Novel Alkaloids from the Sponge *Batzella* sp.: Inhibitors of HIV gp120-Human CD4 Binding

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The Caribbean sponge *Batzella* sp. contains a number of guanidine alkaloids, two of which, batzelladines A (**1**) and B (**2**), inhibit the binding of HIVgp-120 to CD4 and are therefore potential inhibitors of HIV. In addition to the known metabolites ptilomycalin A (**6**), ptilocaulin (**7**), crambescin A (**8**), crambescidin 800 (**9**), and crambescidin 816 (**10**), *Batzella* sp. contains five new alkaloids, batzelladines A–E (**1**–**5**), the structures of which were elucidated by interpretation of spectral data and chemical degradation.

Introduction

The hallmark of acquired immunodeficiency syndrome (AIDS) is a progressive decline in the number of $CD4^+$ cells leading to the demise of immune function and consequent susceptibility to opportunistic infections, the primary cause of morbidity. Human immunodeficiency virus type 1 (HIV-1) is the primary causative agent of AIDS.[1] This highly variable virus shows selective affinity for $CD4^+$ cells which is determined by recognition of the HIV envelope glycoprotein gp120 by the CD4 cell-surface receptor protein. The manner in which HIV infection leads to the slow but progressive decline in $CD4^+$ cells has not been established. Therefore, numerous approaches are being investigated in search of potential anti-HIV drugs and vaccines.

It has been shown that an agent which blocks HIV replication *in vitro*, such as azidothymidine (AZT), also provides a therapeutic benefit *in vivo*. Thus, antagonism of HIV replication is a therapeutic strategy for AIDS. One approach to inhibit HIV replication is to antagonize the interaction of the virus with the host cell. The process of viral infection is initiated by the attachment of HIV to cells through a high affinity interaction between viral envelope and the CD4 receptor on the surface of a T cell. The HIV-envelope gp160 glycoprotein consists of the CD4 binding gp120 and fusogenic gp41 domains. Subsequent to gp120–CD4 binding, the virus enters the host cell by fusion of the viral and cellular membranes facilitated by the gp41 domain. The binding of gp120 to CD4 is well characterized; the primary determinants of the gp120 binding site on CD4 have been localized and the molecular structure of the first two domains of CD4 have been described.[2–5] Compounds that prevent the binding of gp120 to CD4 may inhibit the entry of HIV into the cell and thus inhibit HIV replication, because HIV cannot replicate without using the biosynthetic apparatus of the host cell.

In the last two decades, the search for marine-derived natural products with useful pharmacological properties has been extended to all oceans of the world; the results of this search have been reported in numerous reviews.[6] Sponges have contributed significantly to the array of new structural types derived from marine organisms. Although the pharmacopeia contains a large number of alkaloids isolated from terrestrial plants, relatively few alkaloids have been found from marine sources and so far none have found a clinical use.[7]

Results and Discussion

As part of our continuing search for biologically active natural products with potential utility in the treatment of AIDS,[8] we established a biochemical screen for inhibition of gp120–CD4 binding. This assay measures the binding of soluble CD4 (sCD4) to gp120 that is immobilized on a microtiter plate. Binding of sCD4 was quantified with an anti-CD4 antibody conjugated to horseradish peroxidase. A total of 5000 extracts of terrestrial plants and marine organisms were screened with this plate-based assay. Several inhibitors were found initially, but the majority of these were found to be active only in the presence of ambient light. We subsequently discovered that this affect was due to the presence of photoactive compounds such as porphyrins in the active extracts. Only one example, the methanolic

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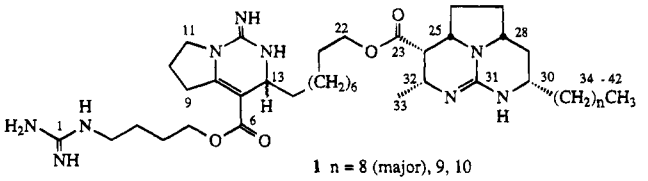
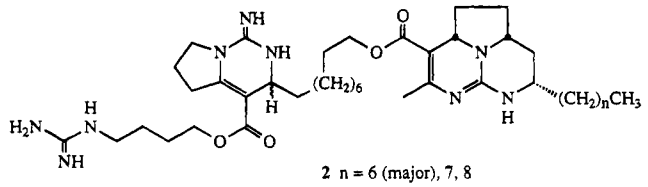
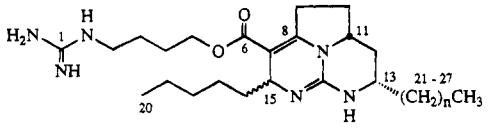
extract of the bright red Caribbean sponge *Batzella* sp.,[9] was found to inhibit gp120–CD4 binding in a light-independent manner. Bioassay-directed fractionation of the dichloromethane-soluble material by column chromatography on Sephadex LH-20 followed by silica gel chromatography yielded several active and inactive fractions. Further purification of these fractions by preparative thin layer chromatography or silica gel column chromatography led to the isolation of a total of 21 compounds that included several known metabolites together with five novel alkaloids, batzelladines A–E (**1**–**5**) from the gp120/CD4 active fraction. The known



