Notes

Crambescidin 826 and Dehydrocrambine A: New Polycyclic Guanidine Alkaloids from the Marine Sponge *Monanchora* sp. that Inhibit HIV-1 Fusion

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Two new polycyclic guanidine alkaloids, crambescidin 826 (**1**) and dehydrocrambine A (**2**), and the known compounds crambescidin 800 (**3**) and fromiamycalin (**4**) were isolated from the marine sponge *Monanchora* sp. The structures of **1** and **2** were elucidated by 2D NMR and mass spectrometry, and relative stereochemistry was established by analysis of coupling constants and ROESY spectra. The pentacyclic guanidine alkaloids **1**, **3**, and **4** inhibit HIV-1 envelope-mediated fusion in vitro with IC_{50}'s of 1−3 μM, while compound **2**, a tricyclic guanidine alkaloid, showed weaker inhibition, with an IC_{50} of ∼35 μM.

Marine sponges are known to be prolific producers of a wealth of diverse secondary metabolites.[1] One unique class of sponge-derived metabolites includes compounds that feature complex polycyclic guanidine alkaloid cores, most of which exhibit diverse biological activities. Members of this class of marine natural products have been isolated predominantly from sponges belonging to the orders Poecilosclerida and Axinellida, with collections of the genera *Batzella* and *Crambe* (poecilosclerids) and *Ptilocaulis* (axinellid) frequently identified.[2]

The first report of a sponge-derived polycyclic guanidine alkaloid described the complex pentacyclic alkaloid ptilomycalin A, isolated from both a Caribbean sample of *Ptilocaulis spiculifer* and a Red Sea collection of *Hemimycale* sp.[3] Extending from the pentacyclic core of ptilomycalin is a fatty acid coupled to a spermidine moiety. Shortly thereafter, the structures of other pentacyclic guanidine alkaloids, isolated from Mediterranean collections of *Crambe crambe*, were elucidated and included crambescidins 800 (**3**), 816, 830, and 844 (where the numbers designate the molecular weight of the alkaloids)[4] and isocrambescidin 800.[5,6] Pentacyclic guanidine alkaloids have also been reported to occur in Brazilian specimens of *Monanchora unguiculata*, which yielded crambescidins 359 and 431, both of which lack the aliphatic chain and spermidine groups;[7] Caribbean collections of *Batzella* spp., which yielded ptilomycalin and crambescidin 800;[8] and curiously, in the starfish *Celerina heffernani* and *Fromia monilis* collected in New Caledonia, which yielded the novel compounds celeromycalin and fromiamycalin (**4**).[9] In addition to pentacyclic alkaloids, the poecilosclerids and axinellids have also yielded metabolites containing bicyclic or tricyclic guanidines, or mixtures of the two. Several examples include crambines A (**5**) and B[10] from *Crambe crambe*, mirabilins A−G[11] from *Arenochalina mirabilis* and *Clathria* sp., and the more complex structures of batzelladines A−I,[8,12] most of which feature either one bicyclic and one tricyclic ring system or two tricyclic guanidine units tethered by aliphatic esters of differing lengths.

Diverse biological activities have been reported for these polycyclic guanidines including cytotoxicity toward the cancer cell lines L1210[4,5] and HCT-16[6] and antifungal, antimicrobial, and antiviral activities.[3,4] As part of an effort to identify small molecules that disrupt protein−protein interactions involved in HIV-1 entry, we investigated a crude extract of the marine sponge *Monanchora* sp. (collection C11883, Natural Products Repository, NCI) because the ethyl acetate-soluble fraction showed significant activity in blocking HIV-1 envelope-mediated cell fusion in vitro.[13] In this paper, we report the isolation and structure elucidation of two new polycyclic guanidine alkaloids, **1** and **2**, from the marine sponge *Monanchora* sp., along with the known compounds crambescidin 800 (**3**) and fromiamycalin (**4**), and report on their inhibition of HIV-1 envelope-mediated fusion in vitro.

