Expedited Articles

Anti-HIV Michellamines from *Ancistrocladus korupensis*[1]

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Here we report details of the isolation and determination of the absolute configurations and comparative anti-HIV activities of novel, atropisomeric naphthylisoquinoline alkaloid dimers, michellamines A, B, and C, from a newly described species of *Ancistrocladus* from the Korup rainforest of Cameroon. We further provide a more extensive analysis of the range of anti-HIV activity of michellamine B, the most potent and abundant member of the series. Michellamine B inhibited HIV-induced cell killing and viral replication in a variety of human cell lines, as well as in cultures of human peripheral blood leukocytes and monocytes. Michellamine B was active against a panel of biologically diverse laboratory and clinical strains of HIV-1, including the AZT-resistant strain G910-6 and the pyridinone-resistant strain A17; the compound also inhibited several strains of HIV-2.

Introduction

The human immunodeficiency virus (HIV), which is generally recognized as the principal causative factor of the worldwide pandemic of acquired immune deficiency syndrome (AIDS), presents an increasingly urgent and challenging target for new drug discovery and development. The propensity for the rapid emergence, in the clinic as well as in the laboratory, of HIV strains highly resistant to available drugs suggests that effective and durable chemotherapy of this disease will require the use of innovative combinations of drugs having diverse mechanisms and ranges of anti-HIV activity. The present report stems from our continuing efforts to exploit a high-flux *in vitro* anti-HIV activity screen for the discovery of new drug development leads from plants and other natural sources.[2]

In a preliminary communication,[3] the novel structures, relative stereochemistry, and anti-HIV activity of a pair of atropisomeric naphthylisoquinoline alkaloid "dimers", michellamines A and B, were described. The compounds were isolated from a Central African plant tentatively identified as *Ancistrocladus abbreviatus*. Subsequently, detailed taxonomic investigations[4] revealed subtle but distinctive differences in the source plant compared to authentic specimens of *A. abbreviatus* and all other known *Ancistrocladus* species. Moreover, our follow-up chemical and bioactivity analyses yielded no evidence of michellamines in any except the original source *Ancistrocladus* species.[4] Thus, it became apparent that the true michellamine-containing plant, now officially named *Ancistrocladus korupensis*,[4] was a species previously unknown to science.

While the *relative* stereostructures of michellamines A and B were firmly established by extensive ^1H and ^{13}C NMR experiments, the arbitrarily depicted configurations in our initial communication[3] were based upon the available literature precedents.[5] Therefore, we have been pursuing the elucidation of the *absolute* configurations of the michellamines by application of a procedure first developed for such configurational analyses of "monomeric" naphthylisoquinolines.[6] A ruthenium-mediated degradation of michellamine B into simple-to-analyze chiral amino acids, alanine and 3-aminobutyric acid, allowed determination of the absolute configurations at C1 and C3 of both halves of the "dimer". We recently communicated those results,[7] which definitively established the absolute configuration of michellamine B as **2**, that is, having a 1*R*,3*R*,5*M*,1‴*R*,3‴*R*,5‴*P* configuration (i.e., opposite to the original, arbitrarily assigned *S*-configurations at C-1, C-3, C-1‴, and C-3‴).

Ongoing studies of this lead in our as well as other laboratories are focusing upon further elucidation of the antiviral activity and mechanism of action. A recent series of studies of michellamine B has implicated a multilevel mode of action including both an inhibition of the viral reverse transcriptase as well as blockage of cellular fusion and syncytium formation (J. McMahon et al., LDDRD, DTP, NCI, unpublished). Compound supply has been problematic, as the only known source at the present time is the rare *A. korupensis* liana, which thus far has been found only in a limited region of the Cameroonian rainforest.[4] Therefore, there has been increasing emphasis

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upon the identification of alternative plant sources of the compounds and the cultivation of *A. korupensis*, as well as further focus upon total synthesis and/or semisynthesis of michellamines and potentially improved (e.g., more potent/less toxic) derivatives thereof. In complement to and in support of these initiatives, we report here (a) further details of the isolation of michellamines A and B from *A. korupensis*; (b) elucidation of a third related dimer, michellamine C; (c) the absolute configurations of the michellamines; (d) a comparison of the *in vitro* anti-HIV activities of michellamines A, B and C; and (e) a more extended evaluation of michellamine B against a wide diversity of HIV strains in a variety of target cell types.

