Bioactive Compounds from *Combretum erythrophyllum*

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A methanol extract of *Combretum erythrophyllum* showed inhibitory bioactivities in a yeast-based microtiter assay for DNA-damaging agents. Bioassay-guided fractionation of this extract yielded two known bioactive compounds, combretastatin A-1 and (−)-combretastatin, and two new bioactive glucosides, combretastatin A-1 2′-*β*-D-glucoside (**1**) and combretastatin B-1 2′-*β*-D-glucoside (**2**). The structures of the new compounds were assigned by ^1H and ^{13}C NMR, DEPT, HMQC, and HMBC spectra.

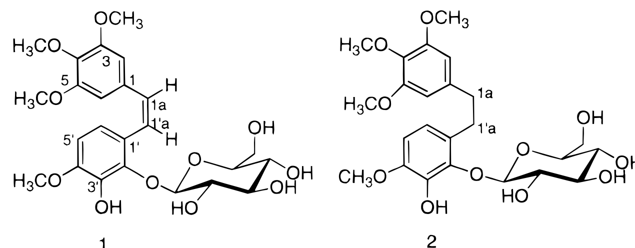
The genus *Combretum,* which occurs in South Africa among other places, belongs to the family Combretaceae. One member of this genus, the South African willow tree *Combretum caffrum*, was found to provide extracts with significant activity in vivo against the murine P-388 lymphocytic leukemia, and the bioactive compounds combretastatin A-1 and B-1 were isolated from the stem wood.[1] The related compound (−)-combretastatin, with activity in an astrocytoma assay and cytotoxicity to P-388 cells, was also isolated from the same plant.[2] In subsequent work the analogue combretastatin A-4 was identified as a promising antiangiogenic agent,[3] and its prodrug form began clinical trials in November 1998.[4]

In our search for anticancer compounds from natural resources[5] we have made extensive use of an agar diffusion yeast bioassay for DNA-damaging agents.[6] This assay has led to the isolation of many interesting compounds,[6b,c] but it has a limited sensitivity due to the nature of the assay. In addition to this limitation some compounds gave "false positives" in this assay. We thus developed a new version of the assay with two significant modifications. In the first place, the agar diffusion format was replaced by a microtiter plate format; this modification increased the sensitivity of the assay significantly. Second, the tester strains were modified so that growth inhibition could be determined by comparison of the growth of the same yeast strain grown under two different conditions. This was accomplished by developing a mutant strain of *Saccharomyces cerevisiae* deficient in the RAD52 recombination repair gene and in topoisomerase I *(Δrad52Δtop1),* in which the RAD52 gene is on a plasmid under the control of the galactose promoter.[7] To do this, a single copy plasmid harboring the yeast RAD52 gene under the control of the GAL1 promoter[8] was introduced into RS321N yeast to give a yeast designated RS321NpRAD52. This resulted in the cells being able to repair DNA on galactose, while being repair-deficient on glucose. To control for the possibility of a compound's interference with transcriptional induction by galactose, a similar single copy plasmid vector without the RAD52 gene[7] was introduced into the RS321N strain, and the resulting cells were used in the assay. With this control strain, designated RS321NYCp50, cells were assayed in the same galactose condition as were the RS321NpRAD52 cells. Thus the differential growth observed here would only be attributed to the difference in DNA repair capabilities and not to carbon source. An extract is selected for investigation if its IC_{50} value for growth inhibition of RS321NpRAD52 when grown on glucose or of RS321NYCp50 grown in galactose is more than 3-fold smaller than its IC_{50} value for RS321NpRAD52 grown on galactose.

Results and Discussion

Using this new assay, it was found that a crude extract of *Combretum erythrophyllum* (Burch) Sond (family Combretaceae) showed reproducible bioactivity, with IC_{50} values of 3.6 μg/mL against RS321NYCp50 grown on galactose, 15.4 μg/mL against RS321NpRAD52 on glucose, and >100 μg/mL against RS321NpRAD52 on galactose. It was thus selected for isolation of its bioactive compounds.