As a result of the observed fusion-blocking activity of a crude organic extract of *Monanchora* sp., the ethyl acetate soluble, and active, fraction of that extract was subjected to bioassay-guided fractionation using Sephadex LH20, followed by reversed-phase HPLC (see below). In addition to the known alkaloids crambescidin 800 (**3**) and fromiamycalin (**4**), HPLC purification led to the isolation of two new alkaloids, **1** and **2**, that were eluted from a C18 column with 56% and 65% aqueous acetonitrile, respectively. HRFABMS of the earlier eluting compound, **1**, gave a molecular ion at *m/z* 827.6389 [$(M + H)^+$], indicating a molecular formula of $C_{47}H_{83}O_6N_6$ (calcd *m/z* for $C_{47}H_{83}O_6N_6$ 827.6374) that requires 10 degrees of unsaturation. A cursory examination of the ^1H and ^{13}C NMR spectra suggested that compound **1** was a crambescidin analogue, while analysis of the ^1H,^{13}C correlation spectra, in particular, established the presence of ^{13}C resonances attributable to one alkene (δ_C 131.1 and 134.2), one imine (δ_C 150.4), one ester carbonyl (δ_C 170.3), and two amide carbonyls (δ_C 176.1 and 176.3). Compound **1** must therefore contain five rings to satisfy the required degree of unsaturation.

A direct comparison of the NMR spectra of **1** with those of crambescidin 800 (**3**) recorded under identical conditions confirmed the presence of the same pentacyclic ring system in both molecules and suggested that the 26 amu difference

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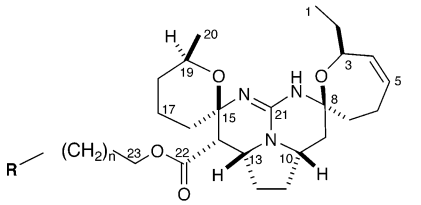

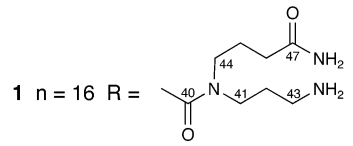

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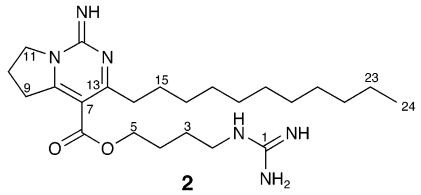

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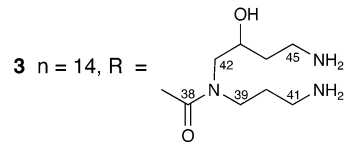

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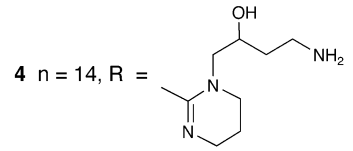

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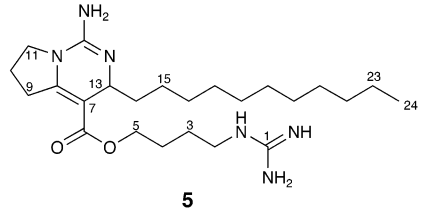

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between the two molecules was at least partially attributed to differences in the spermidine unit. Resonances corresponding to C-42 to C-45 of crambescidin 800 were absent from the spectra for compound **1**, revealing the absence of the C-43 hydroxyl group, and were replaced with resonances with δ_H and δ_C values similar to those for C-39−C-41 of **1**. Because initial HMBC spectra failed to show correlations that were useful for assignment of the structure of the fatty acid and spermidine units in **1**, we recorded a long-range HSQC spectrum with a reduced spectral width of 60 ppm with the carbon carrier set to 160 ppm and T_d set to half a dwell time. In addition to minimization of baseline offset and curvature, setting the initial sampling delay to half a dwell time produces processed spectra having respective zero- and first-order phase corrections of 90° and −180° in F1.[14] Because the 90°/−180° phase correction gives rise to spectra where cross-peaks located within the spectral window are positive while those lying outside the spectral window (i.e., folded peaks) are negative, incorporation of the half dwell delay also facilitates simple interpretation of spectra. In our case, this long-range HSQC spectrum clearly showed the presence of two amide carbonyls at δ_C 176.1 and 176.3 (see Supporting Information). Thus, analysis of the HMBC spectra together with the long-range HSQC spectrum revealed correlations