Chemistry

Isolation of Michellamines from *A. korupensis*. We have used a number of methods to isolate and purify the michellamines from extracts of *A. korupensis*. These include solvent–solvent partitioning, centrifugal partition chromatography, gel permeation chromatography, and HPLC with a variety of bonded phases. A typical procedure is as follows:

Approximately $^1/_2$ kg of the air-dried plant material consisting of leaves, stems, and twigs is first ground to a coarse powder and extracted with MeOH–CH_2Cl_2 (1:1), followed by a second extraction with methanol. These initial crude organic extracts typically amount to a total of approximately 8–10% of the mass of the original dried plant material. A portion of this crude extract is suspended in 5% aqueous HCl and extracted with $CHCl_3$. The aqueous layer is then made basic with concentrated NH_4OH to a pH of 10–11 and extracted with $CHCl_3$–MeOH (4:1) and subsequently with MeOH–$CHCl_3$ (1:1), followed by removal of the solvent. The extract is then dissolved in the lower phase of a biphasic solvent system ($CHCl_3$–MeOH–0.5% aqueous HBr, 5:5:3) and injected onto a Sanki CPC operating in the descending mode. The effluent is monitored at 270 nm. The final peak to elute in descending mode contains the HBr salts of both michellamines A and B. The mixture is further separated with amino-bonded phase HPLC using $CHCl_3$–MeOH/0.075% $(NH_4)_2CO_3$ (43:7) as the solvent. Using this general procedure, the overall yield of michellamines from crude organic extract is about 0.5–2% for michellamine A and 2–10% for michellamine B.

A more specific example is described in detail in the Experimental Section. In that example, a trace amount of yet a third atropisomeric dimer, now named michellamine C, was isolated.

Michellamine C. High-resolution FAB mass spectroscopy analysis of this minor compound gave *m/z* 757.3489 ($M^+ + H$), corresponding to a molecular formula of $C_{46}H_{48}N_2O_8$, the same as michellamine A and B. The 500-MHz ^{1}H NMR spectrum of michellamine C resembled that of michellamine A, in that it contained signals for only half of the protons indicated by HRFABMS. However, the chemical shifts very closely matched those for the "half" of michellamine B disparate from the equivalent "halves" of michellamine A.

The doublet at δ 1.68 was coupled to the quartet at δ 4.73, and the doublet at δ 1.30 showed coupling to the proton at δ 3.65, which was further coupled to the pair of protons at δ 2.62 and 2.35. The aromatic protons were assigned as in michellamines A and B. The ^{13}C NMR spectrum of michellamine C revealed 23 carbon signals, 11 of which were protonated, as determined by DEPT experiments. The chemical shifts of all carbons were very similar to those of half of michellamine B. This, in combination with the ^{1}H NMR data, indicated that michellamine C was a symmetrical, dimeric naphthylisoquinoline alkaloid isomeric to michellamine A. Thus, from the NMR data (Table 1), michellamine C appeared to have opposite relative configurations from michellamine A about the C-5/C-8′ and C-5‴/C-8″ bonds. Variable-temperature NMR experiments failed to show evidence of spontaneous interconversion.

Table 1. NMR Data for Michellamine C[a]

