Sideroxylonal C, a New Inhibitor of Human Plasminogen Activator Inhibitor Type-1, from the Flowers of *Eucalyptus albens*

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Sideroxylonal C (**3**), a new phloroglucinol dimer, was isolated from the flowers of *Eucalyptus albens* through bioassay-guided fractionation. The structure elucidation was based on 1D and 2D NMR experiments, MS analysis, and comparison with sideroxylonals A (**1**) and B (**2**). Sideroxylonal C inhibited human plasminogen activator inhibitor type-1 at 4.7 μM without any significant effect on human tissue plasminogen activator.

Human plasminogen activator inhibitor type-1 (PAI-1), a member of the serine protease inhibitor family, is the main inhibitor of human tissue plasminogen activator (tPA) in plasma. The cleavage of plasminogen by tPA governs the release of the active enzyme plasmin, which degrades the fibrin clot. PAI-1 binds irreversibly to tPA to inhibit tPA activity, and thereby all fibrinolytic activity.[1,2] Clinical studies have shown that high levels of plasma PAI-1 may be related to the pathogenesis of arterial and thrombotic diseases.[2,3] Specific inhibition of PAI-1 should therefore be a novel way to enhance fibrinolysis.

A total of 21 384 extracts of plant and marine organisms was screened for inhibition of PAI-1 in the presence of tPA, with a 1% hit rate. A CH_2Cl_2 extract of the flowers of *Eucalyptus albens* Benth. (Myrtaceae) showed 100% inhibition of PAI-1 at 500 μg/mL (dry wt). Bioassay-guided fractionation of the active extract led to the isolation of known sideroxylonals A (**1**) (0.18%) and B (**2**) (0.03%) along with a new isomer, sideroxylonal C (**3**) (0.06%). Sideroxylonal A (**1**) and its C-10′ epimer, sideroxylonal B (**2**), which belong to the acylphloroglucinol class of compounds, were first isolated from *Eucalyptus sideroxylon*.[4] Acylphloroglucinol compounds are known for their diverse biological activities, including antibacterial, anticancer, antiviral, and granulation-inhibiting activity.[5]

Flash chromatography of the CH_2Cl_2 crude extract of *E. albens* on Si gel yielded four active fractions. Compound **1**, identified as sideroxylonal A by comparison of ^{1}H and ^{13}C NMR data,[4] was the main component of the more polar fractions and was purified by C_{18} HPLC. The less polar fractions were combined and chromatographed on Sephadex LH-20, yielding two active fractions, one of which contained compound **2**, identified as sideroxylonal B by comparison of ^{1}H and ^{13}C NMR data.[4] The other fraction contained compounds **1** and **3**, which were separated by recrystallization.

The ^{1}H and ^{13}C NMR spectra of **3** (Table 1) were consistent with this compound being an isomer of **1** and **2**. The low-resolution negative ion ESMS of **3** displayed peaks at *m*/*z* 499 and *m*/*z* 249 relating to the molecular ions [M − H]$^-$ and [M − 2H]$^{2-}$, respectively, and the molecular formula $C_{26}H_{28}O_{10}$ was confirmed by high-resolution measurements (positive electrospray) *m*/*z* 501.1799 ± 0.003 (calcd for $[C_{26}H_{28}O_{10} + H]^+$ 501.1761). Relevant COSY correlations were observed for **3** from H-7 to H-10 and H-10′, and from H-10′ to H-7′ and H-11′. The coupling constant $J_{H7'-H10'}$ = 10.8 Hz was similar to the H-7′−H-10′ coupling constant in **1** (11.4 Hz), which was due to a *trans*-diaxial arrangement. This contrasts with the small