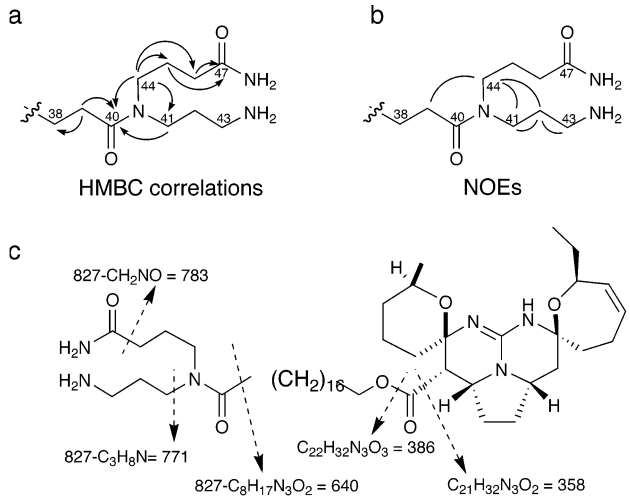

Figure 1. (a) $^{2,3}J_{C,H}$ correlations, (b) NOEs, and (c) fragmentation by mass spectrometry for crambescidin 826 (**1**).

from H-39, H-41, and H-44 to the carbon at δ_C 176.3, establishing it as C-40, and correlations from H-45 and H-46 to δ_C 176.1 established the presence of an amide carbonyl at C-47 within the spermidine unit. These and other $^{2,3}J_{CH}$ correlations illustrated in Figure 1a and summarized in Table 1 were consistent with this structure.

To further validate the presence of the spermidine group in compound **1**, tandem mass spectrometry (FABMS/CID (collision-induced dissociation)/MS/MS) was carried out on *m/z* 827 and gave fragment ions at *m/z* 783, 771, 640, 386, and 358 as shown in Figure 1c. High-resolution FAB mass measurement of the ion at *m/z* 386 gave a value of 386.2448, compatible with a formula of $C_{22}H_{32}N_3O_3$, providing additional support for the presence of the pentacyclic guanidine ring system.

To establish relative stereochemistry, NOESY and ROESY spectra were recorded and analyzed. Observed dipolar couplings between H-9$_\beta$ and H-10, H-10 and H-13, and H-13 and H-14 established a *syn* relationship between H-10, H-13, and H-14, thereby confirming **1** to be a crambescidin rather than an *iso*-crambescidin analogue, and couplings between the more distal protons H$_3$-1 and H-10 and H$_3$-1 and H-19 were consistent with this relative stereochemistry.[3,5] In addition, correlations between H-39 and H-44, H-41 and H-44, and H-42 and H-44 (Figure 1b) corroborate the structure of the sperimidine unit. Compound **1** was given the trivial name crambescidin 826.

The molecular formula of the later eluting compound, **2**, was established by high-resolution FABMS to be $C_{24}H_{42}O_2N_6$ ([M + H]$^+$ calcd, 447.3448; found, 447.3428), indicating seven degrees of unsaturation. Inspection of the ^{1}H and ^{13}C NMR data for **2** (Table 2) showed the presence of signals with chemical shifts similar to those of the aliphatic chains of crambines[10] and crambescins.[15] In particular, the spin system of C-1−C-6 containing the guanidine and ester groups could be assigned on the basis of $^{2,3}J_{C,H}$ HSQC correlations from H-2 to C-1 of the guanidine unit, which was also correlated to C-3 and C-4. The ester carbonyl carbon C-6 could be assigned by long-range HSQC correlations from H-5, which was also correlated to C-3 and C-4. Similarly, resonances for the first and last three carbons of the aliphatic chain were confirmed from COSY and long-range HSQC spectra (Table 2). Other key carbon signals observed in the long-range HSQC spectra of **2**, however, were not in agreement with those of the dihydropyrimidine unit present in other related molecules such as crambine A (**5**). Specifically, no methine

