, as shown by the similarity of ^{13}C NMR signals to **1** (Table 1). Key differences were seen however, at C-5 and C-4′, both methine carbons according to DEPT, indicating the absence of the side chains present in **1** at these positions. The presence of the geranyl-derived side chain was readily established by 2D NMR correlations. Attachment of this group and the subsequent structure determination of the oxygen-containing ring then followed, with essential HMBC correlations observed from H_3-4″ to C-2″, C-3″, and C-5″. The remaining assignments for **2** were determined by 2D NMR in a similar fashion and by comparison with **1**. The stereochemistry at C-3″ unfortunately has yet to be determined at the time of this writing. To our knowledge, the general carbon skeleton of **2** has no close counterpart in the literature.

Pawhuskin C (**3**) had the molecular formula $C_{24}H_{28}O_4$ by HRFABMS, requiring one less unsaturation than compounds **1** and **2**. ^{1}H and ^{13}C NMR signals for the stilbene core of **3** were nearly identical to those for **2**, indicating a similar substitution pattern. The presence of one additional phenolic hydroxyl group and the deshielding of C-6′ relative to **2** suggested the opening of the heterocyclic ring present in **2**. The remaining 2D NMR data for **3** led to the identification of the intact geranyl side chain. HMBC correlations from H_2-1″ to C-5′, C-6′, and C7′ were sufficient to determine the point of attachment of the side chain and to establish the structure of **3** as shown. Efforts to convert compound **3** to **2** under mild conditions, such as extended time in solution, adsorbed to silica gel, or exposed to air, were unsuccessful in producing any detectable amount of **2** by TLC and ^{1}H NMR spectroscopic analysis. Thus, it was concluded that **2** is likely to be of natural origin, although a decisive conclusion is precluded by the complexity of the mixtures present in the crude extracts.

The known compound petalostemumol (**4**) was identified by HRESIMS, analysis of NMR data, chromatographic behavior, and comparison with literature values for these data.[2]

Competition results in the opioid assay, depicted in Figure 1, yielded the following rank order of potency (K_i values) for displacement of specific [^{3}H]-naloxone binding: compound **1** (0.29 ± 0.11 μM) > **3** (4.2 ± 3.6 μM) > **2** (11.4 ± 7.9 μM) > **4** (>100 μM). Statistical analysis did not reveal any significant differences between K_i values ($F_{2,11} = 2.456$; $p = 0.141$), yet compound **1** was over 14 times more potent

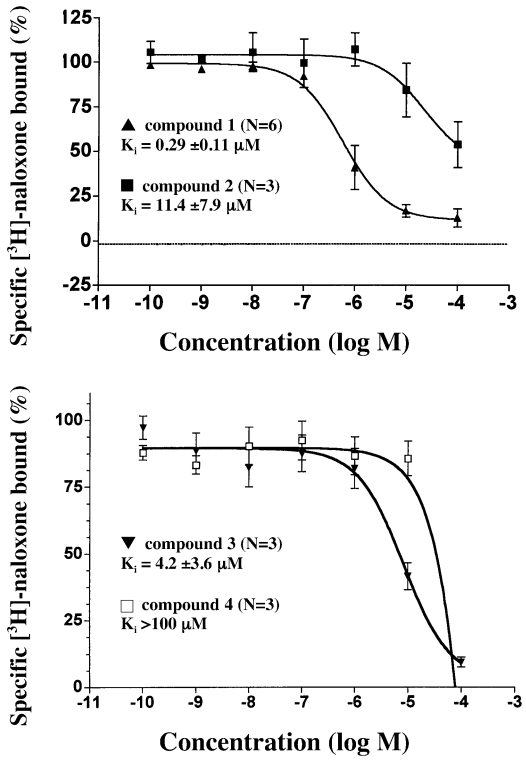

Figure 1. Nonlinear regression analyses of compounds **1** and **2** (top panel) and compounds **3** and **4** (bottom panel) showing displacement of [^{3}H]-naloxone binding to striatal membranes from 4-month-old male Sprague−Dawley rats ($N = 3-6$). Tissue homogenates were incubated with 5 nM [^{3}H]-naloxone and seven concentrations (0.1 nM to 100 μM) of each test compound. Nonspecific binding was determined in the presence of 10 μM naltrexone. Data are expressed as mean ± SEM of three or six experiments run in duplicate.

