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Alkaloids from the Australian Rainforest Tree *Ochrosia moorei*

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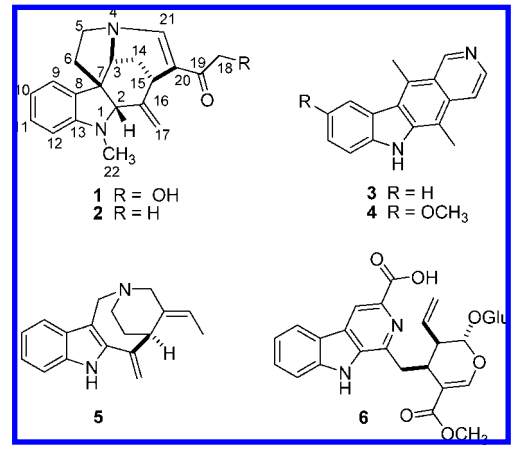
High-throughput screening of a plant and marine invertebrate extract library to find natural products that down-regulate expression of pro-inflammatory genes associated with the glucocorticoid receptor ligand complex led to the identification of bioactive CH_2Cl_2 extracts from stems and leaves of the Queensland tree *Ochrosia moorei*. Bioassay-guided purification of the stem extract enabled the isolation of four alkaloids including two new compounds, ochrosamines A (**1**) and B (**2**), and the known compounds ellipticine (**3**) and 9-methoxyellipticine (**4**). The leaf extract also afforded **3** and **4** as well as apparicine (**5**) and desoxycordifoline (**6**). The structures of the two new compounds were assigned from interpretation of 2D NMR and high-resolution ESIMS data. Ellipticine and 9-methoxyellipticine were the most active components, and both displayed IC_{50} values of 90 μM. Apparicine and desoxycordifoline were only very weakly active, and ochrosamines A and B were inactive.

The glucocorticoid receptor (GR) is a member of a superfamily of ligand-inducible transcription factors, the nuclear hormone receptors. Glucocorticoids play an essential role in maintaining basal and stress-related homeostasis and display potent anti-inflammatory and immunosuppressive properties.[1,2] As a consequence, synthetic glucocorticoids are used widely as drugs to treat inflammatory conditions such as rheumatoid arthritis or dermatitis and as adjunct therapy for conditions such as autoimmune diseases. The GR functions as a transcription factor upon binding of the glucocorticoids (GCS). The GR−GCS complex up-regulates transcription of genes containing the glucorticoid response element (GRE). This results in activation of genes encoding anti-inflammatory proteins, such as secretory leukoprotease inhibitor (SLPI), β_2-adrenoceptors, and CD163, but is also thought to lead to some of the side effects of glucocorticoids. The major anti-inflammatory effects of the GR−GSC complex are, however, a result of the down-regulation of genes via interaction of the complex with other transcription factors such as activator protein-1 (AP-1). The GR−GCS binds to AP-1 via a protein–protein interaction and prevents AP-1 binding to the TPA responsive element (TRE), causing down-regulation of pro-inflammatory genes that are usually activated by AP-1.[3,4] The discovery of compounds that selectively down-regulate pro-inflammatory genes may provide new and improved drug therapies to treat inflammatory conditions.

A high-throughput screening campaign employing a cell-based reporter gene assay was used to find extracts that down-regulate pro-inflammatory genes. For down-regulation, multiple TRE elements were linked to a minimal promoter driving the β-galactosidase gene. Extracts from 7399 plants and 517 marine invertebrates collected in Queensland were screened, and CH_2Cl_2 and MeOH extracts of the leaves and stems of *Ochrosia moorei* (F. Muell.) F. Muell. ex Benth. (Apocynaceae) showed activity in the assay. Bioassay-guided isolation of these extracts led to the isolation of two new alkaloids, ochrosamines A (**1**) and B (**2**), and four known alkaloids, ellipticine (**3**), 9-methoxyellipticine (**4**), apparicine (**5**), and desoxycordifoline (**6**). This paper reports on the isolation, structure determination, and biological activity of these alkaloids isolated from *O. moorei*.

