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ANTI-HIV AND CYTOTOXIC ALKALOIDS FROM *BUCHENAVIA CAPITATA*[1]

JOHN A. BEUTLER, JOHN H. CARDELLINA II, JAMES B. MCMAHON, MICHAEL R. BOYD,*

Laboratory of Drug Discovery Research and Development

and GORDON M. CRAGG

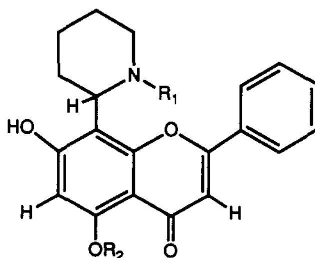
Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Building 1052, Frederick, Maryland 21702-1201

ABSTRACT.—The anti-HIV activity in the organic solvent extract of leaves of *Buchenavia capitata* was traced to a series of known flavonoid alkaloids, which represent a new chemotype for anti-HIV activity. The ^{13}C-nmr assignments for this series of compounds have been revised. *O*-Demethylbuchenavianine [**1**] was the most active compound of the series but produced only moderate cytoprotective effects against HIV in cultured human lymphoblastoid (CEM-SS) cells. Compound **1** was cytotoxic also in the NCI human disease-oriented in vitro tumor screening panel and produced a pattern of modest differential cellular sensitivity.

An organic extract of the leaves of *Buchenavia capitata* (Vahl) Eichl. (Combretaceae) showed cytoprotective activity against the HIV virus in the NCI AIDS-antiviral drug screen (1). An alkaloid partition experiment suggested that alkaloids were the source of the bioactivity. A series of flavonoid alkaloids had been isolated previously from this genus by Ahond *et al.* (2). Our bioassay-guided fractionation of the basic fraction led to three flavonoid alkaloids **1–3**, one of which was confirmed to be the major active principal. Further in vitro screening against the NCI's in vitro panel of human tumor cell lines showed that *O*-demethylbuchenavianine [**1**] was cytotoxic, with the various cell types showing a modest degree of differential sensitivity.

RESULTS AND DISCUSSION

BIOASSAY-GUIDED ISOLATION OF ANTI-HIV CONSTITUENTS.—An initial solvent-solvent partition of the crude extract distributed the total anti-HIV bioactivity among the H_2O, $CHCl_3$, and CCl_4 fractions, with the $CHCl_3$ fraction being the most potent. Alternatively, an acid-base extraction scheme gave enhanced activity in the crude basic fraction, supporting the hypothesis that alkaloids were responsible for the anti-HIV activity. The most efficient initial treatment of the crude organic extract con-



1 R_1=Me, R_2=H
2 R_1=R_2=H
3 R_1=R_2=Me

[1]Part 5 in the series "HIV-Inhibitory Natural Products." for Part 4, see Manfredi *et al.* (14).

sisted of suspension of the crude extract in 30% NH_4OH and MeOH, followed by partition between EtOAc and H_2O. Tlc of the basic organic fraction thus obtained showed several spots staining positively with Dragendorff's reagent.

Flash chromatography of the base fraction on Si gel with a step gradient of $CHCl_3$ and MeOH resulted in isolation of one of the most abundant and less polar alkaloids in relatively pure form. Nmr studies of this material and comparison with the data of Ahond *et al.* (2) suggested that the compound was *O*-demethylbuchenavianine [**1**]. The purification of **1** required two additional steps, a low pressure chromatography on Si gel (hexane/Me_2CO), followed by centrifugal partition chromatography. A series of 2D nmr experiments (HMBC, HMQC) confirmed the structure and clarified the ^{13}C-nmr assignments (2) for this compound (Table 1), which proved to be the most potent antiviral constituent, having an EC_{50} value of 0.26 μM (Table 2). HMBC correlations are shown in Figure 1.