than the next closest compound, **3**. All competition data were fit to both a one-site and a two-site model. The "best fit" of the data was then determined statistically by the *F*-test.[13] All curves were significantly better fit to the simpler one-site model. This suggests identification of a single noninteracting site similar to that identified by [^{3}H]-naloxone. The binding data, using a nonselective antagonist like [^{3}H]-naloxone, does not rule out the presence of multiple sites having similar affinities.

The rationale for the use of [^{3}H]-naloxone is 2-fold; first, [^{3}H]-naloxone binding has been very well characterized in the mammalian CNS, and second, naloxone is an antagonist with similar affinities for the mu, kappa, and delta opioid receptors. One advantage to using an antagonist with similar affinities for different opioid receptors is the ability to screen against each of the three subtypes, without having to consider their different affinity states. Differences observed with naloxone binding could be confirmed by the use of mu-, kappa-, and delta-selective radioligands to determine subtype selectivity of the compounds. Rat striatal tissue is used due to its relatively heterogeneous population of opioid receptors, albeit with different densities. The use of [^{3}H]-naloxone, which is inexpensive and well characterized, along with striatal tissue, allows for investigation of multiple opioid receptor subtypes simultaneously. This results in an initial screen for possible opioid activity before more in-depth, labor-intensive studies are performed.

The affinities of compounds **2** and **3** were quite weak for opioid receptors, and **4** displayed virtually no ability to displace specific [^{3}H]-naloxone binding (Figure 1). Pawhuskin A (**1**) displayed the greatest potency for the displacement of specific [^{3}H]-naloxone binding, although its K_i value (~290 nM) is relatively weak by comparison to other opioid-selective compounds (e.g., morphine at ~2−5 nM). Although a direct comparison is difficult, it is likely that compounds **1**−**3** contribute to the overall activity observed in the crude extract,[8] for which a K_i value was not determined. Collectively, these pharmacological data suggest that compound **1** may provide the most promising prospect for action at opioid receptors. Further studies are needed to evaluate whether **1** is an agonist or antagonist, properties that are not determined in this study. Although the apparent affinity for **1**, based on displacement of [^{3}H]-naloxone, is relatively low, it may still function as an analgesic agent. Additional investigation is currently underway to determine this compound's binding characteristics. These studies will employ subtype-selective compounds, as well as cell lines expressing a homogeneous population of mu, kappa, and delta opioid receptors in order to further our understanding of the action of this compound as a putative opioid agent.

There is a growing body of evidence to suggest that there is a potential for opioid receptor ligands without the functionalities previously thought to be essential for binding, that may bind at alternate sites, or to newly identified receptor subtypes.[14−16] It is hoped that these studies will help to demonstrate the possibility of obtaining new CNS-active chemotypes from natural sources and will also help support the environmental efforts of The Nature Conservancy and similar organizations.

Experimental Section

General Experimental Procedures. NMR spectra were acquired on a Varian UNITY INOVA 600 spectrometer, equipped with an inverse detection probe, and on a Varian Gemini 200 spectrometer. Optical rotations were obtained on a JASCO model P-1010 polarimeter. IR spectra were recorded on a Nicolet Avatar 360 FT-IR spectrophotometer, and UV spectra were acquired on a Hewlett-Packard 8453 diode array spectrophotometer. ESIMS/MS and HRESIMS were obtained in positive ion mode on a Micromass Q-TOF mass spectrometer. HRFABMS (3-NBA matrix) were obtained in positive ion mode on a VG ZAB-E spectrometer. EIMS was obtained on a Shimadzu GCMS-QP5000 equipped with a DI-50 direct sample inlet device.