The ground stems of *O. moorei* were extracted exhaustively with CH_2Cl_2, and the extract was chromatographed on Si gel using a stepped gradient from CH_2Cl_2 to MeOH. An early-eluting fraction contained a mixture of ellipticine (**3**) and 9-methoxyellipticine (**4**). Two later-eluting fractions were each partitioned between CH_2Cl_2 and 1 M HCl, and the aqueous layers were basified with 5 N NH_4OH and then extracted with CH_2Cl_2, yielding ochrosamine A (**1**) and ochrosamine B (**2**). The ground leaves of *O. moorei* were extracted exhaustively with CH_2Cl_2 and MeOH. The CH_2Cl_2 and MeOH extracts were combined and chromatographed on Sephadex LH-20 eluting with MeOH. An early-eluting fraction was chromatographed on diol-bonded Si gel using a stepped gradient from CH_2Cl_2 to MeOH, yielding two alkaloid-containing fractions. These fractions were further purified by HPLC, the first on aminopropyl-bonded Si gel, yielding apparicine (**5**), and the second on C_{18}, yielding desoxycordifoline (**6**). A later-eluting fraction from the Sephadex LH-20 column was purified by HPLC on aminopropyl-bonded Si gel, to yield ellipticine (**3**) and 9-methoxyellipticine (**4**).



The known compounds were identified by 2D NMR analysis and by comparison of spectroscopic data with published results.[5–8]

Accurate mass measurement of the pseudomolecular ion in the (+) HRESIMS at *m/z* 323.1768 (Δ 4.3 ppm) allowed a molecular formula of $C_{20}H_{22}N_2O_2$ to be assigned to ochrosamine A (**1**). IR absorption bands at 3402, 2920, and 1630 cm^{-1} suggested the presence of hydroxyl, amine, and α,β-unsaturated ketone functionalities. UV absorbances at 253 nm, indicative of an indoline chromophore, and at 311 nm, suggestive of a β-amino-α,β-unsaturated ketone, were observed in the UV spectrum.[9]

The ^1H NMR spectrum of ochrosamine A (**1**) (Table 1) exhibited signals characteristic for a 1,2-disubstituted phenyl group (δ_H 6.72,

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Table 1. ^{1}H (600 MHz), ^{13}C (150 MHz), and HMBC NMR Data for Ochrosamines A (**1**) and B (**2**) in C_6D_6

	ochrosamine A (**1**)			ochrosamine B (**2**)		
position	δ_C[a]	δ_H, intrgt, mult (*J* in Hz)	gHMBC	δ_C[a]	δ_H, intrgt, mult (*J* in Hz)	gHMBC
2	76.7	2.74, 1H, br s	C-7, C-3, C-15, C-13, C-6, C-22	76.4	2.85, 1H, s	C-7, C-3, C-15, C-13, C-6, C-22
3	62.9	2.75, 1H, ddd (1.2, 1.2, 3.9)		62.7	2.80, 1H, ddd (1.2, 1.2, 3.9)	
5	53.0	2.52, 1H, ddd (7.8, 11.4, 11.4)	C-21, C-7, C-3	52.9	2.68, 1H, ddd (6.6, 11.4, 11.4)	C-21, C-7, C-3
		2.71, 1H, dd (5.4, 11.4)	C-21, C-7, C-3		2.85, 1H, dd (8.4, 11.4)	C-21, C-7, C-3
6	40.8	1.11, 1H, ddd (5.4, 11.4, 13.2)	C-5, C-7, C-8, C-2	40.8	1.19, 1H, ddd (8.4, 11.4, 13.2)	C-5, C-7, C-8, C-2
		1.74, 1H, dd (7.8, 13.2)	C-8		1.80, 1H, dd (6.6, 13.2)	C-8
7	54.4			54.2		
8	135.1			135.4		
9	121.8	6.72, 1H, m	C-7, C-11, C-13	121.6	6.73, 1H, d (7.8)	C-7, C-11, C-13
10	119.2	6.73, 1H, m	C-8, C-12	119.0	6.76, 1H, dd (7.8, 7.8)	C-8, C-12
11	128.3	7.07, 1H, ddd (7.8, 7.8, 1.8)	C-9, C13	128.3	7.07, 1H, ddd (7.8, 7.8, 1.8)	C-9, C13
12	108.6	6.30, 1H, d (7.8)	C-8, C-10	108.5	6.31, 1H, d (7.8)	C-8, C-10
13	152.2			152.1		
14	23.0	1.22, 1H, ddd (2.6, 3.9, 13.2)		22.9	1.34, 1H, ddd (2.6, 3.9, 13.2)	
		2.15, 1H, ddd (1.2, 2.4, 13.2)			2.24, 1H, ddd (1.2, 3.6, 13.2)	
15	27.8	3.84, 1H, br s	C-14, C-18	27.0	3.94, 1H, br s	C-14, C-18
16	147.8			148.1		
17	119.2	4.65, 1H, br s	C-2, C-15, C-16	118.8	4.68, 1H, br s	C-2, C-15, C-16
		5.71, 1H, br s	C-2, C-15, C-16		5.83, 1H, br s	C-2, C-15, C-16
18	62.9	4.19, 2H, s	C-19, C-20, C-21	24.1	1.97, 3H, s	C-19, C-20, C-21
19	192.5			191.2		
20	120.5			125.1		
21	146.2	6.24, 1H, s	C-3, C-5, C-15, C-20, C-18	145.9	6.50, 1H, s	C-3, C-5, C-15, C-20, C-18
22	32.9	2.29, 3H, s	C-2, C-13	32.7	2.31, 3H, s	C-2, C-13