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data for Sideroxylonal C (in $CDCl_3$) and HMBC Correlations

carbon	^{13}C NMR	^{1}H NMR	$^2J_{CH}$ and $^3J_{CH}$
C-1	112.6		
C-2	165.2		
C-3	109.1		
C-4	168.0		
C-5	106.0		
C-6	168.6		
C-7	26.2	3.24 (ddd, *J* = 2.4, 6.4, 7.6 Hz)	C-1, C-2, C-6, C-10, C-11, C-7′, C-10′, C-11′
C-8	192.4	10.21 (s)	C-3, C-4
C-9	192.9	10.27 (s)	C-1, C-5, C-6
C-10	50.2	1.36 (m)	
		1.45 (m)	C-11, C-12, C-10′
C-11	25.5	1.47 (m)	C-10
C-12	22.7	0.96 (d, *J* = 6.6 Hz)	C-10, C-11, C-13,
C-13	23.1	0.89 (d, *J* = 6.6 Hz)	C-10, C-11, C-12
C-1′	104.2		
C-2′	169.4		
C-3′	104.1		
C-4′	167.3		
C-5′	105.8		
C-6′	168.2		
C-7′	79.3	5.19 (d, *J* = 10.8 Hz)	C-1′, C-2′, C-6′, C-10, C-11′
C-8′	191.4	9.98 (s)	C-3′,C-4′
C-9′	192.4	10.21 (s)	C-3′
C-10′	52.6	2.30 (ddd, *J* = 2.4, 2.4, 10.8 Hz)	C-1, C-7, C-10, C-11, C-1′, C-7′, C-12′, C-13′
C-11′	28.3	1.71 (dq, *J* = 2.4, 6.6 Hz)	C-7, C-7′, C-10′, C-12′, C-13′
C-12′	20.7	1.00 (d, *J* = 6.6 Hz)	C-10′, C-11′, C-13′
C-13′	16.1	0.85 (d, *J* = 6.6 Hz)	C-10′, C-11′, C-12′
C-4-OH		13.47 (s)	C-3, C-4
C-6-OH		13.27 (s)	C-1, C-2, C-5, C-6, C-9
C-2′-OH		13.27 (s)	
C-4′-OH		13.10 (s)	
C-6′-OH		13.52 (s)	C-6′

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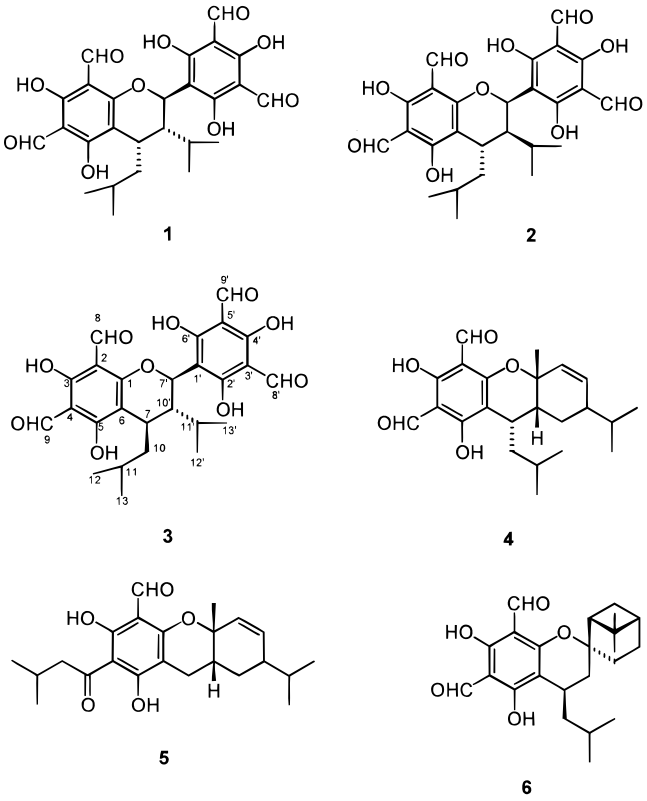
coupling constant in **2**, where $J_{H7'-H10'} = 2.4$ Hz for an axial−equatorial conformation. The coupling constant $J_{H10'-H7}$ was 2.4 Hz in **3**. Molecular modeling of a C-7 epimer of **1** predicted that the lowest energy conformation was one in which the six-membered ring heterocycle in **3** assumed a distorted boat with calculated angles of 154° between H-7′ and H-10′ and 112° between H-10′ and H-7.[6] A coupling constant of 2.4 Hz was also observed between H-10′ and H-11′ indicating restricted rotation around the C-10′−C-11′ bond with the lowest energy conformation, which had a dihedral angle of 70° between H-10′ and H-11′. NOE enhancements were observed from H-7 to H-10a, H-10b, H-12′, and H-13′, and from H-7′ to H-11′, H-12′, and H-13′, which is consistent with the structure proposed for **3**.

