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ANTINEOPLASTIC AGENTS, 122. CONSTITUENTS OF *COMBRETUM CAFFRUM*[1]

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ABSTRACT.—An investigation of the South African tree *Combretum caffrum* (Combretaceae) for antineoplastic constituents was conducted by employing the astrocytoma bioassay (9ASK). By this approach and a combination of solvent partition, steric exclusion, and adsorption chromatographic procedures, a substance designated combretastatin [**1a**] was isolated and found to display significant (71-90% astrocyte reversal at 1-100 μg/ml dose levels) astrocyte reversal and murine P-388 lymphocytic leukemia (PS) cell growth inhibition (ED_{50} 0.011 μg/ml). The structure of (−)-combretastatin was elucidated by X-ray crystal structure analysis and confirmed by total synthesis. The absolute configuration at C-10 was assigned the *R*-configuration on the basis of Horeau esterification techniques. Other prominent, albeit PS- inactive, constitutents were found to be 3,3′,4′-tri-*O*-methylellagic acid [**2**] and acacetin [**3**].

The tropical and subtropical Combretaceae family is composed of some 600 shrubs and trees distributed among 20 genera. Seven species of the genus *Terminalia* and one *Guiera* species have a long history in African and Asian primitive medical treatments for cancer (2). The largest genus of this family is *Combretum*, a group of 250 tropical and deciduous trees (3). While 24 species of *Combretum* are well known in African folk medicine for applications and problems ranging from heart and worm remedies to wound dressings, treatment for the mentally ill, and scorpion sting (4), only the Indian *Combretum latifolium* appears to have been recorded as a folk medical treatment for cancer.[2] However, as part of the U.S. National Cancer Institute's (NCI) world-wide exploratory survey of terrestrial plants, both *Combretum molle* (5) and *Combretum caffrum* (Eckl. and Zeyh.) Kuntze were found to provide extracts significantly active against the murine P-388 lymphocytic leukemia (PS system).

In 1979, in collaboration with the NCI, we undertook a detailed examination of *C. caffrum* collected in South Africa in January of that year. A combination of the leaves, fruit, stems, and twigs were found to give EtOH extracts with a confirmed level of activity against the NCI PS in vivo system. Near the beginning of our studies we found the crude fractions also to show significant activity against the NCI in vitro astrocytoma assay. The basis of this bioassay with immature AC glioma cells derived from a 1974 rat glioma is the ability of a substance to reverse differentiation to astrocytes with a mature morphology. The NCI procedure employs 51-90% astrocyte reversal with cytotoxicity not exceeding 50% as the criteria for activity. When a CH_2Cl_2-MeOH extract of *C. caffrum* was subjected to solvent partition between aqueous MeOH (9:1↦3:2) with hexane↦CCl_4↦CH_2Cl_2, the resulting chlorocarbon fractions were found to exhibit 71-90% astrocyte reversal at 1-100 μg/ml dose levels. The CCl_4 and CH_2Cl_2 fractions also were effective against the PS leukemia and showed, respectively, in vitro (ED_{50} 1.5 and 0.23 μg/ml) and in vivo (27% life extension at 100 mg/kg and 45% at 50 mg/kg) activity. At this juncture the CH_2Cl_2 fraction was selected for careful separation based on the 9ASK system.

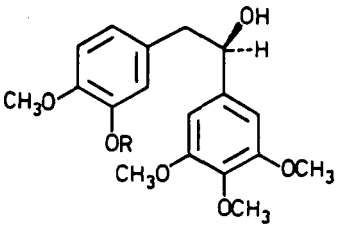
The CH_2Cl_2 fraction in MeOH was further separated by gel permeation chromatography on Sephadex LH-20. The 9ASK active fraction was triturated with Me_2CO, and the solid residue was further separated by a series of Si gel column

[1]Contribution 121 see Piatak *et al.* (1).