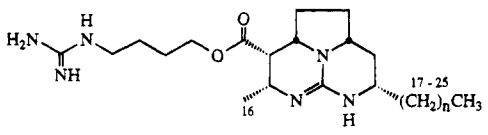
1 n = 8 (major), 9, 10



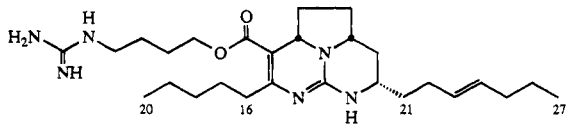
2 n = 6 (major), 7, 8



3 n = 6 (major), 7, 8



4 n = 8 (major), 9, 10



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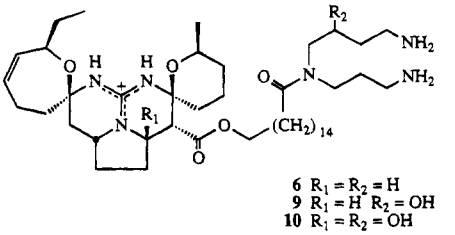
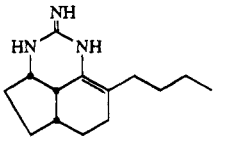
compounds are ptilomycalin A (**6**),[10] the major metabolite of the sponge, ptilocaulin (**7**),[11] crambescin A (**8**),[12,13] crambescidin 800 (**9**),[14] and crambescidin 816 (**10**).[14] Several minor inactive metabolites related to ptilomycalin A (**6**), ptilocaulin (**7**), and crambescin A (**8**), which was



6 $R_1 = R_2 = H$
9 $R_1 = H$ $R_2 = OH$
10 $R_1 = R_2 = OH$



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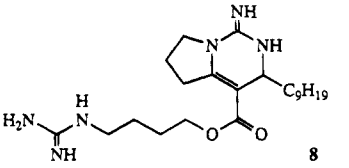

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originally named as crambine A,[12] will be described elsewhere. The batzelladines **1**–**5**, which are the subject of this report, are the first natural products of small molecular weight that have been shown to inhibit the gp120–CD4 interaction.

The alkaloid batzelladine A (**1**) was isolated as an amorphous, water soluble powder, $[\alpha]_D$ +8.9° (*c* 2.3, MeOH). The IR spectrum contained bands at 3600–3100 (NH or OH and CH), 1733 (ester), 1696, and 1684 cm^{-1} (unsaturated ester), and the UV spectrum (λ_{max} 205 and 288 nm) was very similar to that of crambescin A (**8**). The mass spectral data, which included both high-resolution FABMS and fragmentation data resulting from high-energy, collision-induced dissociation of the selected MH^+ precursor ion (four-sector tandem MS) indicated that batzelladine A consisted of a major protonated alkaloid (**1**, n = 8) of molecular formula $C_{42}H_{74}N_9O_4$ together with minor amounts of two higher homologs (**1**, n = 9 and 10). The mass of the fully deuterium-exchanged molecule (m/z 776.6) indicated the presence of 8 exchangeable protons, which was consistent with a ^{13}C NMR spectrum that required 66 attached protons. Batzelladine A has 11 degrees of unsaturation and since the ^{13}C NMR data requires one double bond (δ 151.6 and 103.1), two ester carbonyls (171.0 and 166.5), and three C=N bonds (159.4, 153.3, and 153.1), the molecule must contain five rings.