Table 1. ^{1}H and ^{13}C NMR Data (in ppm) for Crambescidin 826 (**1**) in CD_3OD[a]

position	δ_C	m	δ_H	m (*J* Hz)	$^{2,3}J_{C,H}$ correlations[b]	NOEs[c]
1	10.7	CH_3	0.85	t (7.2)	2, 3	H-3, H-10, H-19
2	30.2	CH_2	1.46 α, 1.56 *β*	m		H-3, H-4
3	72.2	CH	4.40	m	1, 2	H-1, H-2*β*, H-4
4	134.2	CH	5.51	br d (11.0)	3, 6	H-2*β*, H-3, H-5
5	131.1	CH	5.71	ddt (9.6, 7.2, 2)		H-4, H-6α, H-6*β*
6	24.3	CH_2	2.16 α	m		H-5, H-7
			2.42 *β*			H-5, H-7
7	38.0	CH_2	1.98 α	m	5, 6, 8, 9	H-6, H-9α
			2.40 *β*		5, 6, 8, 9	H-6
8	85.3	C				
9	37.7	CH_2	1.42 α	m	7, 10, 11	H-7α, H-7*β*, H-9*β*
			2.64 *β*	dd (13.0, 4.5)	7, 8	H-7α, H-9α, H-10
10	55.4	CH	4.05	m	9	H-9*β*, H-11*β*, H-13
11	31.3	CH_2	1.60 α	m	10	H-12α,*β*
			2.29 *β*			H-10, H-11α
12	27.4	CH_2	1.82 α	m		
			2.34 *β*			H-13
13	54.0	CH	4.37	m	10, 11	H-10, H-12*β*, H-14
14	50.7	CH	3.07	d (4.8)	13, 15, 22	H-12α, H-13, H-16, H-17
15	82.3	C				
16	32.7	CH_2	1.69	m	17	
17	19.2	CH_2	1.83	m		H-14, H18*β*, H-19
18	32.7	CH_2	1.60 α, 1.70 *β*	m		H-17, H-19, H-20
19	68.3	CH	3.83	m	18, 20	H-1, H-17, H-18*β*, H-20
20	21.7	CH_3	1.09	d (6)	19, 18	H-18*β*,[d] H-19
21	150.4	C				
22	170.3	C				
23	66.4	CH_2	4.14	t (6.5)	22, 24, 25	H-24
24	26.7	CH_2	1.37, 1.66	m	23, 25	H-23
25−38	28.2−32.0	CH_2	1.24−1.35	m		
39	33.7	CH_2	2.40	m	38, 40	H-44
40	176.3	C				
41	43.0	CH_2	3.46	m	40, 42, 43, 44	H-42, H-44
42	26.7	CH_2	1.91	m	41, 43	H-41, H-43, H-44
43	38.1	CH_2	2.88	m	41, 42	H-41, H-42
44	46.0	CH_2	3.37	m	40, 41, 45, 46	H-39, H-41, H-42, H-45, H-46
45	26.7	CH_2	1.63, 1.68	m	44, 46, 47	H-44, H-46
46	33.9	CH_2	2.39	m	44, 45, 47	H-44, H-45
47	176.1	C				

[a] Recorded at 500.13 MHz, 300 K, and referenced to CD_3OD at 3.30 ppm. [b] Observed in HMBC or long-range HSQC spectra. [c] Observed in NOESY or ROESY spectra. [d] Could be interchanged with H-16.