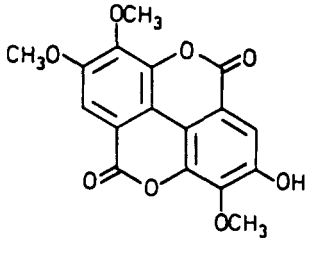
[2]Private communication from Dr. James A. Duke and Kay K. Wain, USDA, Beltsville, MD, December 1985.

chromatograms. The 9ASK active constituent designated combretastatin [**1a**] showed 71-90% astrocyte reversal at dose levels of 1-100 μg/ml and 51-70% at 0.1 μg/ml with only 0-5% cytotoxicity being observed. Combretastatin also inhibited growth of the PS in vitro cell line with ED_{50} 0.011 μg/ml. So far only enough natural combretastatin has been available just to reach the beginning of PS in vivo activity at 5 mg/kg. Because the 9ASK system has shown good selectivity for antimitotic substances, combretastatin was examined at the NCI for such properties and antitubulin activity (6). Subsequently, combretastatin was found to be a very effective antimitotic and to inhibit tubulin polymerization (*cf.* 7,8). Meanwhile, a preliminary report (7) of the combretastatin isolation and X-ray crystal structure determination was prepared followed by total syntheses of natural (8) and racemic (9) combretastatin.

While the isolation and structure elucidation of combretastatin [**1a**] were in progress, an examination of companion constituents in the original CCl_4 and CH_2Cl_2 fractions led to isolation of the known gallic acid derivative 3,3′,4′-tri-*O*-methylellagic acid [**2**] (10,11). Phenol **2** was identical with an authentic specimen provided by Prof. Cambie. Initial separation of the CCl_4 fraction also led to the previously known flavone acacetin [**3**] (12-14). Once the easily isolated methyl ellagic acid and flavone derivatives were removed, it became clear that the remaining fractions contained a considerable number of closely related substances requiring separation guided by the PS leukemia and results of those endeavors will form the basis of future contributions concerning this potentially important *Combretum* sp.



1a R=H
1b R=$Si(CH_3)_2C(CH_3)_3$



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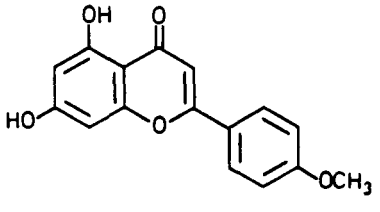

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When combretastatin [**1a**] was found (6) to competitively inhibit the binding of colchicine to tubulin, our original interest in finding the absolute configuration of combretastatin intensified. Because the X-ray crystal structure determination was performed by light atom techniques and the unweighted and weighted residuals for both enantiomorphs were almost identical, it was not possible to define the chiral center at C-10 (7). The more obvious approaches to ester or urethane derivatives of combretastatin by esterification of the C-10 hydroxy group either led to formation of the corresponding stilbene by very ready dehydration or, otherwise, led to unsuitable products from the few milligrams of natural combretastatin available at the time. When larger amounts of natural combretastatin become available by total synthesis (8), a suitable heavy atom and/or chiral derivative will be easily located. An attractive (substance spar-

ing) alternative appeared to be offered by Horeau's esterification procedure (15-20), for assigning absolute configuration to an asymmetric carbon bearing a hydroxyl group. Thus, one of our total synthetic intermediates, 3-[(*tert*-butyl-dimethylsily)oxy]-combretastatin [**1b**] (8) was resolved on a Pirkle column and the (−)-epimer (Figure 1), corresponding to natural (−)-combretastatin, was found very useful for the Horeau method. Reaction of this partially protected (−)-combretastatin [**1a**] with racemic 2-phenylbutyric anhydride led to isolation of (+)-2-phenylbutyric acid with an optical rotation of $[\alpha]^{27}D+19.3°$. An analogous experiment starting with the enriched (+)-enantiomer of combretastatin afforded 2-phenylbutyric acid with $[\alpha]^{27}D-8.9$. As expected, only racemic 2-phenylbutyric acid was obtained when the experiment was repeated with racemic 3-(*t*-butyldimethylsilyl)-combretastatin [**1b**]. Results of these experiments suggested assignment of the *R*(−) configuration to natural (−)-combretastatin. Further support for this conclusion was obtained by repeating the assignment experiments with cholesterol and obtaining 2-phenylbutyric acid with $[\alpha]^{27}D-7.4$ in complete agreement with the well-known *S*-configuration for the C-3 hydroxyl group. A cd comparison of the (+)- and (−)-epimers of 3-(*t*-butyldimethylsilyl) combretastatin [**1b**] has been entered in Figure 1. The cd curve of natural (−)-combretastatin (**1a**) has been included as Figure 2 and nicely illustrates the potential of this technique for distinguishing between *R* and *S* enantiomers of this type. Unless (−)-combretastatin is eventually found to violate the Horeau's rule, the absolute configuration is *R*(−) for natural combretastatin.