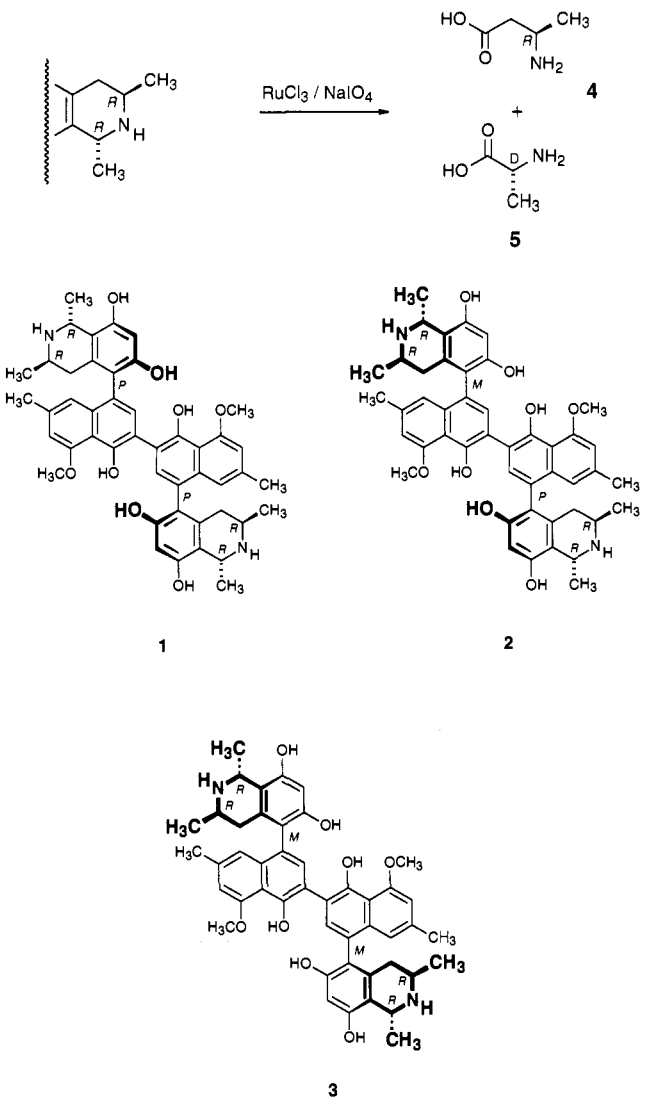
carbon no.	δ (no. attached H)	^{1}H δ (multiplicity) J (Hz)
1/1‴	49.1(1)	4.73 q 7.0
3/3‴	45.0(1)	3.65 ddq 11.5, 5.0, 6.5
4/4‴	34.3(2)	(a) 2.62 dd 17.5, 11.5; (e) 2.35 dd 17.5, 5.0
4a/4a‴	133.5(0)	
5/5‴	120.3(0)	
6/6‴	156.6(0)	
7/7‴	102.0(1)	6.43 s
8/8‴	155.6(0)	
8a/8a‴	113.9(0)	
1′/1″	119.3(1)	6.84 s
2′/2″	137.4(0)	
3′/3″	108.0(1)	
4′/4″	158.0(0)	
4a′/4a″	115.2(0)	
5′/5″	152.2(0)	
6′/6″	119.0(0)	
7′/7″	135.3(1)	7.28 s
8′/8″	124.3(0)	
8′a/8a″	136.4(0)	
OMe/OMe	57.0(3)	4.09 s
Me-3/Me-3‴	19.6(3)	1.30 d 6.5
Me-1/Me-1‴	18.6(3)	1.68 d 7.0
Me-2/Me-2″	2.22(3)	2.36 s

[a] ^{13}C (125 MHz) and ^{1}H (500 MHz) NMR spectra of the free base were recorded in methanol-d_4. The designations a and e for H-4 refer to axial and equatorial, respectively. The number of attached H was determined from DEPT experiments.

Interconversions of Michellamines A, B, and C. A ^{1}H NMR analysis of michellamine A in base (1 mg in 1 mL of MeOH-d_4 and 0.5 mL of 0.5 M NaOD/D_2O) indicated a slow conversion of the compound to a mixture of michellamines A, B, and C (~3:3:1) over a period of 7 days. Likewise, michellamine B under identical conditions was converted to a similar mixture. HPLC analyses confirmed these results. From these observations, additional quantities of michellamine C for further analysis and biological testing were obtained by base-catalyzed conversion from michellamine B, followed by HPLC purification (see the Experimental Section).

Absolute Configurations of Michellamines A and C. As described in our earlier communication,[7] the assignment of the *R*-configurations of both amino acids derived from C-1 and C-3 of michellamine B in the ruthenium-catalyzed oxidation procedure unambiguously established the *R*-configurations at both of these centers in each of the diastereomorphous molecular "halves" of the dimer. Given the relative configuration at the two stereogenic biaryl axes (at C-5/C-8′ and C-5‴/C-8″) of the two molecular moieties) vs the stereocenters, as deduced from the original NOE experiments,[3] the complete absolute stereostructure (**2**) of michellamine B therefore was established as 1*R*,3*R*,5*R* (or *M*), 1‴*R*,3‴*R*,5‴*S* (or *P*). Michellamine A was likewise subjected to the same degradation analysis, again giving stereochemically pure D-alanine (**4**) and (*R*)-3-aminobutyric acid (**5**) (Scheme 1;