Comparison of the ^1H and ^{13}C NMR data of **1**, which were analyzed using, *inter alia* DQFCOSY, COSY45, TOCSY, HMQC, COLOC, HMBC experiments, with those of crambescin A (**8**) allowed the C-1 to C-16 portion of the molecule to be assigned. The major ion peaks at m/z 655, 637, 609, 474, and 114 in the tandem mass spectrum of **1** support this assignment. The presence of prominent ions at m/z 304, 332, and 350 ($C_{20}H_{36}N_3O_2$, C-22 to C-42 fragment) indicate the presence of an O-ester at C-22, and NMR signals corresponding to the linear C-14 to C-22 alkyl chain were assigned, with signals at δ 4.14 (t, 2 H, J = 6.6 Hz) and 66.4 (t) being assigned to H-22 and C-22, respectively. The HMBC

(9) The sponge *Batzella* sp was identified by Dr. Rob van Soest, and recollections were verified by Mary Kay Harper, SIO. Dr. van Soest noted that the sponge resembled a *Ptilocaulis* species except that the spicules were exclusively thin strongyles rather than the expected thick styles. This placed the sponge in the genus *Batzella*, morphologically very similar to *Batzella frutex* Pulitzer-Finali (1982) from the Great Barrier Reef, and it is probably an undescribed species. Since the major metabolite of *Batzella* sp. is ptilomycalin A, Dr. van Soest examined Kashman's voucher specimen of *Ptilocaulis*[10] obtained from Dr. Shirley Pomponi, HBOI) and found the two specimens to be the same. A voucher specimen of the sponge has been deposited in the Zoological Museum of Amsterdam, ZMA registry number POR. 8788.

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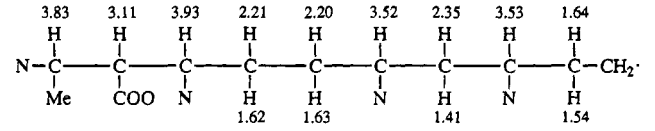
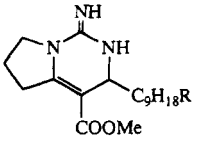


Figure 1. Fragment **a** in batzelladine A (**1**).

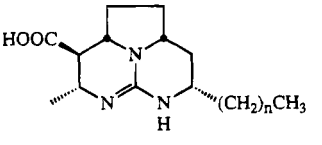
showed significant correlations between H-13 and C-6, C-7, C-8, C-12, C-14, and C-15 and between H-22 and C-20, C-21, and C-23. The remainder of the major homologue (**1**, $n = 8$) must consist of a tricyclic unit of molecular formula $C_{19}H_{34}N_3$ (m/z 304) containing one C=N bond and only one exchangeable proton. This unit must be attached to the C-23 ester carbonyl.

The HOHAHA spectrum revealed a contiguous spin system from H-32 and H-24 to H-30 and is illustrated by fragment **a** (Figure 1). In the HMBC spectrum, correlations were observed between H-25 and C-23, C-26, and C-27 and between H-32 and C-23 and C-33. The ester carbonyl must therefore be attached at C-24. The chemical shifts of the proton signals for H-32 (δ 3.83), H-25 (3.93), H-28 (3.52) and H-30 (3.53) are typical of protons attached to carbons bearing nitrogen, and the tricyclic ring system was therefore constructed by attaching the four carbons to the three nitrogens of a guanidine unit (δ_C 153.1) in the manner shown. The absence of correlations between the C-31 and H-32, H-25, H-28, and H-30 in both the HMBC and COLOC spectra was initially disconcerting until it was noted that the same situation exists for ptilomycalin A (**6**).[10] The remaining NMR signals were assigned to a linear hydrocarbon chain from C-35 to C-42.

Methanolysis of batzelladine A (**1**), using sodium methoxide in methanol at 65 °C for 16 h, gave the methyl ester **11** and acid **12**. The methyl ester **11** was very similar to the methanolysis product **13** obtained from crambescin A (**8**) except that the methyl group at the end of the alkyl chain was replaced by CH_2OH. Examination