Table 2. ^{1}H and ^{13}C NMR Data (in ppm) for Dehydrocrambine A (**2**) in CD_3OD[a]

position	δ_C	m	δ_H	m (*J* Hz)	$^{2,3}J_{C,H}$ HSQC
1	158.9	C			
2	41.9	CH_2	3.23	t (6.6)	1, 3, 4
3	26.2	CH_2	1.74	m	
4	26.4	CH_2	1.83	m	
5	67.0	CH_2	4.39	t (6.6)	3, 4, 6
6	165.0	C			
7	112.5	C			
8	168.3	C			
9	34.8	CH_2	3.56	t (7.5)	8, 7, 10, 11
			3.30	m	
10	20.8	CH_2	2.38	m	
			2.00	m	
11	53.6	CH_2	4.25	m	8, 9, 10
12	152.0	C			
13	180.7	C			
14	37.7	CH_2	3.07	t (7.5)	7, 13, 15, 16
15	27.0	CH_2	1.82	m	
16	30.1	CH_2	1.29	m	
22	32.7	CH_2	1.38	m	
23	23.3	CH_2	1.30	m	
24	14.0	CH_3	0.90	t (6.6)	22, 23

[a] Recorded at 500.13 MHz, 300 K, and referenced to CD_3OD at 3.30 ppm.

signal was observed, and the $^{2,3}J_{C,H}$ HSQC spectra showed the presence of quaternary carbons at 112.5, 168.3, and 180.7 ppm. The downfield signal at 180.7 ppm, along with the seven degrees of unsaturation suggested by the molecular formula, thus suggested that compound **2** was a dehydro-analogue of related bicyclic guanidines. $^{2,3}J_{C,H}$ HSQC correlations from the methylene protons at δ_H 3.07 (H_2-14) to carbons at δ_C 112.5 and 180.7, as well as C-15 and C-16, supported this notion; correlations from H_2-9 to C-7 and C-8 and from H_2-11 to C-8 and C-12 were consistent only with the structure **2**. The bicyclic guanidine portion of **2** is thus similar to the tricyclic guanidine structure found in dehydrobatzelladine C,[7] and the carbon chemical shifts for the guanidine ring systems of each are in close agreement.

Confirmation of the length of the alkyl chains at C-7 and C-13 was also provided by tandem mass spectrometry (FABMS/CID/MS) on *m/z* 447, which gave fragment ions at *m/z* 334, 316, and 114. High-resolution FABMS on each of these ions indicated molecular formulas of $C_{19}H_{32}N_3O_2$ (calcd for $C_{19}H_{32}N_3O_2$, 334.2501; found, 334.2494), $C_{19}H_{30}N_3O$ (calcd for $C_{19}H_{30}N_3O$, 316.2398; found, 316.2389), and $C_5H_{12}N_3$ (calcd for $C_5H_{12}N_3$, 114.1031; found, 114.1031), respectively (Figure 2). The fragment peak at *m/z* 334 ([M − $C_5H_{11}N_3$]$^+$) indicated loss of a molecular ion of 113 Da, which corresponds to 4-guanidino-1-butene resulting from a McLafferty rearrangement, consistent with the observed intense peak at *m/z* 114 ($C_5H_{12}N_3$). Compound **2** was given the trivial name dehydrocrambine A.

A variety of unrelated biological activities have been reported for polycyclic guanidine alkaloids in general. These include cytotoxicity toward the cancer cell lines L1210 and HCT-116, potent calcium antagonist activity in a neuroblastoma hybrid cell line, and antiviral activity

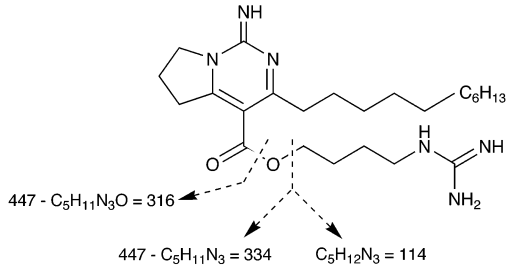

Figure 2. Fragmentation by mass spectrometry of dehydrocrambine A (**2**).