Plant Material. Whole plants of *Dalea purpurea* Vent. (Fabaceae) (syn.: *Petalostemon purpureum*) "Purple Prairie Clover" were collected by G.B., A.F., and S.D. on June 16, 2000, at the Nature Conservancy's Tallgrass Prairie Preserve near Pawhuska, Oklahoma, approximately 5.4 miles from the visitor center using the north approach to the "Bison Loop". A voucher specimen (#11290) was authenticated by Dr. Paul Buck, Professor Emeritus, Department of Biological Science, The University of Tulsa, and deposited in the Barclay Herbarium at the same location. Plants were air-dried on the laboratory benchtop for several days and then stored in a −20 °C freezer prior to extraction.

Extraction and Isolation. Plant material (234 g) was extracted for 24 h with 2.5 L of MeOH to provide, after evaporation, 23 g of crude extract. This material was preadsorbed in MeOH solution onto ca. 10 g of silica gel, the solvent was removed under vacuum, and the resulting powder was subjected to vacuum liquid chromatography (VLC) over a prepacked column bed [14 cm (i.d.) × 5 cm (h)] of TLC-grade silica gel (Selecto Scientific). The column was eluted using a stepwise gradient of solvents (1 L each), beginning with pentane, and continuing with mixtures of EtOAc in pentane (25%, 50%, 75%, 100%), followed by mixtures of MeOH in CH_2Cl_2, up to 30%. The two fractions that eluted with 25% and 50% EtOAc were combined on the basis of TLC analysis (EM Science, silica gel 60, F_{254}, with H_2SO_4/vanillin spray reagent), and the solvents were evaporated. The residue (2.9 g) was further fractionated by Sephadex LH-20 (Sigma) column

chromatography (2.2 cm × 53 cm) eluting with 500 mL of 3:1:1 hexane−toluene−MeOH, followed by 500 mL of MeOH, and collecting ca. 5 mL fractions. Fractions of similar composition as determined by TLC were pooled, resulting in 22 fractions. Further purification of fractions 14−21 of this column resulted in compounds **1**−**4**. Repeated chromatography of fraction 19 (245 mg) on silica gel (2.5 cm × 15 cm, Davison Chemical 100−200 mesh) using linear gradients of MeOH (0−4%) in CH_2Cl_2, and EtOAc (0−35%) in hexane, at flow rates of ca. 20 mL/min afforded compound **1** (39 mg). Chromatography of Sephadex fractions 21 (300 mg) and 20 (55 mg) on silica gel (2.5 cm × 15 cm), using linear gradients of EtOAc (0−40%) in hexane, afforded compounds **2** (25 mg) and **3** (12 mg), respectively. Repeated chromatography of the combined Sephadex fractions 14−18 (452 mg) using similar procedures afforded compound **4** (22 mg).

Pawhuskin A (1): yellow oil; $[\alpha]_D$ +1.4° (*c* 0.25, $CHCl_3$); mp 78−85 °C; UV (MeOH) λ_{max} 208 (ϵ 31 000), 315 (12 000); IR ν_{max} (film on NaCl) 3396 (br OH), 2965, 2924, 2854, 1607, 1497, 1448, 1337, 1288, 1138 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC correlations (acetone-d_6) OH-1 → C-1; H-2 → C-4, 6; H-3 → C-1, 5, 1′; OH-6 → C-6; H_2-7 → C-4, 5, 6, 8; H-8 → C-7, 10; H_3-10 → C-8, 9, 11; H_3-11 → C-8, 9, 10; H-1′ → C-3; H-2′ → C-4, 1′, 4′, 8′; OH-5′ → C-4′, 5′; H-6′ → C-4′, 5′, 8′; OH-7′ → C-6′, 7′, 8′; H-8′ → C-2′, 4′, 6′, 7′; H_2-1″ → C-3′, 4′, 2″, 3″; H-2″ → C-4″, 5″; H_3-4″ → C-2″, 3″, 5″; H_2-5″ → C-3″, 4″, 6″, 7″; H_2-6″ → C-5″; H_3-9″ → C-7″, 8″, 10″; $H_3$10″ → C-7″, 8″, 9″; ESIMS/MS *m*/*z* 471 [(M + Na)$^+$, rel int 100], 415 (4), 383 (7), 227 (8), 195 (3), 181 (4); HRESIMS found *m*/*z* 471.2522 (M + Na)$^+$, calcd for $C_{29}H_{36}O_4Na$, 471.2511.