Sideroxylonals A−C (**1**−**3**) were tested for inhibition of PAI-1 in the presence of Flavigen and tPA and showed IC_{50} values of 3.3, 5.3, and 4.7 μM, respectively. Sideroxylonal A (**1**) bound covalently to PAI-1 as shown by MS examination after incubation with PAI-1 and was shown to bind to immobilized PAI-1 using plasmon resonance. Other phloroglucinol compounds such as euglobal IA2 (**4**),[7] euglobal IIC (**5**),[7] and robustadial A (**6**)[8] were also tested for inhibition of PAI-1 and showed IC_{50} values of 138, 700, and 152 μM, respectively. Compounds **4**−**6** were isolated from various *Eucalyptus* species and identified by comparison of their ^{1}H and ^{13}C NMR data with literature values.[7] Robustadial A (**6**) was identified by comparison of ^{1}H and ^{13}C NMR data with those of its dimethyl derivative.[8] Compounds **1**−**3** were optically inactive, indicating all three were isolated as racemic mixtures.



Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Varian Unity INOVA at 599.926 MHz for ^{1}H and 149.98 MHz for ^{13}C. ^{1}H and ^{13}C were referenced to the solvent peak ($CDCl_3$ δ 7.26 and 77.3 ppm or DMSO-d_6 δ 2.49 and 39.5 ppm, respectively). Standard parameters were used for 1D and 2D NMR spectra obtained, which included ^{1}H, ^{13}C, DEPT, gradient COSY, HMQC, HMBC, and NOESY. UV spectra were recorded on a GBC 916 UV−vis spectrometer, and IR spectra were recorded on a Perkin−Elmer 1725X FTIR spectrometer. The optical rotation was measured on a JASCO P-1020 polarimeter. HRMS was carried out at Astra Draco, Lund, Sweden, and LRESMS were measured on a Fisons VG Platform II. Rainin 3-μm C_{18} Microsorb (50 × 4.6 mm and 50 × 10 mm) HPLC columns were used for analytical and semipreparative chromatography. A Waters 600 pump with a 996 PDA detector, 717 autosampler, and Waters fraction collector were used for analytical and semipreparative HPLC separations. Davisil Si gel 30−40 μm (36 × 140 mm) and Sephadex LH-20 (36 × 600 mm) (Pharmacia Biotech) were used for chromatography in open glass columns.

Plant Material. The sample of *Eucalyptus albens* flowers was collected on 5 May 1994, by Paul Grimshaw. A voucher sample (P. Grimshaw AQ 600913) is lodged at the Queensland Herbarium, Brisbane, Queensland, Australia.

Extraction and Isolation. The air-dried flowers (16.7 g) of *E. albens* were ground and exhaustively extracted with CH_2Cl_2 to afford a crude extract (1.417 g). This extract was chromatographed on Si gel using stepwise gradient elution from hexane to CH_2Cl_2, and then from CH_2Cl_2 to EtOAc, to afford 10 fractions of which the four most polar were active. The two most polar active fractions were combined and purified by reversed-phase C_{18} HPLC gradient elution from 55% MeOH−buffer (CH_3COOH 10%, pH 2.85) to 100% MeOH and yielded the previously reported sideroxylonal A (**1**). The two less polar active fractions were combined and chromatographed on Sephadex LH-20 (MeOH), yielding two active fractions, one of which contained pure sideroxylonal B (**2**). The other fraction contained compounds **1** and **3**, which were separated by recrystallization in MeOH: sideroxylonal A (**1**) crystallized out of the solution to leave sideroxylonal C (**3**) in the supernatant.