TABLE 1. ^{13}C-nmr Assignments for *Buchenavia* Alkaloids.[a]

Carbon	Compound		
	1	**2**	**3**
C-2	163.0	166.2	166.3
C-3	105.8	106.4	107.0
C-4	182.4	184.1	184.0
C-4a	104.6	105.5	106.4
C-5	161.2	159.2	164.3
C-6	100.4	95.1	97.2
C-7	164.7	163.3	165.0
C-8	105.2	107.8	102.2
C-8a	153.9	160.8	165.0
C-1′	131.68[b]	132.3	132.4
C-2′, -6′	126.0 ×2	127.6 ×2	127.8 ×2
C-3′, -5′	129.2 ×2	130.4 ×2	130.5 ×2
C-4′	131.74[b]	133.4	133.4
C-2″	61.9	54.0	63.8 br
C-3″	31.6	28.7	30.4
C-4″	24.0	23.3[b]	23.9
C-4″	25.4	24.1[b]	24.9
C-6″	55.9	47.3	58.6
N-Me	44.0	—	42.4
O-Me	—	—	57.6

[a]125 MHz, $CDCl_3$ for **1**, CD_3OD for **2** and **3**. All assignments based on HMBC and HMQC experiments.
[b]Could not be distinguished.

The more polar alkaloid fractions had less bioactivity but were pursued to obtain congeners of the lead compound for structure-activity comparisons. A Si gel partition system was useful in partially purifying those polar alkaloids with weak activity. High-speed countercurrent chromatography resolved two alkaloids from the nonalkaloidal material. *N,O*-Didemethylbuchenavianine [**2**] was isolated in pure form and characterized. It was less potent in the anti-HIV screen than **1** (Table 2). Further countercurrent partitioning led to the isolation of buchenavianine [**3**] which also had relatively low potency (Table 2). 2D Nmr experiments with **2** and **3** also resulted in revised carbon

TABLE 2. Anti-HIV Activity of *Buchenavia* Alkaloids.

Compound	EC_{50} (μM)[a]	IC_{50} (μM)[b]
O-Demethylbuchenavianine [**1**]	0.26	0.66
N,O-Didemethylbuchenavianine [**2**]	142	142
Buchenavianine [**3**]	n.d.[c]	5.7

[a]HIV-inhibitory activity.
[b]Cytotoxicity to CEM-SS host cells.
[c]Less than 50% protection obtained.

assignments (Table 1). The remaining minor alkaloids were not characterized due to their lack of biological activity. The use of centrifugal partition chromatography (cpc) to purify these alkaloids proved critical, since all but the least polar were poorly separated via normal or reversed-phase chromatography. In contrast, cpc was most effective in separating the most polar of the alkaloids, a task for which normal-phase chromatography was unsuitable.

The aqueous extract of wood from the same collection of *B. capitata* also showed cytoprotective activity against HIV. However, a preliminary test of the aqueous extract for alkaloids was negative, and the distribution of activity in a solid-phase extraction protocol indicated that the anti-HIV principle was anionic and of high mol wt. Polymeric phenolics (tannins) may be the active agents (3); some support for this was obtained by passing the aqueous material through a polyamide column (4); no anti-HIV active fractions could be eluted from the column with MeOH. The aqueous phase from the basic partition of the organic extract of the leaves of the plant was found to contain similar bioactive material, also presumably tannins.

CONFIRMATORY ANTI-HIV EVALUATIONS.—The purified alkaloids were evaluated in further detail using a battery of correlative anti-HIV assays (5). Multiple aliquots from single wells of 96-well microtiter plates were analyzed in parallel for cell viability, reverse transcriptase (RT) levels, DNA content, viral core protein p24 levels, and syncytium formation (see Experimental). In this multiparameter assay system, **1** produced only partial cytoprotection against HIV, and the cytotoxicity of the compound against the CEM-SS lymphoblastoid host cells used in the assay resulted in a low therapeutic index (Figure 2).