After partition of crude extract between different organic solvents, bioactivity was concentrated in the CH_2Cl_2 fraction with IC_{50} values of 1.7 μg/mL in RS321NYCp50(gal), 41.3 μg/mL in RS321NpRAD52(gal), and 6.3 μg/mL in RS321NpRAD52(glu). The EtOAc fraction was similarly active with IC_{50} values of 3.8, >100, and 12.1 μg/mL, respectively. The CH_2Cl_2 fraction was subjected to CC on silica gel with the solvent CH_2Cl_2−MeOH (100:3), and the bioactivity was concentrated in fraction 3. From this fraction, combretastatin A-1 and (−)-combretastatin were isolated by PTLC on silica gel (CH_2Cl_2−MeOH, 100:3) and PTLC on RP-18 with the solvent MeOH−H_2O (6:4) and MeOH−H_2O (7:3), respectively. The EtOAc extract was subjected to CC on RP-18 with the solvent MeOH−H_2O (6:4), to PTLC on a silica gel plate with the solvent CH_2Cl_2−MeOH (8:2), and finally to PTLC on RP-18 with the solvent MeOH−H_2O (6:4). These procedures led to the isolation of the active compound **1** and its analogue **2**.



Combretastatin A-1, $C_{18}H_{20}O_6$, was identified by comparison of its EIMS fragmentation pattern and its ^1H and ^{13}C NMR spectra with those reported in the literature.[1] (−)-

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Table 1. Bioactivities of Compounds Combretastatin A-1, (−)-Combretastatin, and Compounds **1** and **2**

	bioactivity in the microtiter plate assay (IC_{50}, μg/mL)					agar diffusion assay (IC_{12}, μg/mL)		
sample	Ycp(gal)	pRAD52(gal)	pRAD52(glu)	phTOP1(gal)	phTOP1(glu)	RS188 (WT erg6)	RS321 (Rad52.erg6.top1)	RS322 (Rad52.erg6)
MeOH extract	4	>100	15	NT	NT	NT	NT	NT
CH_2Cl_2 fraction	2.0 ± 0.6	34 ± 4	31 ± 17	3.3	4.3	73.7	5.9	>100
EtOAc fraction	4	>100	12	NT	NT	NT	NT	NT
combretastatin A-1	1.8 ± 0.2	53 ± 10	18 ± 6	1.6	1.9	80.1	2.2	33.5
(−)-combretastatin	6.0 ± 2	>100	>100	6.6	7.4	>100	45.1	>100
compound **1**	13	>100	3	NT	NT	NT	NT	NT
compound **2**	25	>100	>100	68.7	>100	>100	>100	>100

Combretastatin, $C_{18}H_{20}O_6$, was also identified by comparison of its EIMS and ^1H and ^{13}C NMR data with the literature data.[9]

Compound **1** had the molecular formula $C_{24}H_{30}O_{11}$ as determined by HREIMS, and it gave signals for 14 sp^2 carbons in its ^{13}C NMR spectrum. It was optically active, with $[\alpha]^{23}_D$ +40.1° (*c* 0.037, MeOH), and UV absorbing, with λ_{max} (log ϵ) 220 (4.28) and 295 (3.94). Its ^1H NMR spectrum showed the presence of four methoxy groups [δ_H 3.61 (3H, s), 3.61 (3H, s), 3.71 (3H, s), and 3.82 (3H, s)], four singlet proton signals [δ_H 6.65 (2H, s) and δ_H 6.50 (2H, s)], and two olefinic protons [δ_H 6.84 (1H, d, 12.12 Hz) and δ_H 6.49 (1H,s, 12.12 Hz)]. On the basis of these spectroscopic data, compound **1** was determined to be a stilbene derivative. The presence of signals for an anomeric carbon (δ_H 4.75 and δ_c 106.9) indicated the presence of a sugar moiety, and the *J*-values of the protons in this moiety suggested it to be glucosyl. The $J_{G1,G2}$ value of 7.91 Hz further indicated that the glucosyl moiety was connected to the aglycone by a β-linkage.