toward herpes simplex and human immunodeficiency viruses. Indeed, many of the crambescidins were discovered by the Rinehart group because of their anti-HSV activity.[4,5] In this study, we tested compounds **1**−**4** in a quantitative HIV-1 fusion assay for their ability to inhibit HIV-1 envelope-mediated fusion. Compounds were tested against a T-cell tropic strain (HIV-1 LAV and coreceptor CXCR4) and a macrophage tropic strain (HIV-1 SF-162 and coreceptor CCR5). The pentacyclic guanidine alkaloids crambescidins 826 (**1**) and 800 (**3**) and fromiamycalin (**4**) all inhibit HIV-1 envelope-mediated fusion with IC_{50}'s of ~1−3 μM for both strains, whereas dehydrocrambine A (**2**) showed weaker inhibition, with an IC_{50} value of ~35 μM. The events that mediate HIV-1 membrane fusion include binding of the viral envelope protein gp120 to the cellular receptor CD4, followed by gp120 binding to the appropriate coreceptor via a newly exposed epitope created by CD4 binding. Previous studies by the Smith−Kline group showed several of the batzelladines to block gp120-CD4 interactions.[8] Consistent with those observations, we observed increases in the extent of HIV-1 fusion in time-delayed experiments with **3** where crambescidin 800 was added to the mixture of HIV-1 envelope-expressing cells (effectors) and CXCR4 or CCR5 expressing cells (targets) after 15, 30, 45, 60, and 90 min. When fusion proceeded for 60 min or more prior to addition of **3**, no protection was observed (CAB and LC, unpublished data).

In this study, the new alkaloids **1** and **2** were not tested for cytotoxicity toward mammalian cells over an extended period of time.[16] However, other groups have reported high toxicity toward target cells for polycyclic guanidines such as batzellins A−E,[8] crambescidin 800,[4] fromiamycalin, and ptilocmycalin A.[9] We anticipate that crambescidin 826, in particular, would exhibit comparable cytotoxicity, thereby limiting its utility, at least as an oral fusion inhibitor. Nonetheless, molecules that can block gp120-CD4 interactions have important potential as HIV entry inhibitors. Concerted efforts by several groups have yielded engineered proteins or peptides, all based on the CD4 receptor, that block gp120-CD4 interactions and HIV-1 Env-mediated fusion.[17−20] On the other hand, there are few examples of small molecules that specifically block gp120-CD4 binding.[21] Thus, this class of polycyclic guanidines may aid in the rational design or optimization of small molecule HIV-1 fusion inhibitors.

Experimental Section

General Experimental Procedures. UV and IR spectra were obtained with a Beckman DU-600 spectrophotometer and a Bio-Rad FTS-45 FT-IR spectrophotometer. NMR experiments were recorded on a Bruker DMX500 spectrometer equipped with an *xyz*-shielded gradient triple resonance probe. FABMS was performed on a JEOL-SX102 and CIMS on a Finnigan 4500. Reversed-phase column chromatography was carried out using C-18 packing material from Baker, and reversed-phase HPLC was performed on a GBC system equipped with a photodiode array detector (210 nm detection) using a Waters μBondapak C_{18} column (7.8 × 300 mm) run with a flow rate of 3 mL/min.

NMR. The long-range HSQC spectrum was recorded at 500 MHz using an HSQC sequence with a half dwell time delay introduced before initial sampling. Thus, the initial delay was set to

$$t_1(0) = 1/(2SW) - 4\tau_{90(C)}/\pi - \tau_{180(H)}$$

where $\tau_{90(C)}$ and $\tau_{180(H)}$ are durations of the 90° carbon and 180° proton pulses, respectively. A total of 1024 complex points in F2 and 256 complex points in F1 were collected, with respective spectral widths of 10.0 and 60.0 ppm. The carbon carrier was set to 160 ppm to center on the amide carbonyl region, and the delay Δ was set to 31.25 ms. Spectra were processed using NMRPipe with zero-filling once in each dimension to yield spectra of 2048 × 512 data points.

Sponge Material. *Monanchora* sp. (1.2 kg collection, C011883) was collected in August 1993 in Palau, immediately frozen, and shipped to NCI for processing (Frederick, MD). Following aqueous extraction of the frozen sponge at 4 °C, the material was lyophylized and extracted successively with CH_2Cl_2−MeOH (1:1) and MeOH. The combined organic extracts were evaporated in vacuo and stored at −30 °C. Voucher specimens were deposited at the Queensland Museum, Brisbane, and the Smithsonian Sorting Center, Suitland, MD.