Scheme 1



also see the Experimental Section). By this, the absolute stereostructure of michellamine A was established similarly as 1, with 1*R*,3*R*,5*S* (or *P*), 1‴*R*,3‴*R*,5‴*S* (or *P*) configuration. Since the NMR data had indicated opposite relative configurations at the C-5/C-8′ (and C-5‴/C-8‴) linkages compared with the stereocenters in michellamine A versus C, and due to the fact that michellamine C was obtained from michellamine A or B by base-catalyzed atropisomerization (see above), the absolute stereostructure 3 of michellamine C was deduced as 1*R*,3*R*,5*R* (i.e., *M*), 1‴*R*,3‴*R*,5‴*R* (i.e., *M*). Thus, differing from most other Ancistrocladaceae alkaloids,[5] the michellamines have an oxygen function at C-6, but *R*-, not *S*-configuration at C-3.

Biology

Comparison of Anti-HIV Activities of Michellamines. The acetate salts of michellamines A, B, and C were evaluated side-by-side for their anticytopathic effects against HIV-1 (RF strain) and HIV-2 (CBL-20 strain) in CEM-SS cells using the XTT assay (see the Experimental Section). All three compounds were fully protective against both HIV-1 and HIV-2; Table 2 shows the respective EC_{50} values (μM). A separate study of michellamines A and B (HBr salts) against a different strain of HIV-2 (NIH-DZ) revealed a somewhat inferior activity

Table 2. Comparison of Anticytopathic Activity[a] of Michellamines A, B, and C against HIV-1 (RF Strain) and HIV-2 (CBL-20 Strain) in CEM-SS Human Lymphoblastoid Target Cells

michellamine	HIV-1 EC_{50} (μM)	HIV-2 EC_{50} (μM)
A	10	10
B	10	2
C	13	2

[a] The activity was measured with the XTT assay (see the Experimental Section); repeated tests of michellamines A and B using this assay typically yielded mean EC_{50}'s having standard errors of less than 20% of the respective means. Michellamine C was tested only once in this study, due to the very limited amounts of compound.

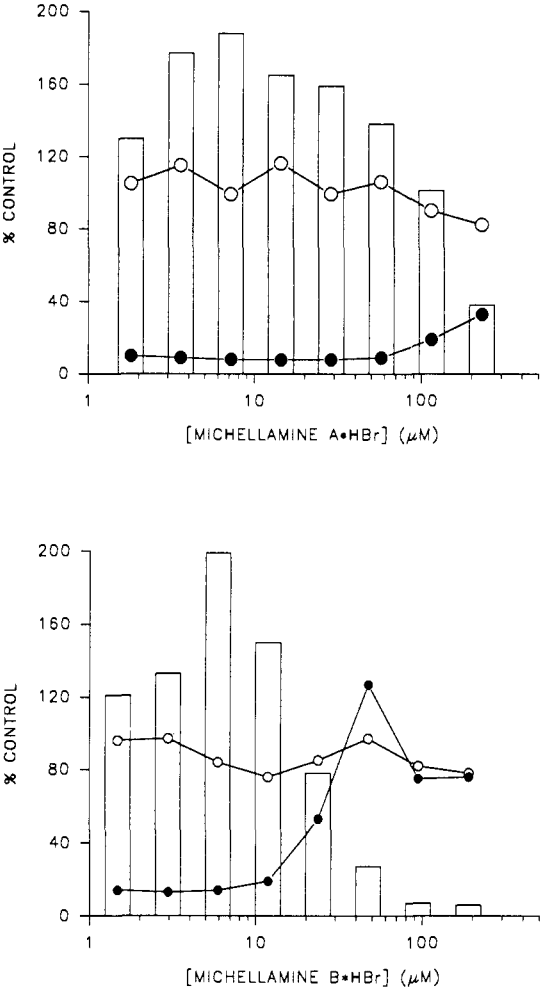


Figure 1. The upper composite graph shows the effects of a range of concentrations of michellamine A (HBr salt) upon uninfected MT-2 cells (○) and upon MT-2 cells infected with the NIH-DZ strain of HIV-2 (●) as determined after 6 days in culture using the XTT assay.[16] The open bars show the corresponding supernatant reverse transcriptase activities. The lower composite graph shows the results of the same experiment performed with michellamine B (HBr salt). In both graphs, all data points are represented graphically as the percent of the respective controls.

of michellamine A compared to B (Figure 1); concentrations of michellamine A as high as 250 μM gave only partial protection (20–40%) of MT-2 cells against HIV-2 (NIH-DZ). Similar results were obtained when the compounds were compared in their free base forms (data not shown). Michellamine C was not included in either of the latter studies due to limited compound supply.