Long-range correlations in the HMBC spectrum of **1** indicated that the four methoxy groups were connected at C-3, C-4, C-4′, and C-5. The long-range correlation between H-1′a (δ_H 6.84) and C-2′ (δ_C 144.8) and between the anomeric proton (δ_H 4.75) and C-2′ (δ_C 144.8) showed the glucosyl moiety to be at C-2′. The observation of NOE between H-G_1 (δ_H 4.75) and H-1′a (δ_H 6.84) in NOESY and NOEDS spectra supported this result. The positions of the four methoxy groups at C-3, C-4, C-4′, and C-5 were also confirmed by the corresponding NOESY spectrum. Although $J_{1a,1'a}$ was 12.1 Hz, the presence of an NOE between H-2 and H-6′ indicated that the stereochemistry of the double bond must be *Z*, as is the case for similar compounds in the literature.[1,2] All of these data demonstrated that compound **1** is combretastatin A-1 2′-β-D-glucoside. The ^1H NMR signals of H-5′ and H-6′ appeared as a singlet due to accidental chemical shift equivalence; the corresponding ^{13}C NMR signals appeared as separate signals at δ_C 109.5 and 121.1.

Compound **2** had the molecular formula $C_{24}H_{32}O_{11}$, as deduced by HRMS, and it too was optically active with $[\alpha]^{23}_D$ +36.8° (*c* 0.027, MeOH). Its UV spectrum [λ_{max} (log ϵ) 213 (4.38) and 274 (3.43)] was similar to that of **1** but with a shift to shorter wavelengths, consistent with the loss of some conjugation. In agreement with this, its ^{13}C NMR spectra showed signals for only 12 sp carbons. Its ^1H NMR spectrum was very similar to that of compound **1** except for the lack of signals for two sp^2 protons and the presence of two ortho-coupled protons [δ_H 6.69 (1H, d, J = 8.46 Hz), 6.58 (1H, d, J = 8.46 Hz)] instead of the two-proton singlet observed for compound **1**. In addition to the signals corresponding to a glucosyl moiety, two proton signals [δ_H 6.47 (2H, s)] and two methylene signals [δ_H 3.11 (1H, m), 2.92 (1H, m) and 2.84 (2H, m)] were observed. The long-range correlations in the HMBC spectrum and $J_{G1,G2}$ = 7.91 Hz indicated that compound **2** is combretastatin B-1 2-β-D-glucoside.

Combretastatin A-1, (−)-combretastatin, and compounds **1** and **2** were tested in the microtiter assay for DNA-damaging agents at both Virginia Tech and SmithKline Beecham Pharmaceuticals. Combretastatin A-1, (−)-combretastatin, and compound **1** all showed reproducible and selective inhibitory activity against the DNA repair-deficient strain pRAD52.glu, with combretastatin A-1 and compound **1** showing the greatest activity. Compound **2** was inactive in these assays. Combretastatin A-1, (−)-combretastatin, and compound **2** were also tested in the initially used agar diffusion assay; in this assay only combretastatin A-1 showed significant activity, illustrating the greater sensitivity of the microtiter plate assay (Table 1). Compound **1** was evaluated further in a standard mammalian cell growth inhibition assay; it inhibited growth of two human cell lines—HeLa, derived from a cervical carcinoma, and A549, from lung adenocarcinoma—with 50% inhibitory concentrations (IC_{50} values) of 1.5 and 7.0 μg/mL, respectively. For comparison, the IC_{50} values for AC-7739, a combretastatin analogue in clinical development, were 0.001 and 0.004 μg/mL, respectively, in these cell lines.

Combretastatin A-1, (−)-combretastatin, and compounds **1** and **2** were tested for inhibition of topoisomerase 1 in an assay using isogenic yeast strains with the gene for topoisomerase 1 under the control of the galactose promoter (phTOP1.glu and phTOP1.gal). In this assay there was no significant difference in activity between the two strains for any of the compounds, indicating that they do not act as inhibitors of topoisomerase 1 (Table 1).