Sideroxylonal A (1): light yellow oil (18 mg) and white crystals (16.7 mg) (0.18%); (−) LRESMS *m*/*z* 499 $[C_{26}H_{28}O_{10}-H]^-$ (20), 249 $[C_{26}H_{28}O_{10}-2H]^{-2}$ (100).

Sideroxylonal B (2): yellow oil (5.5 mg, 0.03%); (−) LRESMS *m*/*z* 499 $[C_{26}H_{28}O_{10}-H]^-$ (20), 249 $[C_{26}H_{28}O_{10}-2H]^{-2}$ (100).

Sideroxylonal C (3): yellow oil (9.7 mg, 0.06%); $[\alpha]^{22}_D$ (MeOH) ± 0°; UV (MeOH) λ_{max} (ϵ) 280 (6764), 379 (1361) nm; IR ν_{max} (KBr disk) 3423, 1636, 1438, 1302, 1177 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; (−) LRESMS *m*/*z* 499 $[C_{26}H_{28}O_{10}-H]^-$ (20), 249 $[C_{26}H_{28}O_{10}-2H]^{-2}$ (100); (+) HRESMS Q-TOF (PEG) *m*/*z* 501.1799 ± 0.003 $[C_{26}H_{28}O_{10}+H]^+$ (calcd for $[C_{26}H_{28}O_{10}+H]^+$ 501.1761).

PAI-1 Inhibitor Assay. Assays were performed in 96-well microplates (Nunclon) containing final reagent concentrations in 200 μL: 3 nM guanidine-activated, purified human PAI-1 (Astra Hässle, Umeå, Sweden), 250 μM Flavigen (Biopool, Sweden), 4 nM two-chain tPA (Biopool, Sweden), and unknowns. Extracts, fractions, and compounds were constituted in neat DMSO and assayed at 2% DMSO using diluent (50 mM Tris, 100 mM NaCl, and 0.1 g/L Tween 80, pH 7.4). After 60 min at 23 °C, absorbance at 405 nm (A_{405}) was measured. Compound IC_{50} values (concentration required to increase A_{405} to 50% of control) were estimated from the average of triplicate measurements using nonlinear, least-squares analysis (GraphPad Prism 2.0, San Diego, CA). Sideroxylonals A, B, and C (**1**−**3**) did not alter A_{405} measured in the absence of PAI-1, up to 100 μM.

Plasmon Resonance. A BIAcore2000 analytical system from Pharmacia Biosensor AB (Uppsala, Sweden) was used, and the optical phenomenon observed was expressed in resonance units (RU). PAI-1 and tPA were immobilized on the dextran surface of the BIAcore CM5 sensor chip via its primary amine groups. After coupling of the proteins, the surface was washed with buffer. All injections were made with 5 μL/min at 37 °C. To detect dissociation, a pulse of either tPA or PAI-1 was made, followed again by buffer, to quantify unmodified protein, after injection of compound **1** followed by buffer. It

showed a very tight binding of approximately 8 mol/mole PAI-1. Subsequent injection of tPA gave rise to a very small change in RU, indicating that tPA could no longer form a complex with PAI-1.

ESMS of PAI-1 and Sideroxylonal A (1) Complex. Unglycosylated PAI-1 (1.1 μM) was incubated with compound **1** (151 μM) in buffer. Subsequently, the excess of compound **1** was separated from the protein by buffer exchange on a NAP-5 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated in H_2O (1% HOAc). Mass spectra were recorded using a Finnigan TSQ 700 (San Jose, CA) triple quadrupole mass spectrometer equipped with a Finnigan electrospray ion source. The sample was infused at 1 μL/min after mixing with 3 μL/min of a modifier solution (30% MeCN−H_2O) directly in the spray needle using two Harvard Syringe pumps (Harvard Apparatus, South Natick, MA). The capillary temperature was 200 °C and the spray voltage 3.5 kV. The spectrometer was calibrated in the high mass range using myoglobin and PEG 2000. ESMS of unglycosylated PAI-1 modified by incubation with excess of compound **1** for 10 min at 37 °C indicated complete modification and the formation of a variety of species [*m*/*z* 43259 (100), 43757 (90), 43917 (70), 44946 (60)], of higher mass than that of untreated protein.[9]

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