Range of Anti-HIV Activity of Michellamine B. The relatively favorable supply of michellamine B permitted a broader evaluation of this compound against different strains of HIV in a variety of target cells. A

Table 3. Anti-HIV Activity (EC_{50}'s, μM) of Michellamine B against Different HIV Strains

HIV-1 Laboratory Strains (vs CEM-SS cells)		HIV-2 Strains (vs CEM-SS cells)	
IIIB	1	ROD	18
RF	12	MS	7
LAV	4	NIH-DZ	5
		CBL-20	2
HIV-1 Clinical Isolates (vs CEM-SS cells)			
promonocytropic		lymphotropic	
214	10	205	11
SKI	12	G	3
TP1	6	MCK	3
MN	13	PM16	12
Drug Resistant HIV-1 (vs MT-2 cells)			
A17	9		
G9106	5		

Table 4. Anti-HIV Activity (EC_{50}'s) and Cytotoxicity (IC_{50}'s) of Michellamine B in Different Host Cell Types

cell lines/virus strains	EC_{50} (μM)	IC_{50} (μM)
CEM-SS/HIV-1 (RF)	13	120
CEM-SS/HIV-1 (NIH-DZ)	1	42
MT-2/HIV-1 (RF)	40	200
MT-2/HIV-2 (NIH-DZ)	6	100
C-344/HIV-1 (RF)	39	160
C8166/HIV-1 (RF)	88	>240
LDV-7/HIV-1 (RF)	13	100
174×CEM/HIV-1(III_B)	23	>125
AA5/HIV-1(III_B)	33	>125
fresh cell preparations/virus strains		
PBLs/HIV-1 (WEJO)	5	>240
PMCs/HIV-1 (Ba-L)	10	>240

remarkable diversity of strains of both HIV-1 and HIV-2 were inhibited in a wide spectrum of cell types (Tables 3 and 4). The activity was measured either as an inhibition of viral cell killing (XTT assay) or in noncytopathic infections (e.g., in PBL's and PMC's) as a decrease in viral replicative indices (p24; RT). The various T-cell lines were protected at concentrations of michellamine B ranging from approximately 1 to 90 μM. Furthermore, the compound completely protected CEM-SS cells from 11 different strains of HIV-1 (EC_{50}'s 1–13 μM). Also noteworthy was the complete protection of MT-2 cells from drug-resistant HIV-1, including the AZT-resistant G910-6 strain[8] and the pyridinone-resistant A17 strain.[9] The cytotoxic concentrations (IC_{50}'s) varied among the different cell lines, ranging from about 40 to >240 μM. Michellamine B did not alter HIV replication in the chronically infected (HIV-III_B) H9 cell line (data not shown).

Combination Anti-HIV Activity of Michellamine B with AZT. Michellamine B was tested for anti-HIV activity in combination with AZT. Five concentrations of michellamine B were tested in all combinations with eight concentrations of AZT. The antiviral assays and data analyses were performed as described in the Experimental Section. Effects of the drug combinations were calculated on the basis of the activity of the two compounds when tested alone. The calculated additive antiviral protection was subtracted from the experimentally determined antiviral activity at each combination concentration resulting in a positive value (synergy), a negative value (antagonism), or zero (additivity). Data were analyzed by the most stringent statistical means by assuming the compounds inhibited HIV replication by action at the same site (mutually exclusive). The results of these assays demonstrated that the combined antiviral activity was equal to that predicted based on additivity. Slight synergistic interactions were detected at some combination concentrations; no antagonism was detected with any combination concentrations. The three-dimensional, Prichard–Shipman antiviral synergy plot[10] of the data demonstrated a predominantly flat plane of activity, consistent with an additive interaction with AZT (data not shown).