[a] ^{13}C NMR chemical shifts were assigned from HMQC and HMBC correlations.

m; 6.73, m; 7.07, ddd, $J = 7.8, 7.8, 1.8$ Hz; 6.30, d, $J = 7.8$ Hz), an exocyclic double bond (δ_H 4.65, br s; 5.71, br s), a trisubstituted double bond (δ_H 6.24, s), a terminal alcohol (δ_H 4.19, s), and an *N*-methyl group (δ_H 2.29, s). The COSY spectrum also indicated that the molecule contained the partial structures $-CH_2CH_2N-$ and $-CHCH_2CH-$. The H-2 (δ_H 2.74) and H-15 (δ_H 3.84) resonances correlated to the 17-CH_2 double-bond methylene protons, indicating that these methines flanked either side of the exocyclic double bond. An HMQC spectrum was used to provide the carbon chemical shifts for all of the directly bound protons. HMBC correlations from δ_H 2.29 (22-CH_3) to the carbons at δ_C 76.7 (C-2) and 152.2 (C-13) indicated that the molecule contained an *N*-methylaminophenyl group. Correlations from δ_H 2.74 (H-2) and 6.72 (H-9) to a quaternary carbon at δ_C 54.4 (C-7) extended the structure to include a 2,3-dihydro-*N*-methylindole. A cyclohexyl group was attached to C-2 and C-7 of the indole since correlations were observed between δ_H 2.74 (H-2) and δ_C 62.9 (C-3) and 27.8 (C-15). The methylene protons at δ_H 2.52 and 2.71 (CH_2N-5) showed correlations to carbons at δ_C 62.9 (C-3) and 146.2 (C-21), and the methine proton at δ_H 6.24 (H-21) correlated to δ_C 53.0 (C-5) and 62.9 (C-3). This indicated that the amino group N-4 was tertiary, with C-3, C-5, and C-21 each attached to N-4. A correlation between δ_H 6.24 (H-21) and δ_C 27.8 (C-15) suggested that the double bond C-20−C-21 was attached to C-15. A correlation to a ketone carbonyl carbon at δ_C 192.5 (C-19) from H-21, H-15, and 18-CH_2 indicated that a methyl ketone alcohol was substituted at C-20 of the double bond. The relative configurations of the four stereogenic centers in ochrosamine A (**1**) were assigned on the basis of analysis of ROESY correlations and H−H coupling constants. A strong ROESY correlation was observed between H-2 and H-6a, indicating a 2β-H, 7β configuration. It then followed that H-15 and H-3 were both α since the rigid tetracyclic framework dictates that their configurations are dependent on the configuration at C-7. Small couplings between H-3 and H-14a and H-14b and a *W* coupling between H-3 and H-15 were consistent with this stereochemical assignment. The absolute configurations of C-2 (2*S*) and C-7 (7*R*) were deduced from comparison of the CD spectrum of ochrosamine A (**1**) with that of strychnozairine, a compound similar in structure and also containing a 2β-H, 7β configuration.[9] Both compounds had a negative Cotton effect near 200 nm and positive Cotton effects near 250 and 300 nm. The structure **1** was therefore assigned to ochrosamine A.

Ochrosamine B (**2**) was assigned the molecular formula $C_{20}H_{22}N_2O$ by high-resolution positive electrospray mass measurement of the MH^+ ion (Δ +1.3 ppm). The ^{1}H NMR spectrum of ochrosamine B (**2**) (Table 1) was very similar to that of ochrosamine A (**1**). In fact, the only difference was the replacement of the two-proton oxygenated methylene at C-18 with a methyl singlet at δ_H 1.97. Ochrosamine B (**2**) was therefore the 18-deoxy derivative of ochrosamine A. Analysis of 2D NMR (gCOSY, gHMQC, and gHMBC) and CD spectra confirmed this assignment.