Experimental Section

General Experimental Procedures. Optical rotations were recorded with a Perkin-Elmer 241 Polarimeter. NMR spectra were recorded in $CDCl_3$ on a JEOL Eclipse+ 500 instrument at 500.1624 MHz for ^1H and 125.7778 MHz for ^{13}C, and a Varian Unity 400 NMR instrument at 399.951 MHz for ^1H and 100.578 MHz for ^{13}C, using standard pulse sequences programs. The exact mass measurements were obtained on a VG 7070E-HF mass spectrometer in the Department of Biochemistry, Virginia Polytechnic Institute and State University. UV spectra were measured on a Shimadzu UV1201 spectrophotometer instrument. Other conditions were as previously described.[10]

Plant Materials. The wood of *C. erythrophyllum* (Burch) Sond. (Combretaceae) was collected in March 1998 from branches of a young tree at the University of Natal, Durban, South Africa. After collection, the wood and associated bark were allowed to dry at ambient temperature for about one week and were then milled and immediately extracted. Voucher specimens are deposited in the Herbarium, University of Natal, Durban (No. 272).

Table 2. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2**

	compound **1** (in CD_3OD)				compound **2** (in CD_3OD)	
position	δ_C	δ_H	HMBC	NOE correlation	δ_C	δ_H
1	134.6				137.1	
2	107.5	6.50 (1H, s)	C-1, C-3, C-4, C-1a	3-OCH_3, 6′-H	107.0	6.47 (1H, s)
3	154.0				154.1	
4	138.2				134.0	
5	154.0				154.1	
6	107.5	6.50 (1H, s)	C-1, C-4, C-5, C-1a	5-OCH_3	107.0	6.47 (1H, s)
1′	125.9				129.7	
2′	144.8				145.2	
3′	140.9				140.7	
4′	149.6				148.5	
5′	109.5	6.65 (1H, s)	C-1′, C-3′	4′-OCH_3	109.9	6.69 (1H, d, 8.46 Hz)
6′	121.1	6.65 (1H, s)	C-1′, C-2′, C-4′	2-H	120.6	6.58 (1H, d, 8.46 Hz)
1a	130.6	6.49 (1H, d, 12.12)	C-1′, C-1, C-2, C-6		38.4	2.84 (2H, m)
1′a	127.7	6.84 (1H, d, 12.12)	C-1, C-2′, C-6′	H-G1	32.8	2.92 (1H, m); 3.11 (1H, m)
G1	106.9	4.75 (1H, d, 7.91)	C-2′	1′a-H, H-G3, H-G5	107.1	4.59 (1H, d, 7.91)
G2	75.4	3.51 (1H, dd, 7.91, 9.01)			75.6	3.52 (1H, dd, 7.91, 9.10)
G3	77.9	3.38 (1H, dd, 9.01, 9.19)		H-G1	71.1	3.40 (1H, dd, 8.00, 9.10)
G4	71.0	3.44 (1H, dd, 9.19, 7.90)			78.1	3.42 (1H, dd, 8.00, 6.98)
G5	78.5	3.18 (1H, m)		H-G1	78.5	3.23 (1H, m)
G6	62.3	3.69 (1H, dd, 11.95, 4.60); 3.75 (1H, m)			62.4	3.65−3.90 (2H, m)
3-OCH_3	56.3	3.61 (6H, s)	C-3	H-2	56.6	3.78 (6H, s)
4-OCH_3	56.8	3.71 (3H, s)	C-4		61.1	3.71 (3H,s)
5-OCH_3	56.3	3.61 (6H, s)	C-5	H-6	56.6	3.78 (6H, s)
4′-OCH_3	61.1	3.81 (3H, s)	C-4′	H-5′	56.8	3.80 (3H, s)

Plant Extraction. The dried milled sample (238 g) was extracted with methanol on a shaker for 48 h, and the crude extract (8.63 g) was obtained.

Isolation of Combretastatin A-1, (−)-Combretastatin, and Compounds 1 and 2. The crude extract of *C. erythrophyllum* (Burch) Sond. (8 g) was bioactive in the microtiter assay (Table 1) and was partitioned between *n*-hexane and 4:6 H_2O−MeOH. The aqueous MeOH extract was extracted with CH_2Cl_2 to give a CH_2Cl_2 extract and was then diluted with H_2O to 75:25 H_2O−MeOH and extracted with EtOAc to give an EtOAc extract. The CH_2Cl_2 and EtOAc extracts were bioactive (Table 1). The CH_2Cl_2 fraction (479 mg) was subjected to CC on silica gel with the solvent CH_2Cl_2−MeOH (100:3), and the bioactivity was found to be concentrated in fractions 3 and 5. Combretastatin A-1 (4.1 mg) was isolated from fraction 3 by RP-PTLC on a C-18 plate with the solvent MeOH−H_2O (6:4), and (−)-combretastatin (2.2 mg) was obtained from fraction 5 by RP-PTLC on C-18 with the solvent MeOH−H_2O (7:3).