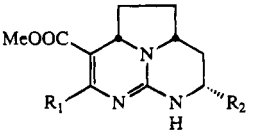
11 R = OH
13 R = H



12 n = 8 (major), 9, 10

of the ^{1}H NMR spectrum of **12**, which is derived from the right-hand portion of **1**, revealed that the stereochemistry of **12** at the carbon adjacent to the carboxylic acid group (C-2) differs from that in **1** (C-24). Under the basic conditions, the axial carboxylic ester in **1** has epimerized to the equatorial configuration in **12**, as indicated by the coupling constants of the H-2 signal at δ 2.01 in pyridine-$d5$ solution (t, 1 H, J = 10.5 Hz) compared to the H-24 signal in **1** at 3.11 (dd, 1 H, J = 3.6, 4.6 Hz). Thus H-25 and H-32 are both axial with respect to the six-membered ring. The coupling constants of the H-29 signals in **1** [δ 2.53 (ddd, 1 H, J = 12, 5, 3 Hz) and 1.42 (dt, 1 H, J = 12, 11 Hz)] indicated that H-28 and H-30 must also be axial with respect to the other six-membered ring. The five-membered ring must be cis fused to the two six-membered rings. Although determination of the relative stereochemistry of the tricyclic ring system of batzelladine A (**1**) was relatively straightforward, we were unable to define the relative stereochemistry at C-13, as this requires determination of the absolute stereochemistry of both ring systems.

The major component ($n = 6$) of batzelladine B (**2**), which was isolated as a white amorphous powder, had the molecular formula $C_{40}H_{68}N_9O_4$ [m/z = 738.5356 (M + H)$^+$] and was accompanied by small quantities of two higher homologs ($n = 7, 8$), the presence of which could only be detected by mass spectrometry. Considering only the major metabolites, batzelladine B has two methylene units less and one degree of unsaturation more than batzelladine A. The UV absorption at λ_{max} 289 and 340 nm indicated that the additional degree of unsaturation is involved in a conjugated chromophore and the IR bands at 1691 and 1686 cm^{-1} suggested that both ester groups were conjugated. Comparison of the NMR data for **1** and **2** clearly indicated that the molecules were identical from C-1 to C-22 and hydrolysis of **2** with sodium methoxide in methanol gave the same methyl ester **11** that had been obtained from **1**, together with methyl ester **14**. The presence of the 24,32-olefinic bond was supported by the following NMR data: the new olefinic signals at δ 102.5 (C-24) and 144.7 (C-32) are typical of carbon signals for an α,β-unsaturated ester that has a basic nitrogen at the β-position,[15] the ^{1}H NMR spectrum contains signals at δ 2.30 (s, 3 H, H-33) and 4.50 (dd, 1 H, J = 6, 4 Hz, H-25), both of which show less coupling and are shifted downfield compared to the corresponding signals in **1**. Analysis of the HMBC data fully supports the proposed structure of **2**; for example, the H-25 signal at δ 4.50 showed correlations to 102.5 (C-24), 144.7 (C-32), 166.8 (C-23), and 33.8 (C-26). The close similarity of all aspects of the NMR data for batzelladines A (**1**) and B (**2**), except in the vicinity of C-24 and C-32, requires the same tricyclic ring system and stereochemistry for both molecules. Analysis of the mass spectral fragmentation patterns revealed that the alkyl side chain attached at C-30 in **2** was shorter by two methylene units than that of **1** and that the additional methylene groups in the minor homologs were in the same side chain. The structure of the methanolysis product **14** also supported this assignment.



14 R_1 = Me R_2 = n-heptenyl (major)
15 R_1 = n-pentyl R_2 = n-hept-3-enyl (major)

Batzelladine C (**3**), $[\alpha]_D = -3.7°$, was isolated as a white powder of molecular formula $C_{27}H_{49}N_6O_2$ [m/z = 489 (M − H)$^+$, major homolog]. The mass spectrum contained smaller peaks at m/z = 503 and 517 due to two higher homologues. The IR spectrum contained bands at 3600 (NH), 1700, and 1688 cm^{-1} (unsaturated ester), and the UV spectrum, with absorptions at λ_{max} 206 and 287 nm, was similar to that of crambescin A (**8**). The high-energy CID tandem mass spectrum revealed a loss of 113 Da (m/z = 376), due to loss of the 4-guanidino-n-butyl group, followed by losses of H_2O, CO_2, and HCOOH, indicating the adjacent ester linkage. An intense peak at m/z = 304 could be interpreted as the loss of C_5H_{12} from the peak at m/z = 376. The NMR

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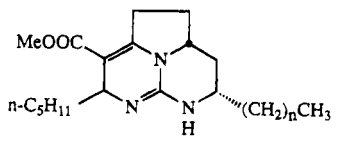
Table 1. ^{1}H NMR Data (MeOH-d_4) for Batzelladines A–E (1–5) and Crambescin A (8)