Pawhuskin B (2): white powder; $[\alpha]_D$ +21.2° (*c* 0.25, $CHCl_3$); mp 90−96 °C; UV(MeOH) λ_{max} 215 (ϵ 13 000), 347 (8200); IR ν_{max} (film on NaCl) 3364 (br OH), 2966, 2925, 2854, 1609, 1570, 1539, 1474, 1340, 1273 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC correlations (acetone-d_6) OH-1 → C-1, C-2; H-2 → C-4, 6; H-3 → C-1, 5, 1′; H-5 → C-1, 3, 1′; OH-6 → C-1, 5; H-1′ → C-3, 5, 3′; H-2′ → C-4, 3′, 4′; H-4′ → C-2′, 5′, 6′, 8′; OH-7′ → C-6′, 7′, 8′; H-8′ → C-2′, 4′, 6′, 7′; H-1″ → C-5′, 7′, 3″; H-2′ → C-6′, 3″, 4″; H_3-4″ → C-2″, 3″, 5″; H_2-5″ → C-3″, 4″, 6″; H_2-6″ → C-5″, 7″, 8″; H-7″ → C-9″, 10″; H_3-9″ → C-7″, 8″, 10″; H_3-10″ → C-7″, 8″, 9″; ESIMS/MS *m*/*z* 401 [(M + Na)$^+$, rel int 100], 313 (25), 269 (17), 255 (27), 197 (33), 181 (47), 137 (34), 123 (62); HRESIMS found *m*/*z* 401.1742 (M + Na)$^+$, calcd for $C_{24}H_{26}O_4Na$, 401.1729.

Pawhuskin C (3): yellow solid; $[\alpha]_D$ +5.29° (*c* 0.25, $CHCl_3$); mp 148−152 °C; UV(MeOH) λ_{max} 204 (ϵ 17 500), 222 (16 000), 300 (13 500), 329 (17 500); IR ν_{max} (film on NaCl) 3385 (br OH), 2921, 1653, 1576, 1521, 1437, 1355, 1269 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HMBC correlations (acetone-d_6) H-2 → C-4, 6; H-3 → C-1, 1′; H-5 → C-1, 3, 1′; H-1′ → C-3, 5, 2′, 3′; H-2′ → C-1′, 4′, 8′; H-4′ → C-2′, 5′, 6′; H-8′ → C-2′, 6′, 7′; H_2-1″ → C-5′, 6′, 7′, 2″, 3″; H-2″ → C-1″, 4″, 5″; H_3-4″ → C-2″, 3″, 5″; H_2-5″ → C-2″, 3″, 4″, 6″; H_2-6″ → C-5″, 7″, 8″; H-7″ → C-6″, 9″, 10″; H_3-9″ → C-7″, 8″, 10″; H_3-10″ → C-7″, 8″, 9″; ESIMS/MS *m*/*z* 380 (M$^+$, rel int 1), 257 (100), 239 (3), 229 (3), 211 (12), 183 (7), 171 (5), 123 (4); HRFABMS (3-NBA) found *m*/*z* 380.1922 (M$^+$), calcd for $C_{24}H_{28}O_4$, 380.1987.