The active EtOAc extract was subjected to CC on a C-18 column with the solvent MeOH−H_2O (6:4), and fractions 4−16 (367.7 mg) were bioactive. The most bioactive fractions, 6−13 (218.6 mg), were combined and purified by PTLC on silica gel with the solvent CH_2Cl_2−MeOH (8:2). The most bioactive fraction from this step was further purified by RP-PTLC on C-18 with the solvent MeOH−H_2O (6:4). These procedures led to the isolation of the active compounds **1** (3.5 mg) and **2** (1.7 mg).

Compound 1: $[\alpha]^{23}_D$ +40.1° (*c* 0.037, MeOH); UV (MeOH) λ_{max} (log ϵ) 220 (4.28) and 295 (3.94); ^{1}H and ^{13}C NMR data see Table 2; HRCIMS *m/z* 494.1774 (M^+) (calcd for $C_{24}H_{30}O_{11}$ 494.1788).

Compound 2: $[\alpha]^{23}_D$ +36.8° (*c* 0.027, MeOH); UV (MeOH) λ_{max} (log ϵ) 213 (4.38) and 274 (3.43); ^{1}H and ^{13}C NMR data see Table 2; FABMS *m/z* 497 (MH^+) (calcd for $C_{24}H_{33}O_{11}$ 497).

Bioassay. The mutant strain, RS321NpRAD52, used in this assay is deficient in recombination repair (RAD52) and topoisomerase I, but the strain carries a plasmid containing the RAD52 repair pathway gene under the control of a galactose promoter. The RS321pRAD52 yeast was split into two separate sterile flasks. Galactose was added to one flask, while glucose was added to the other, both to a final concentration of 2 wt %. RS321NYCp50, the same yeast strain as RS321pRAD52 but without the transformed RAD52 gene, was also combined with galactose at a final concentration of 2% in a separate flask.

An aliquot of 90 μL of the selected yeast suspension was added to each well of a 96-well microtiter plate. The test sample was prepared by accurately weighing approximately 0.5 mg of test sample (either fraction or pure compound) and dissolving it in sufficient 10% DMSO to give a solution at 10 times the desired final concentration; a 10 μL aliquot was then added in triplicate to each microtiter plate. Column six of the microtiter plate was used for the control wells. Rows A−D were the positive growth wells, containing 90 μL of yeast/sugar/media suspension and 10 μL of 10% DMSO, while rows E−H were the negative growth wells, containing 90 μL of blank minimal media and 10 μL of 10% DMSO. Each plate was then vortexed for 30 s and incubated in a high-humidity chamber at 30 °C for 48−72 h. The microtiter plates were read when the positive growth wells had reached an OD of 0.15−0.25. At this point, the growth inhibition was calculated by using the formula $1 - (OD_{test\ well} - OD_{blank})/(OD_{plate\ median} - OD_{blank})$.

Any agent that was cytotoxic to the yeast in the RS321-pRAD52 glucose and RS321NYCp50 galactose plate (>65% inhibition) but was not cytotoxic in the RS321pRAD52 galactose plate (<35% inhibition) was considered an active agent.

Combretastatin A-1, (−)-combretastatin, and compound **2** were also evaluated in the agar diffusion assay used previously in this work.[11] Bioassay results for fractions and pure compounds are given in Table 1.

Mammalian cell cytoxicity assays were performed on compound **1** by standard methods with HeLa and A549 cells, using the XTT protocol for visualization.[12]

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Supporting Information Available: Figure 1 showing selected HBMC correlations for compounds **1** and **2**, and Figure 2 showing NOEDS correlations for compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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