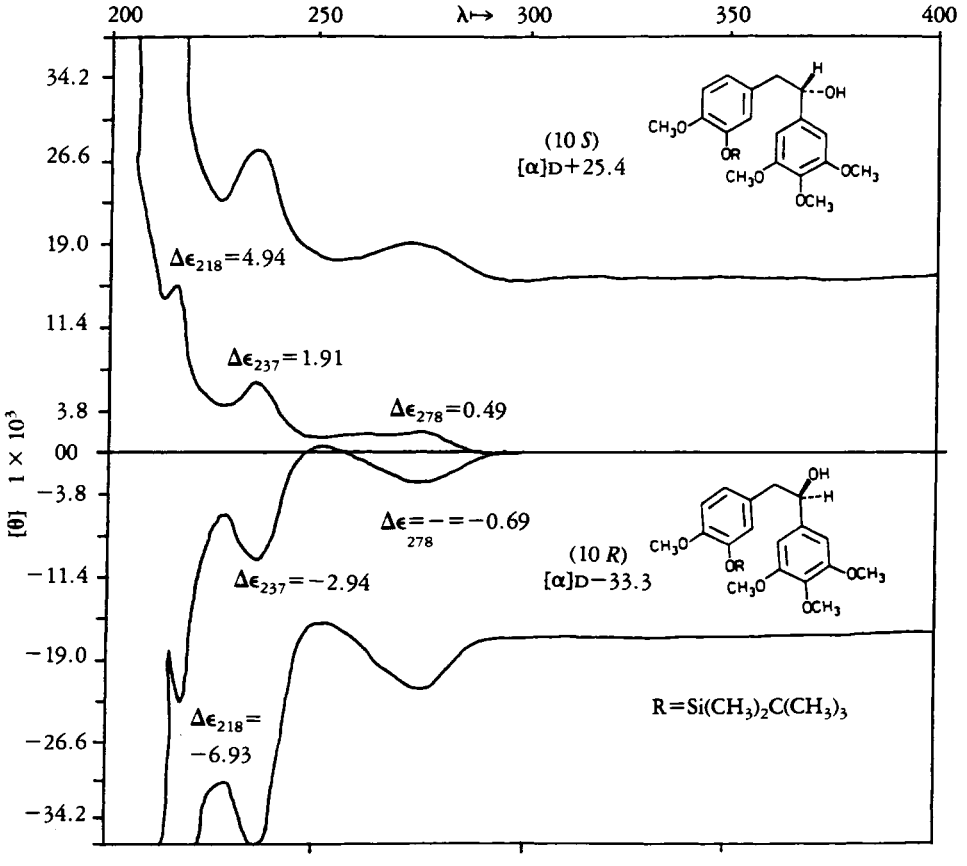

FIGURE 1. Circular dichroism spectra of 3-[(*tert*-butyl-dimethylsilyl)oxy]-*R* and *S*-combretastatin [**1b**]

Present biological results suggest that combretastatin and/or closely related substances will become, at the least, useful biochemical probes. For example, Ludueña and colleagues (21) have found (−)-combretastatin [**1a**] to inhibit alkylation of tubulin by iodo [^{14}C] acetamide, as observed with colchicine and podophyllotoxin (22). When viewing the interesting biological properties so far uncovered for combretastatin and certain other constituents of *C. caffrum* now being pursued, the Zulu practice of using root bark from this tree as a charm for harming an enemy seems particularly appropriate when applied to cancer, a most serious enemy indeed.