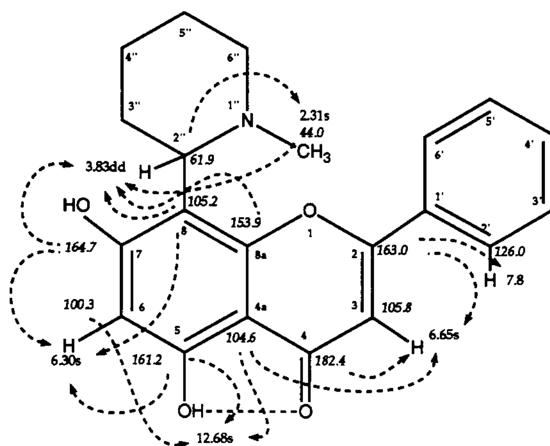


FIGURE 1. Selected HMBC correlations for **1**.

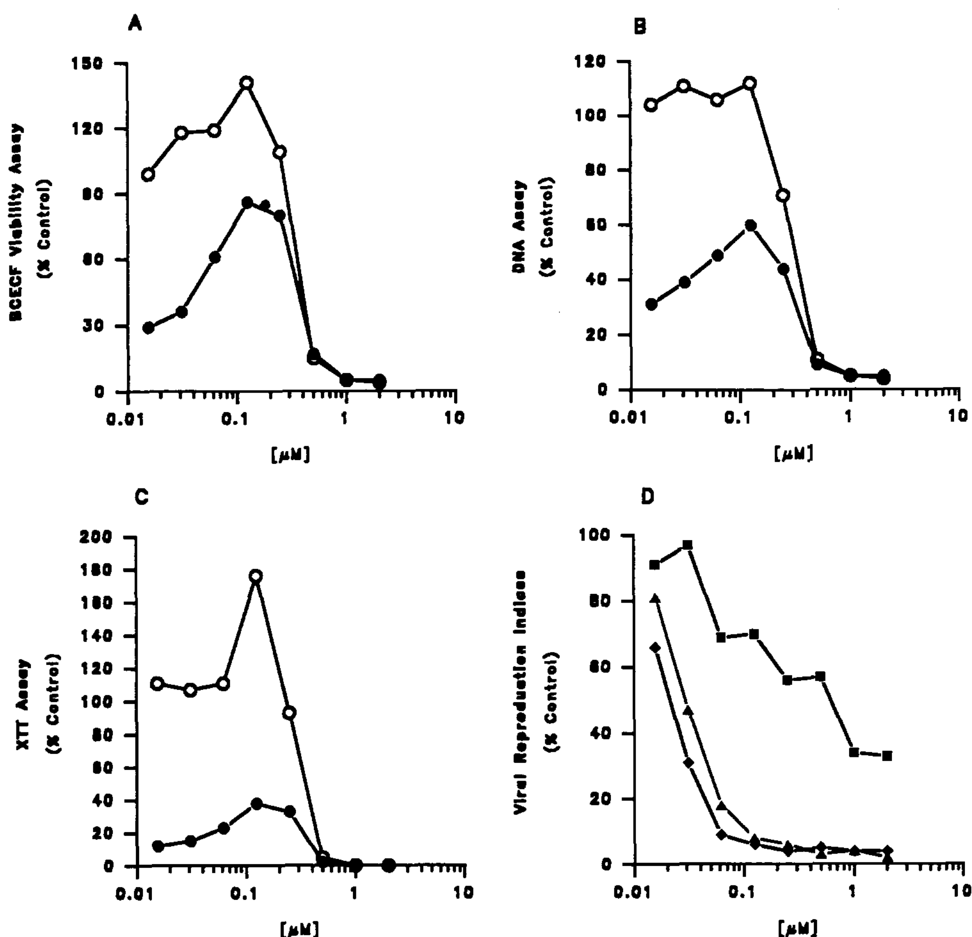


FIGURE 2. Anti-HIV activity of **1**. Graphs A, B and C show the effects of a range of concentrations of **1** upon uninfected CEM-SS cells (○) and upon CEM-SS cells infected with HIV-1 (●), as determined after 6 days in culture. These graphs depict the relative numbers of viable CEM-SS cells, as assessed (see Experimental) by a metabolic fluorescence assay (BCECF, graph A), a DNA assay (DAPI, graph B) and a tetrazolium metabolic assay (XTT, graph C). Graph D shows the effects of a range of concentration of **1** upon indices of infectious virus or viral replication in HIV-infected CEM-SS cells; these indices include viral reverse transcriptase activity (▲), production of viral core protein p24 (◆) and syncytium-forming units (■). In graphs A, B, and C, the data points are represented as the percent of the respective uninfected, non-drug-treated control values. In graph D, the data points are represented as the percent of the respective infected, non-drug-treated control values.

Compound **1** was tested also in other biological assays (data not shown). It did not protect MT-2 lymphocytes from the HIV-1 strain RF nor did it have cytoprotective activity against HIV-2 strain NIH-DZ; it did not protect the human lymphoblastic cell line C-8166 from HIV-1, and, likewise, it had no activity against chronic HIV infection in the H-9/IIIB cell system. It failed to inhibit purified HIV-1 or HIV-2 reverse transcriptases and HIV-1 protease, indicating that the apparent inhibition of viral replication was not due to a direct effect on these key viral enzymes. The crude base fraction of *B. capitata* appeared to inhibit HIV-1 and HIV-2 RT (data not shown), but the inactivity of the purified compounds would seem to indicate that the crude extract exhibited a nonspecific effect due to polyphenolics in the extract (3,4).