H no.	8[a]		1[b]		2[b]		3[c]		4[a]		5[a]	
2	3.21	t, 7.1	3.21	t, 7.2	3.21	t, 7.1	3.21	t, 7.2	3.21	t, 7.0	3.21	t, 7.1
3	1.69	m	1.69	m	1.69	m	1.69	m	1.69	m	1.69	m
4	1.73	m	1.76	m	1.75	m	1.72	m	1.75	m	1.75	m
5	4.20	t, 6	4.20	t, 6.9	4.20	t, 6	4.20	t, 6.2	4.21	t, 6.5	4.18	t, 6.0
7									3.12	dd, 4, 3.5		
8									3.93	m	4.45	dd, 6.1, 4
9a	2.95	m	2.95	m	2.96	m	2.84	ddd, 14, 8, 6	1.60	m	1.65	m
9β	3.31	m	3.31	m	3.32	m	3.35	dd, 14, 6	2.24	m	2.59	m
10α	2.11	m	2.11	m	2.11	m	1.70	m	1.41	m	1.55	m
10β	2.21	m	2.21	m	2.21	m	2.44	m	2.35	m	2.10	m
11α	3.65	m	3.65	m	3.63	m						
11β	3.81	m	3.81	m	3.79	m	3.86	m	3.55	m	3.78	m
12α							1.48	m	1.42	m	1.32	m
12β							2.39	m	2.35	m	2.40	m
13	4.38	t, 5.3	4.38	t, 6.3	4.38	t, 6	3.61	m	3.54	m	3.78	m
14	1.56	m	1.56	m	1.55	m						
15	1.48	m	1.48	m	1.47	m	4.45	dd, 6, 3.5	3.83	m		
16	1.29	br s	1.29	br s	1.29	br s	1.63	m	1.28	d, 6.5	2.72	m
											2.61	m
17	1.29	br s	1.29	br s	1.29	br s	1.29	br s	1.55	m	1.55	m
									1.64	m		
18	1.29	br s	1.29	br s	1.29	br s	1.29	br s	1.29	br s	1.45	m
19	1.29	br s	1.29	br s	1.29	br s	1.29	br s	1.29	br s	1.29	br s
20	1.29	br s	1.42	m	1.42	m	0.90	t, 6.5	1.29	br s	0.92	t, 6.9
21	1.29	br s	1.64	m	1.63	m	1.72	m	1.29	br s	1.75	m
							1.58	m			1.65	m
22	0.89	t, 6.6	4.14	t, 6.9	4.14	m	1.36	m	1.29	br s	2.22	br q, 7
23							1.29	br s	1.29	br s	5.39	m
24			3.11	dd, 4.6, 3.6			1.29	br s	1.29	br s	5.42	m
25			3.93	m	4.50	dd, 6, 4	1.29	br s	0.89	t, 6.5	2.05	m
26α			1.62	m	1.67	m	1.29	br s			1.35	m
26β			2.21	m	2.48	m						
27α			1.63	m	1.57	m	0.90	t, 6.5			0.91	t, 7
27β			2.20	m	2.10	m						
28			3.52	m	3.75	m						
29α			1.42	m	1.32	m						
29β			2.53	m	2.37	m						
30			3.53	m	3.46	m						
32			3.83	m								
33			1.27	d, 6.3	2.30	s						
34			1.54	m	1.60	m						
			1.64	m	1.60	m						
35			1.30	m	1.30	m						
36			1.29	br s	1.29	br s						
37			1.29	br s	1.29	br s						
38			1.29	br s	1.29	br s						
39			1.29	br s	1.29	br s						
40			1.29	br s	0.88	t, 6						
41			1.29	br s								
42			0.89	t, 6.5								

[a] Spectra recorded at 400 MHz. [b] Spectra recorded at 500 MHz. [c] Spectra recorded at 600 MHz.