Extraction and Isolation. Crude organic extract (2.0 g) was partitioned between EtOAc and H_2O to give 0.92 and 0.54 g of organic and aqueous extracts, respectively. Both the EtOAc- and water-soluble extracts inhibited HIV Env-mediated fusion with concentrations less than 20 μg/mL. The organic extract was applied to a Sephadex LH-20 column and eluted with 1:2 $CHCl_3$−MeOH to give 10 major fractions. Active fractions F-3, F-4, and F-6 were combined and further purified by flash column chromatography using a C_{18} column equilibrated in water eluting with increasing concentrations of MeOH (10% increases) to give 12 fractions. Final purification of the active components was accomplished by isocratic RP-HPLC eluting with 56% aqueous CH_3CN in 0.05% TFA to give compounds **1** (t_R 27.3 min; 5.6 mg; 0.00047% of wet weight), **3** (t_R 20.0 min; 24 mg; 0.002% wet weight), and **4** (t_R 36.0 min; 6 mg; 0.0005% wet weight). The water-soluble extract was chromatographed similarly over a C-18 column eluting with increasing amounts of MeOH to give eight major fractions. Active fraction F-2 was further purified by RP-HPLC eluting with 65% aqueous CH_3CN in 0.05% TFA to yield a pure sample of **2** (t_R 24.5 min; 1.0 mg; 0.00008% of wet weight).

Crambescidin 826 (1): glassy solid; $[\alpha]_D^{20}$ −7.7° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 207 (4.04), 274 (3.24); IR (film) ν_{max} 3360−3228, 2927, 2850, 1729, 1651, 1610, 1442 cm^{-1}; ^1H and ^{13}C NMR data for **1** in CD_3OD, see Table 1; HRFABMS (NBA) *m/z* 827.6389 $[M + H]^+$, calcd for $C_{47}H_{83}O_6N_6$, 827.6374.

DehydrocrambineA (2): brown gum; $[\alpha]_D^{20}$ −12.1° (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (3.83), 259 (3.53), 289 (3.18); IR (film) ν_{max} 3335−3193, 2932, 1675, 1650, 1443, 1202, 1133 cm^{-1}; ^1H and ^{13}C NMR data for **2** in CD_3OD, see Table 2; HRFABMS (NBA) *m/z* 447.3428 $[M + H]^+$, calcd for $C_{24}H_{43}O_2N_6$, 447.3447.

Crambescidin 800 (3): glassy solid; UV, IR, ^1H and ^{13}C NMR spectra data were in agreement with published data.[4]

Fromiamycalin (4): glassy solid; UV, IR, ^1H and ^{13}C NMR spectra data were in agreement with published data.[9]

Bioassays. Cell fusion assays were conducted as previously described using soluble CD4 and recombinant vaccinia viruses expressing genes for HIV-1 envelopes derived from LAV or SF-162, CXCR4 or CCR5, T7 polymerase, and β-galactosidase (β-Gal). (See ref 22 for a detailed description of assay conditions and sources of all reagents.) Briefly, BS-C-1 and NIH-3T3 cells were used for effector and target cell populations, respectively. Effector cells were co-infected with vaccinia viruses encoding HIV-1 Env and β-Gal, and target cells were co-infected with CXCR4 or CCR5 and T7 polymerase-encoding viruses at an MOI of 5. For inhibition studies, extracts or pure compounds

were added to an appropriate volume of DMEM 2.5% and PBS (to yield identical buffer compositions in 100 μL per well of a 96-well plate), followed by addition of 1×10^5 effector cells in 50 μL of media and 1×10^5 target cells in 50 μL of media containing soluble CD4. Following a 2.5 h incubation at 37 °C, % fusion was measured as a function of β-Gal activity on the substrate chlorophenol-red-β-D-galactopyranoside (A_{570}, Molecular Devices 96-well spectrophotometer).

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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