Petalostemumol (4): pale yellow solid; $[\alpha]_D$ −17.36° (*c* 0.125, $CHCl_3$); mp 99−126 °C (dec); UV(MeOH) λ_{max} 212 (ϵ 73 000), 297 (17 500), 345 (5344); IR ν_{max} (film on NaCl) 3397 (br OH), 2968, 2924, 1636, 1448, 1375, 1282, 1183, 1076, 996 cm^{-1}; ^1H and ^{13}C NMR data for **4** have been previously reported[2]; EIMS *m*/*z* 508 (M$^+$, rel int 1), 304 (2), 257 (5), 221 (10), 203 (5), 189 (5), 177 (6), 165 (32); HRESIMS found *m*/*z* 531.2389 (M + Na)$^+$, calcd for $C_{30}H_{36}O_7Na$, 531.2359.

Opioid Receptor Assay. Striatal (rich in opioid receptors) tissue from 4-month-old Sprague Dawley rats was obtained following sacrifice by decapitation. Whole brains were removed, washed in ice-cold PBS, and then placed in a prechilled (4 °C) zinc-alloy brain matrix-dissecting block (Activational Systems, Inc., Warren, MI). A coronal cut was made to remove the frontal cortex and expose the rostral pole of the striatum. A posterior coronal cut was placed 4 mm caudal to the first cut.[17] Surrounding cortical and septal tissues were removed, and dorsal striatum was separated from the nucleus accumbens via a horizontal cut at the level of the anterior commissure. Tissue was frozen in liquid nitrogen and stored at −80 °C until assayed.

Frozen tissue was then homogenized in approximately 20 mL/g tissue of TRIS-HCl (pH = 7.4) and centrifuged at 24000*g* for 15 min at 4 °C. The pellet was resuspended in approximately 250 mL/g tissue of assay buffer consisting of 50 mM TRIS-HCl and 100 mM NaCl (pH = 7.4). Tissue homogenate (400 μL) was added to binding tubes, followed by 50 μL of either buffer (50 mM TRIS-HCl + 100 mM NaCl, pH = 7.4; total binding), 10 μM naltrexone (nonspecific binding), or designated concentrations of extract. The binding reaction was initiated by the addition of 50 μL of 5 nM [^{3}H]-naloxone (specific activity = 42 Ci/mmol, Amersham), and the binding reaction was allowed to proceed to equilibrium for 60 min at room temperature. Binding was terminated by filtration under reduced pressure using the Brandel Tissue Harvester (Gaithersburg, MD) onto GF/B fiberglass filters presoaked in 0.3% polyethylenimine to reduce nonspecific binding. Radioligand bound was then determined by scintillation spectrophotometry.

To determine the displacement of [^{3}H]-naloxone in the initial studies, 5 nM [^{3}H]-naloxone was incubated in the presence of 1, 10, or 100 μg/tube based on the dry weight of the fraction being tested. Data were then expressed as percent of control values ([^{3}H]-naloxone in the absence of any test compound, 324 ± 27 fmol/mg protein for the striatal tissue examined). Once a molecular weight of the unknown compound was determined, more robust pharmacological analyses could be performed. To determine the inhibition constant (K_i value) for each of the unknown compounds, each of the homogenate samples was incubated with one of seven concentrations of active fraction from 100 pM up to 100 μM. Data were then analyzed by the iterative nonlinear least-squares curve-fitting program GraphPAD-PRISM (GraphPAD Inc., San Diego, CA). Curves were fit to both a one-site and two-site model, and best fit was determined by the sum of squares *F*-test.[13] From each curve the total displacement of [^{3}H]-naloxone was determined as well as the corrected K_i value.[18,19]

Acknowledgment. Funding for G.N.B. was generously provided by The Research Corporation. Funding for D.R.W. was provided by NIH DA13137. The authors would like to express thanks to Bob Hamilton and the Nature Conservancy for granting a research permit. Additional thanks to Dr. Larry Russon of the University of Oklahoma, Department of Chemistry and Biochemistry, Mass Spectrometry Facilities, and Dr. Feng Qui of the Oklahoma State University, Department of Chemistry, Statewide NMR Facility.

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NP030258D