spectra clearly indicated the presence of the 4-guanidino-*n*-butyl ester moiety. The ^{1}H NMR spectrum contained a contiguous series of signals from the allylic methylene group at C-9 [δ 3.35 (dd, 1 H, J = 14, 6 Hz), 2.84 (ddd, 1 H, J = 14, 8, 6 Hz)] to the C-13 methine signal at 3.61 (m, 1H). A TOCSY experiment revealed a long range coupling between the H-9 proton signals and the H-15 signal (at δ 4.45 m, 1 H). There were long range correlations from the H-9 and the H-15 signals to the C-7 (δ 101.2) and C-8 (150.4) signals. The chemical shift of the C-7 signal indicated that it must be adjacent to the ester carbonyl (δ 166.3) but the carbonyl signal was coupled only to the H-5 signal at δ 4.20 (t, 2 H, J = 6 Hz). In the HMQC-TOCSY spectrum, the H-15 signal was coupled to carbon signals at δ 38.2 (C-16), 24.3 (C-17), 32.6 (C-18), 23.7 (C-19), and 14.3 (C-20). This defined the alkyl group attached at C-15 as *n*-pentyl. The remaining *n*-heptyl chain was attached at C-13; both the TOCSY and HMQC-TOCSY spectra support the spectral assignments in Tables 1 and 2. Hydrolysis of batzelladine C (**3**) with sodium methoxide in methanol gave the methyl ester **16** [^{1}H NMR, δ 3.70 (OMe)] with the molecular formula, $C_{23}H_{39}N_3O_2$.



16 n = 6 (major), 7, 8

Batzelladine D (**4**), $[\alpha]_D = -1.24°$, was isolated as a white powder of molecular formula $C_{25}H_{47}N_6O_2$ (major homolog). The mass spectrum contained smaller peaks at m/z = 477 and 491 due to two minor higher homologues. The IR spectrum contained bands at 3600–3100 (NH and CH) and 1731 (ester) cm^{-1}. The base peak in the tandem mass spectrum at m/z = 350 ($C_{20}H_{36}N_3O_2$), also seen as a prominent peak in the mass spectrum of **1**, together with a peak at m/z = 114, due to an *n*-butyl

Table 2. ^{13}C NMR Data (MeOH-d_4) for Batzelladines A–E (1–5) and Crambescin A (8)

C no	5	8	1	2	3	4
1	158.8	158.8	158.7	158.7	158.7	158.7
2	42.0	42.0	42.0	42.0	42.0	42.0
3	26.6	26.6	26.6	26.7	26.6	26.7
4	27.0	27.0	27.0	27.0	27.0	27.1
5	65.1	65.1	65.1	65.0	65.4	64.5
6	166.2	166.2	166.2	166.3	170.6	167.1
7	103.1	103.1	103.1	101.2	45.6	100.7
8	152.8	152.8	152.8	150.4	57.7	58.3
9	31.9	32.0	31.9	31.5	29.3	34.4
10	22.9	22.9	22.9	30.3	31.4	27.7
11	48.6	48.8	48.8	58.6	57.3	57.1
12	153.2	153.2	153.2	32.9	34.2	34.4
13	51.1	51.3	51.4	53.5	53.2	50.8
14	37.5	37.5	37.5	149.9	151.5	147.8
15	25.2	25.2	25.2	52.2	49.8	153.1
16	*	*	*	38.2	18.4	32.9
17	*	*	*	24.3	37.0	29.5
18	*	*	*	32.6	26.2	32.8
19	*	*	*	23.7	*	23.6
20	33.0	27.0	27.1	14.3	*	14.3
21	23.6	29.7	29.7	35.8	*	34.9
22	14.5	66.0	65.5	25.9	*	24.0
23		170.7	166.9	*	33.0	129.3
24		45.6	102.6	*	23.7	132.1
25		57.7	58.9	32.6	14.3	30.3
26		29.3	34.0	23.6		23.9
27		31.4	27.6	14.4		14.1
28		57.3	57.1			
29		34.2	33.9			
30		53.2	51.1			
31		151.6	147.6			
32		49.8	144.8			
33		18.4	17.5			
34		36.9	34.7			
35		26.2	25.9			
36		*	*			
37		*	*			
38		*	33.0			
39		*	23.7			
40		33.0	14.4			
41		23.7				
42		14.4				

*Overlapping signals from δ 30.3 to 30.6.

guanidine residue, suggested that batzelladine D (**4**) might consist of a 4-guanidino-*n*-butyl ester of the C-23 to C-42 portion of batzelladine A (**1**). This proposed structure was completely supported by the ^1H and ^{13}C NMR data that was assigned with the aid of the $^1H-^1H$ COSY and HMBC data as shown in Tables 1 and 2. In particular, the H-7, H-8, H-11, H-13, H-15, and Me-16 signals for **4** are virtually identical in chemical shift and multiplicity to the H-24, H-25, H-28, H-30, H-32, and Me-33 signals of **1** as are the ^{13}C NMR signals for the corresponding carbon atoms. Hydrolysis of batzelladine D (**4**) with sodium methoxide in methanol gave the same acid **12** that was obtained under similar conditions from batzelladine A (**1**).