Conclusion and Perspective

Chemically, the michellamines are unique not only due to their unprecedented "dimeric" naphthylisoquinoline alkaloid structure, but also to the exceptionally high polarity resulting from their three phenolic OH and one secondary amino groups per constituent monomer "half". They are also unusual in having the rare C-5/C-8′ coupling between the two ring systems in the monomer units; this linkage is otherwise presently known only in the "monomeric" naphthylisoquinoline alkaloid, ancistrobrevine B.[6]

With respect to biological activity, the michellamines represent a novel new antiviral chemotype. These compounds, either as the free bases or the water-soluble acid salts, are capable of essentially complete inhibition of the replication and cytopathic effects of HIV in human lymphoblastoid host cells. Furthermore, the anti-HIV activity encompasses an exceptionally broad range of strains of both HIV-1 and HIV-2, the two major families of human HIV presently known. Preliminary pharmacokinetic studies[11] in rodents and dogs indicated that *in vivo* concentrations of michellamine B in excess of the *in vitro* effective anti-HIV concentrations could be achieved nonlethally by iv bolus administration.

Based substantially on the above findings, the U.S. National Cancer Institute has committed michellamine B to INDA-directed preclinical development. Ongoing toxicology and pharmacology studies by NCI are focusing upon 4–6-week continuous drug infusions in anticipation of use of similar clinical drug administration protocols in early phase I clinical investigations. Continuous, 14-day infusions of the drug in dogs have been accomplished at nontoxic dose levels, yielding sustained *in vivo* blood concentrations comparable to the *in vitro* effective concentrations (J. Tomaszewski et al., DTP, NCI, unpublished).

Experimental Section

Isolation of Michellamines. The original samples of the plant in which anti-HIV activity was detected were collected on March 25, 1987, in the Korup National Park, west of Mundemba Town in Cameroon's Southwest Province (5°01′N; 8°51′E; 60 m elevation above sea level). Subsequent recollections in that same general area provided additional material for isolation efforts.

The leaves and stems of dried *Ancistrocladus korupensis* (449 g) were ground in a Wiley mill and extracted with MeOH–CH_2Cl_2 (1:1) in a Kimax percolator. The ground material was allowed to steep in the solvent overnight. After removal of the MeOH–CH_2Cl_2 solution, the plant material was extracted further with MeOH. The solvent was removed from the combined extracts by filtration and evaporated at reduced pressure to give 36.62 g of crude organic extract.

A portion (2.107 g) of this extract was suspended/dissolved in 330 mL of 5% aqueous HCl and extracted with four 100-mL aliquots of $CHCl_3$. The extracts were combined and the solvent removed at reduced pressure to give 0.657 g of extract, which was inactive against HIV. The remaining aqueous layer was treated

with concentrated NH_4OH until the pH of the solution was between 10 and 11. The basic aqueous phase was extracted with five 100-mL aliquots of $CHCl_3$–MeOH (4:1). The extracts were combined, and the solvent was removed at reduced pressure to give 0.3195 g of extract. The anti-HIV screen revealed the material to be active. The remaining aqueous layer was extracted further with three 100-mL aliquots of MeOH–$CHCl_3$ (1:1). The extracts were combined, and the solvent was removed at reduced pressure to give 0.2534 g of extract, which was also active.

NMR and TLC analyses of the two active extracts indicated that both samples contained the same compounds. An aliquot of extract from the $CHCl_3$–MeOH (4:1) procedure (264.1 mg) was dissolved in a small amount of the lower phase of a MeOH–$CHCl_3$–0.5% aqueous HBr (5:5:3) biphasic system. This sample was injected into a Sanki centrifugal partition chromatograph (CPC) operating in the descending mode with 12 analytical cartridges (400 rpm, 3.0 mL/min). The effluent was monitored at 270 nm using a Linear UV/vis 200 monitor. Eight fractions (A–H) were collected while the instrument was operating in the descending mode, and a ninth fraction (I) was collected when the instrument operation was reversed to the ascending mode. Fractions A, C, E, and F were inactive. Fractions B (14.4 mg), D (9.0 mg), and I (31.8 mg) showed relatively little activity. The majority of the anti-HIV activity was found in fractions G (72.7 mg) and H (45.4 mg).