FURTHER CYTOTOXICITY EVALUATION.—The marked cytotoxic activity of **1** to CEM-SS cells used in the anti-HIV assay led us to test the alkaloids in the NCI in vitro human-disease-oriented tumor panel. Details of the rationale, experimental protocol, and current data analysis methodologies for this screen are provided elsewhere (6–9). The cytotoxicity data were generally unremarkable. Compounds **2** and **3** were only weakly cytotoxic, with **1** being somewhat more potent, but producing only a modest degree of differential cytotoxicity. Some of the most sensitive (melanoma) lines gave LC_{50} values in the 10^{-5}–10^{-6} M range; however, there was no definitive pattern of subpanel specificity by currently applied statistical criteria (8). A COMPARE analysis (9) of the cytotoxicity profile of **1** showed weak correlations to screening profiles produced by several known DNA-damaging agents, such as actinomycin D, mithramycin, and adriamycin. However, testing of **1** in the Biochemical Induction Assay (BIA) (10) and Inducible DNA Repair Assay (SOS) (11) protocols, which are designed to detect DNA-damaging compounds, revealed no activity in **1** at 1 mg/ml.

CONCLUSIONS.—These studies have established that *O*-demethylbuchenavianine [**1**] is the principal anti-HIV constituent in organic solvent extracts from *B. capitata*. However, at sub-cytotoxic concentrations, the compound produces only partial protection against the cytopathic effects of HIV in cultured CEM-SS target cells. Additional evaluation of the compound in the NCI in vitro antitumor screen revealed only a very modest degree of differential cytotoxicity against the diverse human tumor cell lines in the screening panel. Thus, neither the anti-HIV activity nor the cytotoxicity of *O*-demethylbuchenaviamine provides compelling support for drug development. However, the compound does represent a new anti-HIV chemotype that may merit further synthetic and/or semisynthetic efforts aimed at structure-activity optimization.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Cpc was carried out with a Sanki model NMF with 12 analytical cartridges (volume 300 ml). The solvent system used was $CHCl_3$-MeOH-0.5% aqueous HCl (5:5:3), in the descending mode, at 400 rpm and 500 psi. Nmr experiments were conducted on a Varian VXR-500S in $CDCl_3$ and CD_3OD at 500 MHz and 125 MHz for ^1H nmr and ^{13}C nmr, respectively. The HMBC spectra were optimized to detect 8 Hz couplings.