Batzelladine E (**5**), $[\alpha]_D = 87°$, was isolated as a gummy solid of molecular formula $C_{27}H_{46}N_6O_2$. The mass spectrum did not contain any peaks for higher or lower homologs. The IR spectrum contained bands at 3415 (NH) and 1683 cm^{-1} (unsaturated ester). The peak at m/z = 114 in the mass spectrum together with the corresponding signals in the ^1H and ^{13}C NMR spectra indicated that **5** contained a 4-guanidino-*n*-butyl ester group. Analysis of the NMR data suggested that **5** contained the same tricyclic ring system as that present in **2** except that in place of the vinyl methyl group in **2** there was an alkyl group. The methylene group adjacent to the ring gave rise to ^1H NMR signals at δ 2.72 and 2.61 that showed a strong HMBC correlation to the C-7 signal at δ 100.7. Examination of the COSY and HMBC experiments suggested that an *n*-pentyl chain was attached at C-15. The *n*-3-heptenyl chain attached at C-13 was revealed by careful assignment of the NMR data. The COSY experiment revealed that the terminal methyl signal at δ 0.91 (t, 3 H, J = 7 Hz) was coupled to a methylene signal at 1.41 (m, 2 H) that was in turn coupled to a vinyl methylene signal at 2.05 (br q, 2 H, J = 7 Hz). The vinyl methylene group at the other side of a *Z*-olefin (δ 5.40, 5.42) gave rise to a signal at δ 2.22 (br q, 2 H, J = 7 Hz) that was coupled to signals at 1.75 (m, 1 H) and 1.65 (m, 1 H) and, most significantly, gave a correlation in the HMBC experiment to the C-13 signal at δ 50.8. All NMR signals were assigned as shown in Tables 1 and 2. Methanolysis of batzelladine E (**5**) with methanolic 25% sodium methoxide gave the expected methyl ester **15** of molecular formula $C_{23}H_{37}N_3O_2$.

Table 3. Inhibition of HIV-1 gp120 Binding to CD4 by Batzelladines A–D and Crambescin A

	IC_{50} (μM)	
compound	gp120-CD4 ELISA[a]	cell-based assay[b]
batzelladine A (**1**)	29 ± 4	10
batzelladine B (**2**)	31 ± 12	25
batzelladine C (**3**)	>100	>100
batzelladine D (**4**)	72 ± 2	>100
crambescin A (**8**)	>100	>100

[a] The ELISA measures the binding of soluble CD4 (sCD4) to immobilized gp120 (see Experimental Section). Values shown are mean ± standard deviation (n = 3). [b] The cell-based assay measures the binding of gp120 to CD4-positive T-cells.

Biological Results

Batzelladines A–D and crambescin A were evaluated in an ELISA-based assay that measures the association of soluble CD4 (sCD4) to immobilized recombinant gp120.[16,17] Batzelladines A and B were active in this assay with IC_{50} values of approximately 30 μM (Table 3). Batzelladines C and D and crambescin A were either inactive or significantly less active in the ELISA-based gp120-CD4 assay. This biochemical screen was followed up with a cell-based assay that measures the binding of gp120 to $CD4^+$ T cells. Consistent with the results in the primary assay, only batzelladines A and B were active in the cell-based assay (Table 3). These results suggest that both the tricyclic ring system (tridecaazaline) and crambescin A bicyclic unit must be present in the same molecule for maximal activity.

Batzelladines A and B were evaluated in a 7-day cell-based assay for inhibition of HIV-1 infectivity. However, antiviral activity could not be assessed as the compounds were toxic to host cells. We subsequently found that batzellidines A and B are highly active against certain other ligand–receptor interactions and enzymes. For example, batzellidines A and B inhibited protein kinase C activity as well as the binding of interleukin-8 (IL8) and calcitonin gene-related peptide (CGRP) binding to their respective receptors (Table 4). As was found for gp120–CD4 binding, batzellidines C and D and cramb-

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Table 4. Activity of Batzelladines 1–4 and Crambescin A in Additional Bioassays

compound	IC$_{50}$ (μM)				
	PKC[a]	IL8a[b]	IL8b[c]	CGRP[d]	cytotoxicity[e]
batzelladine A (**1**)	1.4	4.7	7.8	1.7	1.6
batzelladine B (**2**)	1.5	2.6	6.5	1.7	1.8
batzelladine C (**3**)	6.8	9.4	9.4	4.3	1.1
batzelladine D (**4**)	11	15	14	26	0.5
crambescin A (**8**)	9.6	47	41	7.1	0.7