Ochrosamines A (**1**) and B (**2**) are new members of the large group of *Strychnos* alkaloids characterized by strychnine, tubifolidine, and akuammicine.[10] The unusual oxidation pattern characterized by the C-20−C-21 double bond in ochrosamines A and B is rare, with only a few examples such as strychnozairine[9] and dimeric alkaloids panganensine R, S, X, and Y[11] appearing in the literature.

Ellipticine (**3**) and 9-methoxyellipticine (**4**) were the most active compounds in the pro-inflammatory gene down-regulation assay. Both compounds showed IC_{50} values of 90 μM. Apparicine (**5**) and desoxycordifoline (**6**) were only weakly active, showing 54% and 30% activity at 100 μM, respectively, while ochrosamines A (**1**) and B (**2**) were both inactive at 10 μM. The reference compound dexamethasone had an IC_{50} of 5 nM. Since **3** and **4** are known to be potent cytotoxic agents,[12] a neutral red cytotoxicity assay was used to distinguish if the observed activity for ellipticine (**3**) and 9-methoxyellipticine (**4**) was due to cell toxicity or pro-inflammatory gene down-regulation. Cells used in the pro-inflammatory gene down-regulation assay were incubated with 100 μM of either **3** or **4** for 24 h and then treated with neutral red. No toxicity was observed for either compound, and this result indicated that the compounds down-regulated the glucocorticoid gene.

Experimental Section

General Experimental Procedures. All solvents used were Omnisolv HPLC grade, and the H_2O used was Millipore Milli-Q PF filtered. Optical rotations were measured on a JASCO P-1020 polarimeter (10 cm cell). UV spectra were recorded on a CAMSPEC M501 UV/vis spectrophotometer, and IR spectra were recorded on a Bruker Tensor 27 spectrometer. CD spectra were recorded on a

JASCO J-715 spectropolarimeter. NMR spectra were recorded on Varian Inova 600 MHz NMR spectrometer. Samples were dissolved in benzene-d_6, and chemical shifts were calculated relative to the proto-deutero solvent peak (C_6D_6) at δ_H 7.10 and δ_C 128.0. 2D NMR spectra were recorded at 30 °C using the standard Varian pulse sequences, gCOSY, gHMQC, gHMBC, and ROESY. HRESIMS were recorded on a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source. Alltech Davisil 30–40 μm 60 Å Si gel or Alltech Davisil 30–40 μm 60 Å diol-bonded Si gel was used. Pharmacia Sephadex LH-20 was used for gel permeation chromatography. HPLC separations were achieved using either a Rainin Microsorb C_{18} semipreparative column (3 μm; 10 mm × 50 mm) or a Rainin Microsorb aminopropyl-bonded Si gel column (3 μm; 10 mm × 50 mm).

Plant Material. The stems and leaves of *O. moorei* were collected by one of the authors (P.I.F.) in March 1995 from Natural Arch National Park, McPherson Range, in South East Queensland. A voucher specimen, AQ634427, is deposited at the Queensland Herbarium.

Extraction and Isolation. The air-dried ground stems of *O. moorei* (35 g) was extracted exhaustively with CH_2Cl_2 (800 mL), yielding a dark green gum (139 mg). The CH_2Cl_2 extract was chromatographed on Si gel with a stepped gradient from CH_2Cl_2 to MeOH, yielding 11 fractions. Fraction 2 (11.6 mg) was a mixture of ellipticine (**3**) and 9-methoxyellipticine (**4**). Fraction 3 was partitioned between CH_2Cl_2 and 1 M HCl, and the aqueous layer, basified with 5 N NH_4OH and extracted with CH_2Cl_2, yielding ochrosamine B (**2**) (4.9 mg, 0.014%). Fractions 4 and 5 were combined and partitioned between CH_2Cl_2 and 1 M HCl, and the aqueous layer was basified with 5 N NH_4OH and extracted with CH_2Cl_2, yielding ochrosamine A (**1**) (6 mg, 0.017%).