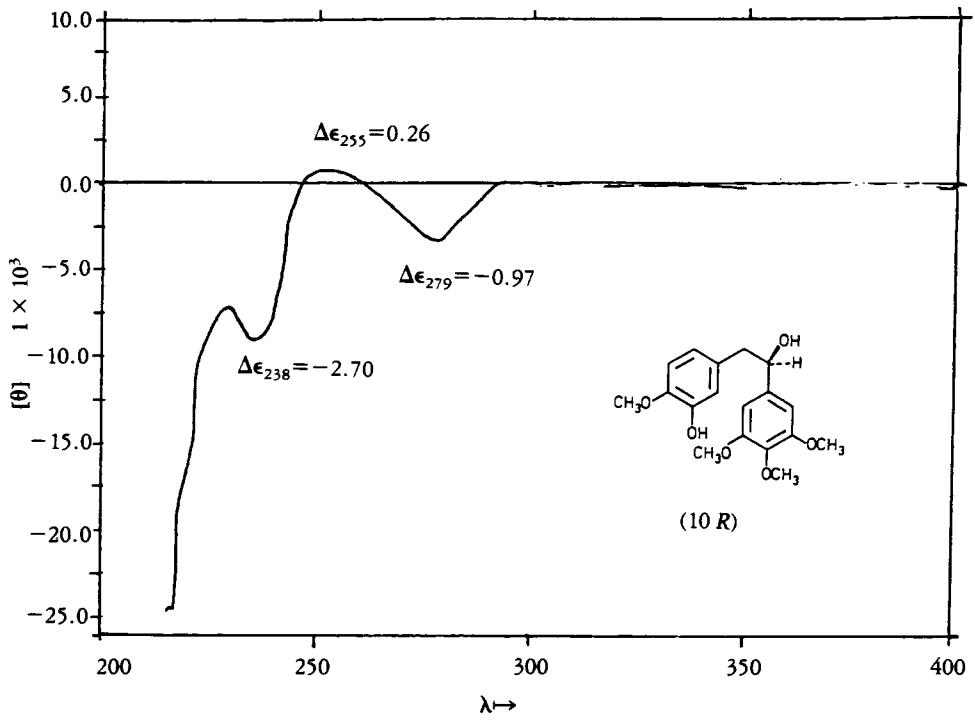

FIGURE 2. Circular dichroism spectrum of natural (−) combretastatin [**1a**]

EXPERIMENTAL

GENERAL METHODS.—For general experimental methods, except as noted below, refer to Pettit *et al.* (23). Optical rotations were measured using a Perkin-Elmer Model 241 automatic Polarimeter. The ^{1}H nmr and ^{13}C- spectra ($CDCl_3$ solution and TMS internal standard) were determined with a Bruker WH-90 and Varian XL-100 spectrometers. All ^{13}C nmr spectra were measured at 22.63 MHz. Mass spectra were obtained using a MAT 312 spectrometer. Elemental analyses were determined by Dr. A.W. Spang of the Spang Microanalytical Laboratory, Eagle Harbor, MI. Comparison ir (KBr), and ^{1}H-nmr spectra and tlc were employed to establish the mutual identity of isolated products and authentic samples.

C. caffrum (*B817373*).—A 61-kg re-collection (1979) of the South African Combretaceae also known as *Combretum solicifolium* E. was obtained through the Economic Botany Laboratory, Agricultural Research Center-East, USDA, Beltsville, MD, as part of a collaborative NCI-USDA research program directed by Drs. John D. Douros, Matthew I. Suffness, and James A. Duke.

EXTRACTION.—The dried and chipped twigs, leaves, and fruit (55 kg) were extracted with MeOH-CH_2Cl_2 (1:1; 120 liters for 10 days) at ambient temperature. H_2O (30 liters) was added to cause separation of the CH_2Cl_2 phase. The aqueous phase was adjusted by addition of MeOH and CH_2Cl_2 in the ratio, aqueous phase MeOH-CH_2Cl_2 (4:1↦5:15) and the plant parts were re-extracted for an additional 5 days. Decantation and addition of 15% by volume H_2O separated the CH_2Cl_2 phase, which was combined with the first CH_2Cl_2 fraction and evaporated to give a combined extract (1.5 kg).