PLANT MATERIAL.—Leaf material of *B. capitata* was collected in the Dominican Republic (voucher J. Pimental *et al.* 993, deposited at the Botany Department, Museum of Natural History, Smithsonian Institution). Organic extracts of other plant parts from this collection were not active in the anti-HIV screen. The aqueous extract of the wood showed anti-HIV activity.

ISOLATION.—The isolation process was guided throughout by the results of the anti-HIV assay (1). The plant material was ground and extracted by the standard NCI plant protocol (12); 495 g of plant material yielded 53.09 g of organic extract. Organic extract (5.05 g) was made basic with 30% NH_4OH and sufficient MeOH to solubilize the material, diluted with 1 liter of H_2O, and extracted with EtOAc (3 × 1 liter). Evaporation of the EtOAc layer gave 1.07 g of the organic base fraction.

The organic base fraction was chromatographed on flash Si gel (3.5 × 21 cm) in $CHCl_3$-MeOH (100:0, 98:2, 97:3, 95:5, 90:10, 80:20, 50:50, and 0:100) (500 ml each). Compound **1** was located in the fractions with anti-HIV bioactivity and identified by ^1H and ^{13}C nmr. Fractions eluting later had minimal bioactivity but also reacted positively with Dragendorff's reagent.

Fractions containing **1** were chromatographed further on a second flash Si gel column (1.5 × 19 cm) with a step gradient of hexane-Me_2CO (8:2, 7:3, 1:1) to give 34.4 mg of impure **1**, which was finally purified by cpc in the system noted above to give 20.8 mg of the HCl salt.

A more polar fraction (130 mg) from the initial column was flash-chromatographed using a partition chromatography system on Si gel [$CHCl_3$-MeOH-HOAc-H_2O (9:1:0.05:0.05)] to give 16.3 mg of **2** contaminated by a yellow pigment. Cpc (system as noted) yielded 6.0 mg of pure **2**.

The partition chromatography also yielded 66.5 mg of a third alkaloidal fraction. Cpc of this material yielded 8.4 mg of pure **3**.

O-Demethylbuchenavianine [**1**].—[α]D −31° (MeOH, *c* = 0.54); uv (MeOH) λ max 210 nm (log ε = 4.5), 273 (4.34), 326 (3.85); (+NH_4OH) 213, 275, 346; (+HCl) 214, 269, 308 sh; ir (film from $CHCl_3$)

ν max 1657, 1652, 1615, 1588, 1428, 1368, 1296, 1250, 1186, 1111, 923, 852, 755 cm^{-1}; ^1H nmr ($CDCl_3$, 500 MHz) δ 12.68 (s, 1H, 5-OH), 7.83 (m, 2H, H-2′, -6′), 7.56 (m, 3H, H-3′, -4′, -5′), 6.66 (s, 1H, H-3), 6.30 (s, 1H, H-6), 3.83 (dd, J = 3.7, 11.5, 1H, H-2″), 3.20 (d, J = 11.8, 1H, H_{eq}-6″), 2.31 (s, 3H, N-Me), 2.26 (dd, J = 2.8, 12.1, 1H, H_{ax}-6″), 1.93 (m, 1H, H-3″), 1.87 (m, 1H, H_{eq}-4″), 1.65–1.83 (m, 3H, H-3″, H-5a″, -5b″), 1.45 (m, 1H, H_{ax}-4″); ^{13}C nmr see Table 1; eims m/z (%) $[M]^+$ 351 (87), 336 (9), 322 (9), 309 (17), 308 (9), 294 (27), 280 (42), 261 (46), 98 (100), 70 (64); hreims (calibration perfluorokerosene, PFK) m/z 351.1444 (calcd for $C_{21}H_{21}NO_4$, 351.1471), 309.1025 (calcd for $C_{18}H_{15}NO_4$, 309.1001), 280.0635 (calcd for $C_{16}H_{10}NO_4$, 280.0610).