Fraction H (45.4 mg) was dissolved in 500 μL of $CHCl_3$–MeOH (43:7) and injected onto a Waters DeltaPrep HPLC using a Rainin Dynamax-NH_2 column (2.1 × 25 cm, equipped with a guard column). The sample was eluted with $CHCl_3$–MeOH/0.075% $(NH_4)_2CO_3$ (43:7) at a flow rate of 13 mL/min and monitored at 260 nm. Six fractions were collected and tested for HIV-inhibitory activity. Fractions 1 (retention time = 10 min, 1.1 mg), 2 (retention time = 19 min, 4.3 mg), 4 (retention time = 26 min, 4.6 mg), and 5 (retention time = 31.5 min, 1.0 mg) were found to be inactive. Fraction 3 proved to be michellamine A (retention time = 22 min, 10 mg), and fraction 6 proved to be michellamine B (retention time = 36 min, 14.4 mg).

Fraction G was treated in a similar manner, except that it was dissolved in a 1.5 mL of solvent and placed on the column in three 500-μL injections. From this sample, 5.0 mg of michellamine A and 39.5 mg of michellamine B were obtained; 3.0 mg of an inactive, unidentified compound were also collected.

The sample obtained from the MeOH–$CHCl_3$ (1:1) extract described above (251 mg) was placed on the Sanki CPC under the same conditions as the 4:1 extract. In this case, seven fractions were collected while the instrument was operated in the descending mode (A–G) and one fraction collected during the ascending mode (H).

Fractions A, B, C, D, and H were inactive in the anti-HIV assay, while E, F, and G were all active. Preparative HPLC of fraction E (72.4 mg), under the identical conditions as above, afforded 0.8 mg of michellamine A, 44.5 mg of michellamine B, and 6.3 mg of an inactive tetrahydroisoquinoline compound. Fraction F (18.8 mg) afforded 2.8 mg of michellamine A and 8.1 mg of michellamine B along with two minor inactive compounds (<2 mg). Fraction G (18.2 mg) afforded 10.1 mg of michellamine A and 2.1 mg of an unknown inactive substance. A third, minor compound, michellamine C (HRFABMS 757.3489 obsd, 757.3487 calcd); $[\alpha]_D = -16.8°$ (*c* 0.14, MeOH) was isolated on one occasion as a shoulder on the michellamine B chromatographic peak. It was not encountered in subsequent, more rapidly processed material. The overall yield of the active fractions from starting crude extract was 1.4% for michellamine A and 5.0% for michellamine B.

Preparation of Michellamine C from Michellamine B. Michellamine B (82 mg, free base) was dissolved in 10 mL of MeOH in a 50-mL round-bottom flask; 120 μL of 0.5 M KOH solution were then added. The mixture, which was allowed to stir at room temperature for 6 days, turned progressively darker brown. Thin-layer chromatography (EM Science amino-bonded phase) with 7:3 CH_2Cl_2–MeOH w/0.1% $(NH_4)_2CO_3$ was used to monitor the progress of the reactions (michellamine A, $R_f = 0.63$; michellamine B, $R_f = 0.55$; michellamine C, $R_f = 0.37$).

The dark brown reaction mixture was concentrated and redissolved in 5 mL of MeOH. Insoluble residues were removed using a 0.25-μm HPLC filter, and the filtrate was subjected to HPLC purification on an amino-bonded phase column (Rainin, 2.1 × 25 cm) using CH_2Cl_2–MeOH w/0.1% $(NH_4)_2CO_3$ (22:3), eluting at 12 mL/min and monitoring at 254 nm. Repeated HPLC purification using either a 2.1- × 25-cm or a 1- × 25-cm amino column provided pure michellamine A, B, and C in 8.7, 15.6, and 3.0 mg quantities, respectively.

Isolated michellamine A and B showed identical ^1H and ^{13}C NMR spectra as authentic samples.