The air-dried ground leaves of *O. moorei* (12.2 g) were extracted exhaustively with CH_2Cl_2 (800 mL) followed by MeOH (800 mL), and the extracts combined to yield a dark green gum (1.25 g). The combined extract was chromatographed on Sephadex LH-20 using MeOH. An early-eluting fraction from the Sephadex LH-20 column was fractionated on diol-bonded Si gel with a stepped gradient from CH_2Cl_2 to MeOH, yielding 20 fractions. Fractions 8 to 11 were combined and purified by HPLC on an aminopropyl-bonded Si gel, eluting with 95:5 *n*-hexane−IPA (containing 1% NH_4OH), yielding apparicine (**5**) (2.7 mg, 0.02%). The 14th and 15th fractions from the diol-bonded Si gel column were combined and chromatographed by C_{18} HPLC elution with MeOH−H_2O (2:3), yielding desoxycordifoline (**6**) (15.9 mg, 0.13%). A later-eluting band from the Sephadex LH-20 column was chromatographed by aminopropyl-bonded Si gel HPLC, eluting with 9:1 hexane−IPA, yielding ellipticine (**3**) (2.3 mg, 0.018%) and 9-methoxyellipticine (**4**) (1.9 mg, 0.016%).

Ochrosamine A (1): yellow gum; $[\alpha]_D^{17}$ +80.3 (*c* 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.80), 253 (3.32), 311 (3.42) nm; CD (MeOH) λ_{ext} ($[\theta]$) 200 (−9570), 248 (1756), 264 (2857), 300 (16838) nm; IR (KBr) ν_{max} 3402 br, 2920, 1658, 1642, 1630, 1441, 1408, 1022 cm^{-1}; ^{1}H (600 MHz, C_6D_6 and ^{13}C NMR (150 MHz, C_6D_6), Table 1; (+)-HRESIMS *m*/*z* 323.1768 $[M + H]^+$ (calcd for $C_{20}H_{23}N_2O_2$, 323.1754), 345.1589 $[M + Na]^+$ (calcd for $C_{20}H_{22}N_2O_2Na$, 345.1573), 667.3259 $[2M + Na]^+$ (calcd for $C_{40}H_{44}N_4O_4Na$, 667.3255).

Ochrosamine B (2): yellow gum; $[\alpha]_D^{17}$ +78.5 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 205 (3.69), 255 (3.28), 311 (3.44) nm; CD (MeOH) λ_{ext} ($[\theta]$) 200 (−4470), 250 (6504), 268 (3608), 312 (13173); IR (KBr) ν_{max} 3425 br, 2918, 2850, 1631, 1463, 1442, 1119 cm^{-1}; ^{1}H (600 MHz, C_6D_6 and ^{13}C NMR (150 MHz, C_6D_6), Table 1; (+)-HRESIMS *m*/*z* 307.1809 $[M + H]^+$ (calcd for $C_{20}H_{23}N_2O$, 307.1805), 329.1626 $[M + Na]^+$ (calcd for $C_{20}H_{22}N_2ONa$, 329.1624), 635.3365 $[2M + Na]^+$ (calcd for $C_{40}H_{44}N_4O_2Na$, 635.3356).

Glucocorticoid Gene Down-Regulation Assay. The rat cell line R1 was co-transfected with a vector containing an oligomeric TRE in front of a minimal promoter and the reporter gene β-galactosidase (lacZ). Stably transfected R1 cells were selected with the antibiotic Geneticin (G148). The stable R1 clone 4:1:6S9 (R1 down) was cultured in Dulbecco's modified Eagle medium (containing L-glutamine, 4500 mg/L D-glucose, and sodium pyruvate), nonessential amino acids, penicillin (50 units/mL), streptomycin (50 mg/mL), and 10% fetal bovine serum. Cells were cultured in a humidified 5% CO_2 atmosphere at 37 °C. R1 down cells were plated at 4.5 × 10^4 cells/mL into a flat-bottom, 96-well microtiter plate and incubated for three days at 5% CO_2 atmosphere at 37 °C. Either extracts, fractions, or pure compounds dissolved in DMSO were then added and the cells and were incubated for 24 h. A final DMSO concentration of 2% was used in the assay. A β-galactosidase assay to determine glucocorticoid gene regulation was then performed.

***β*-Galactosidase Assay.** Cells were washed with PBS and then lysed by a 10 min incubation with 50 μL of 0.5% Triton X-100 in 0.1 M sodium phosphate pH 7.5, at rt. To each well was added 50 μL of reaction mixture containing 2 mM $MgCl_2$, 0.65% β-mercaptoethanol, and 1.76 mg/mL *o*-nitro-β-D-galactopyranose in 0.1 M sodium phosphate, pH 7.5, and the mixture was incubated for 60 min at 37 °C. Then, 100 μL of stop buffer containing 300 mM glycine pH 11.3 and 15 mM EDTA was added to each well. The absorbance for each plate was read at 450 nm in a THERMOmax microplate reader. IC_{50} values for the isolated compounds were obtained by testing four wells per concentration.

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