SOLVENT PARTITION.—The CH_2Cl_2 residue (1.5 kg) was dissolved in MeOH-H_2O (9:1; 9 liters) and extracted with hexane (bp 60-65°, 3×6 liters). The MeOH-H_2O phase was adjusted to 4:1 and 3:2 while extracting, respectively, with CCl_4 (5×2.5 liters) and CH_2Cl_2 (7×3 liters). By this means PS and 9ASK inactive hexane (520 g) and active CCl_4 (210 g, PS ED_{50} 1.5 μg/ml, T/C 127 at 100 mg/kg, 9ASK 71↦90 at 100, and 51↦70 at 10 μg/ml) and CH_2Cl_2 (268 g, PS ED_{50} 0.23 μg/ml, T/C 145 at 50 mg/kg, 9ASK 71↦90 at 100 and 51↦70 at 10 μg/ml) fractions were obtained.

ISOLATION OF (−)-COMBRETASTATIN [**1a**].—An aliquot (159 g of CH_2Cl_2-soluble fraction) was chromatographed in MeOH on Sephadex LH-20 (2 kg). Elution volumes 6200 to 7500 ml gave a 9 ASK active fraction. Trituration of this fraction with Me_2CO allowed a less-soluble fraction (10.3 g) to separate. Si gel (200 g) chromatographic separation of the 10.3 g fraction, by gradient elution with CH_2Cl_2 to 49:1 CH_2Cl_2-MeOH (1.5 liter total), followed by elution with 97:3 CH_2Cl_2-MeOH (1.5 liters) gave another 9ASK active fraction (1.42 g). Further chromatographic separation using a Lobar B column and elution with CH_2Cl_2 (300 ml), followed by 99:1 CH_2Cl_2-MeOH afforded 0.452 g of (−)-combretastatin. Recrystallization from Me_2CO/hexane yielded a pure specimen as needles: mp 130-131° and $[\alpha]^{26}D$ −8.51° (*c* 1.41, $CHCl_3$). The uv, ir, ^1H- and ^{13}C-nmr and mass spectrum of combretastatin was recorded in the preliminary communication (8). *Anal.* calcd for $C_{18}H_{26}O_6$, C, 64.67; H, 6.58. Found: C, 64.76, H, 6.69.

ISOLATION OF 3,3′4′-TRI-*O*-METHYLELLAGIC ACID [**2**].—Continued elution with MeOH of the Sephadex LH-20 column containing the CH_2Cl_2 active fraction (see above), between volumes 9 to 13.5 liters gave a fraction that upon trituration with MeOH and recrystallization from EtOH/$CHCl_3$, yielded 3,3′,4-trimethylellagic acid (0.40 g): mp 303-306 [lit (10), mp 293-294]; Acetylation of phenol **2** (20 mg), using 2 ml of Ac_2O-pyridine (1:1), led to the corresponding acetate (20 mg). Recrystallization from CH_2Cl_2/MeOH yielded a pure specimen melting at 269-272°. Spectral data (uv, ir, ^1H nmr and ms) of ether **2** and its monoacetate were identical with the corresponding literature data (11). The monoacetate exhibited the following ^{13}C nmr (22.63 MHz, $CDCl_3$) 168.55, 158.98, 158.09, 155.29, 144.80, 144.50, 142.16, 141.77, 141.03, 120.26, 112.98, 112.20, 108.05, 79.87, 74.28, 62.00, 56.90, 20.54 ppm; and hreims *m/z* 386.0625 ($C_{19}H_{14}O_9$ requires 386.0612).

ISOLATION OF ACACETIN [**3**].—A 190-g portion of the CCl_4-soluble fraction was chromatographed in CH_2Cl_2-MeOH (2:3) on Sephadex LH-20 (2.5 kg). Elution between volumes 5 to 10 liters and concentration of the fraction gave a buff-colored solid (0.95 g). Recrystallization from MeOH afforded acacetin [**3**], identical with an authentic sample, as buff-colored needles: mp 274-275° [lit. (13) mp 260°]; spectral data (uv, ir, ^1H nmr; ^{13}C nmr, and ms) were identical with the literature values (12,14).