Absolute Configuration of Michellamine A. Michellamine A (10.3 mg, 13.7 μmol) was added to 4 mL of a mixture of MeCN/CCl_4/aqueous phosphate buffer (pH = 6) (1:1:2) under stirring at room temperature, followed by $RuCl_3 \cdot 3H_2O$ (0.1 mg) and $NaIO_4$ (100 mg). After 2.5 h, the aqueous phase was separated and lyophilized. The residue was extracted with dry MeOH. The resulting solution, which contained the product amino acids, was saturated with gaseous HCl at 0 °C and stirred at room temperature overnight to yield the corresponding methyl esters. After evaporation of the solvent, the residue was suspended in dry CH_2Cl_2 (1 mL). Subsequently, NEt_3 (0.2 mL) and (*R*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid chloride (*R*-MTPA-Cl) (46 μmol, prepared from the corresponding (*S*)-acid) were added to give the Mosher derivatives of the esters after stirring at room temperature for 1 h. These were analyzed by FID-GC and found to be derived from D-alanine ($t_R = 18.1$ min) and (*R*)-α-aminobutyric acid ($t_R = 24.5$ min). These assignments were confirmed by co-injection with the corresponding racemic as well as enantiomerically pure amino acid standards. The FID-GC data were obtained on an OV1-column (0.33 mm × 30 m); temperature program: from 140 °C (1 min) to 155 °C (1 min) at 1 °C/min, from 155 °C (1 min) to 160 °C (1 min) at 0.5 °C/min.

Cells and Viruses. Unless indicated otherwise, the tissue culture lines used in these studies were obtained from the NIAID AIDS Research and Reference Reagent Program, NIH. The CEM-SS lymphocytic cell line was obtained from Peter Nara (NCI). All cells were maintained in RPMI 1640 medium without phenol red and supplemented with 5% fetal bovine serum, 2 mM L-glutamine, and 50 μg of gentamicin/mL (complete medium). All incubations were routinely performed at 37 °C in an atmosphere containing 5% CO_2.

Whole blood was obtained from the American Red Cross (Baltimore, MD). Peripheral blood lymphocytes and macrophages were isolated following Ficoll-Hypaque centrifugation as described.[12] The HIV-1 isolates used were the common laboratory strains IIIB, LAV, RF, and MN as well as a panel of HIV-1 clinical isolates obtained from patients at Duke University Medical Center, Durham, NC. The biological and biochemical properties of these isolates have been described previously.[12,13] HIV-2 isolate ROD (no. I-532) was obtained from Luc Montagnier (Pasteur Institue, Paris, France). AZT-resistant HIV-1 G910-6[8] was obtained from the National Institute of Allergy and Infectious Diseases, and pyridinone-resistant HIV-1 A17[9] was obtained from Emilio Emini (Merck, West Point, PA).

Anti-HIV Assays. Cells were seeded into each well of a 96-well microtiter plate at a density of 5×10^3 cells/well. The cells were infected with virus at a multiplicity of infection (MOI) previously determined to give complete cell killing (e.g., CEM-SS, MT2, C-344, C-8166, AA5) or maximal levels of virus production (e.g., PBL's, macrophages) at 6 days postinfection (MOI of 0.01–0.05). The panel of virus isolates was pretitered to induce equivalent infections (based on cell killing or virus production) in these assays. Serial, half-log aqueous dilutions of the alkaloids (as their acetate salts) were added to appropriate wells in triplicate to evaluate their ability to inhibit HIV infection. Controls for each assay included drug colorimetric control wells (drug only), drug cytotoxicity control wells (cells plus drug), virus control wells (cells plus virus), and cell viability control wells (cells only). AZT and ddC were run in parallel as positive control drugs. Following 6 days of incubation at 37 °C, the viability of the cells in each well was determined spectrophotometrically using the metabolic reduction of XTT[14,15] to a soluble, colored formazan. In noncytopathic infections, the results were analyzed by quantitation of either RT activity or p24 core protein in cell-free supernatants derived from each well of the microtiter plate; the anti-HIV assays on freshly isolated target cells were performed as previously described.[15] Supernatant RT and p24 levels were measured as described.[15,16] Antiviral and cytotoxicity data are

reported as the concentrations of compound required for 50% inhibition of virus-induced cell killing or virus production (EC_{50}) or 50% decrease in viable cell number (IC_{50}), respectively.

Effect on Chronically Infected Cells. Chronically infected cells (H9-III_B) were obtained from the outgrowth of HIV-infected, virus-producing cells following acute infection of H9 cells. These cells were cultured in the presence of serial, one-log dilutions of michellamine B (acetate salt). Cell-free supernatant samples were obtained on a daily basis and analyzed for virus content by RT assay, p24 ELISA, and CEM-SS infectivity assay described above.

Combination Studies with AZT. An analysis of the antiviral activity of a range of combination concentrations of michellamine B was performed the same as described elsewhere.[14] The statistical evaluations of the data were performed according to Prichard and Shipman.[10]

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