N,O-*Didemethylbuchenavianine* [**2**].—[α]D −2° (MeOH, c = 0.4); uv (MeOH) λ max 213 nm (log ε = 4.4), 268 (4.3), 333 sh (3.8); (+NH_4OH) 212 nm, 271, 325 sh; (+HCl) 214 nm, 267, 290 sh, 330 sh; ir (film from $CHCl_3$) ν max 3415, 2948, 1651, 1621, 1588, 1449, 1352, 1243, 1178, 1108, 847, 754 cm^{-1}; ^1H nmr (CD_3OD, 500 MHz) δ 8.00 (m, 2H, H-2′, -6′), 7.58 (m, 3H, H-3′, -4′, -5′), 6.82 (s, 1H, H-3), 6.62 (s, 1H, H-6), 4.70 (dd, 1H, H-2″), 3.47 (m, 1H, H-6″), 3.15 (m, 1H, H-6″), 2.35 (m, 1H, H-3″), 1.70–2.04 (m, 5H, H-3″, -4″, -5″); ^{13}C nmr see Table 1; eims m/z (%) $[M]^+$ 337 (61), 320 (30), 308 (54), 295 (7), 292 (14), 281 (45), 268 (79), 267 (31), 254 (100), 253 (4), 98 (3), 97 (14), 84 (9), 83 (43); hreims (calibration PFK) m/z 337.1309 (calcd for $C_{20}H_{19}NO_4$, 337.1314).

Buchenavianine [**3**].—[α]D −8° (MeOH, c = 0.76); uv (MeOH) λ max 214 nm (log ε = 4.5), 243 (3.93), 272 (4.13), 336; (+NH_4OH) 215 nm, 240, 277, 361; (+HCl) 214 nm, 247, 269, 336; ir ν max 2939, 1651, 1608, 1589, 1492, 1450, 1427, 1371, 1332, 1251, 1209, 1166, 1122, 1111, 1032, 1011, 959, 920, 850, 752 cm^{-1}; ^1H nmr (CD_3OD, 500 MHz) δ 8.01 (m, 2H, H-2′, -6′), 7.62 (m, 3H, H-3′, -4′, -5′), 6.89 (s, 1H, H-3), 6.68 (s, 1H, H-6), 4.08 (s, 3H, OMe), 2.74 (s, 3H, N-Me); nOe OMe to H-6 10%. ^{13}C nmr see Table 1; eims m/z (%) 365 (49), 350 (17), 336 (3), 323 (15), 308 (34), 294 (27), 294 (27), 280 (16), 279 (50), 267 (4), 98 (100); hreims m/z 365.1649 (calcd for $C_{22}H_{23}NO_4$, 365.1627), 350.1378 (calcd for $C_{21}H_{20}NO_4$, 350.1392), 98.0976 (calcd for $C_6H_{12}N$, 98.0970).

BIOLOGICAL TESTING.—The XTT-tetrazolium anti-HIV primary screening assay was performed on crude extracts, chromatographic fractions, and pure compounds as previously described (1). Confirmatory anti-HIV, RT, viral protein p24, syncytium-forming assays, and range of activity experiments were performed as described in detail elsewhere (5). In the cytoprotection studies, cellular viability was estimated by two different types of metabolic assay, as well as by a correlative DNA assay. The metabolic assays included an adaptation (5) of the original XTT-tetrazolium method (1), and a method using the fluorescent probe, 2′-7′-biscarboxyethyl-5-(6)-carboxyfluoresceinacetoxymethyl ester (BCECF) (5). The latter compound is a nonfluorescent molecule which readily enters viable cells where it is hydrolyzed by cellular esterases to a fluorescent molecule. Total cellular DNA content was measured (5) with the dye 2-diamidinophenylindole (DAPI), which fluoresces when intercalated at A-T specific sites in chromatin.

HIV-protease inhibition assays were performed as published (13). Human tumor cell line cytotoxicity testing and data analyses were performed as described elsewhere (6–9). SOS tests were performed as described (10,11).

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