Acacetin (25 mg) was acetylated employing 2 ml of Ac_2O-pyridine (1:1) at room temperature (48 h). The reaction mixture was diluted with MeOH, and the solvent was evaporated (reduced pressure). The acetate was crystallized from Me_2CO/hexane to provide 20 mg melting at 202-204° [lit. (13) mp 203°]; ir (KBR) ν max 1768, 1643, 1629, 1606, 1513, 1369, 1183, 1137, 1101, 1032 cm^{-1}; ^1H nmr ($CDCl_3$, 90 MHz) δ 2.35 (3H, s, OAC), 2.44 (3H, s, OAc), 3.89 (3H, s, OCH_3), 6.57 (1H, s, H-3), 6.83 (1H, d, *J*=2.4 Hz, H-6), 7.01 (2H, d, *J*=9.2 Hz, H-3′, 5′), 7.34 (1H, d, *J*=2.4 Hz, H-8), 7.81 (2H, d, *J*=9.2 Hz, H-2′, 6′); and hreims *m/z* (rel int. %) 368.0898 (0.71%, M^+ calcd for $C_{20}H_{16}O_7$, 368.0896), 326.0791 (44.11% calcd for $C_{18}H_{14}O_6$, 326.0790, M^+ - CH_3CO), 284.0684 (100%, calcd for $C_{16}H_{12}O_5$, 284.0684, M^+ - 2 + $COCH_3$), 152.0088 (4.73%, $C_7H_4O_4$), 132.0575 (11.98%, C_9H_8O), 124.0159 (2.96%, $C_6H_4O_3$), 123.0083 (3.63%, $C_6H_3O_3$).

Horeau Esterification of 3-[(*tert-butyl-dimethylsilyl*)*oxy*]-(+) *and* (−)-*combretastatins* [**1b**].—A solution prepared from synthetic (8) 3-[(*tert*-butyl-dimethylsilyl)oxy]-(−)-combretastatin [**1a**] (10.1 mg, 0.023 mmol), 2-phenylbutyric anhydride (42.9 mg, 0.138 mmol), and pyridine (20 drops) was stirred 20 h at room temperature. After adding H_2O (4 ml), stirring was continued for 2 h. C_6H_6 (3 ml) was added, and the mixture was titrated with 0.1 N NaOH to the phenolphthalein end point. The aqueous layer was extracted with C_6H_6 (2×3 ml), acidified with dilute HCl to pH-1, and again extracted with C_6H_6 (3×5 ml). The latter C_6H_6 solution was dried, and solvent was removed to give 2-phenylbutyric acid (6.2 mg) which displayed $[\alpha]D$ + 19.3 (*c*=0.62, C_6H_6).

The preceding experiment was repeated with synthetic 3-[(*tert*-butyl-dimethylsilyl)oxy]-combretastatin [**1b**] (22.6 mg), containing primarily the (+)-isomer. The unnatural (+)-enantiomer was obtained following major removal of the (−)-epimer from the synthetic racemate using a chiral *N*-(3,5-dinitrobenzoyl)-D-phenylglycine bonded to a silica hplc column (10×500 mm). Elution was performed isocratically using hexane-iPrOH (9:1) with a flow rate of 2.5 ml/min. After extraction with Et_2O, 10.1 mg of 2-phenylbutyric acid was isolated, which exhibited $[\alpha]^{27}D$ −8.9 (*c*=1.122, C_6H_6).

In a similar experiment using racemic [**1b**], the recovered 2-phenylbutyric acid showed no optical rotation. In addition, repeating the reaction (20 h) with cholesterol (16 mg), 2-phenylbutyric acid (72.8 mg), and pyridine (1.5 ml), afforded 12.1 mg of 2-phenylbutyric acid with $[\alpha]^{27}D$ −7.4 (*c*=1.21, C_6H_6), suggesting the already well established *S*-configuration for the C-3 hydroxyl group of cholesterol.

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