[a] Protein kinase C enzyme assay using rat brain enzyme and histone protein as substrate. [b] Binding of interleukin-8 to the non-permissive receptor; radioligand binding assay. [c] Binding of interleukin-8 to the permissive receptor; radioligand binding assay. [d] Binding of calcitonin gene-related peptide to porcine lung membranes; radioligand binding assay. [e] Cytotoxicity to proliferating Vero cells; 72 h exposure with an XTT read.

escin A were somewhat less active in these other bioassays as compared to batzellidines A and B. Furthermore, all five compounds demonstrated potent cytotoxicity against proliferating Vero cells. As such, additional work to separate gp120–CD4 inhibitory activity from other activities is required for these compounds to be considered as potential leads for HIV.

Experimental Section

Biological Assays. For the HIV-1 gp120–CD4 ELISA, an Immulon-2 microtiter plate was incubated overnight at 4 °C with 2 μg of goat anti-mouse IgG in 100 μL of PBS (0.01 M phosphate buffer, 0.15 M NaCl), pH 6.8, and 0.005% glutaraldehyde. The solution was removed and the plate was washed three times with PBS, pH 7.4. Mouse anti-gp120 antibody (0.2 μg) was added in 100 μL of PBS-Tween-BSA (0.5% BSA, 0.05% Tween-20) and incubated overnight at room temperature (rt). The antibody was removed from the plate, the plate was washed, and gp120 (0.1 μg) in 100 μL of PBS-Tween-BSA was added and incubated at 37 °C for 1 h. The plate was washed and blocked with PBS-Tween-BSA for 1 h at room temperature and washed again, and then 50 μL of sCD4 (100 ng/mL) plus 50 μL of test substance was added and incubated for 1 h at rt. The plate was washed, and 100 μL of peroxidase-conjugated rabbit anti-sCD4 IgG was added and incubated for 1 h at rt. The bound sCD4 was quantified using a colorimetric enzyme reaction with *o*-phenylenediamine as a substrate. The cell-based assay measuring the binding of gp120 to CD4-positive T cells was performed as described.[18] Cell-associated gp120 was detected by treatment with an anti-gp120 antibody followed by addition of FITC-conjugated goat anti-mouse antibody, analysis was done by flow cytometry.[18] The IL8 and CGRP assays measured the binding of ^{125}I-labeled ligands to their respective receptors (for IL8, Chinese hamster ovary cell membranes containing recombinant IL8 receptors; for CGRP, porcine lung membranes) using standard filter-based radioligand binding assays. The Vero cell toxicity[19] and protein kinase C[20] assays were performed as described.

Collection, Extraction, and Isolation. The sponge *Batzella* sp. was collected by hand using SCUBA at a depth of 10–30 meters at Chub Cay, Bahamas, in June 1988 and December 1990. Specimens were frozen immediately and kept at −20 °C until extraction. The freeze–dried sponge (2.5 kg) was extracted with MeOH–CH_2Cl_2 (1:1) to give a dark red solid (270 g) which was triturated with EtOAc, CH_2Cl_2, and MeOH to give 62, 171, and 19 g extracts, respectively. The CH_2Cl_2 extract (30 g) which showed activity in the gp120/CD4 binding assay was applied to a column of Sephadex LH-20 and eluted with MeOH:CH_2Cl_2:hexane (1:1:1). The gp120/CD4 active fractions were monitored by bioassay and pooled. Combined active fractions (11.27 g), which contained several compounds, were chromatographed over a column of silica gel (800 g, Kieselgel-60, 230–400 mesh) eluting with a solvent gradient system from MeOH:CH_2Cl_2:H_2O:HCOOH (10:90:1.5:2.5) to 25:75:2.5:3.5. Fractions with the same TLC profile were combined to yield individual fractions which were further purified by extensive preparative thin layer chromatography (PTLC) employing several different solvent systems to give several compounds in pure form. Thus, the initial fractions (0.119 g) after repeated PTLC on silica gel using MeOH:CH_2Cl_2:H_2O:HCOOH (12:88:1:1.5) as eluent yielded ptilocaulin (**7**, 0.057 g) and ester **11** (0.039 g). The following combined fractions (5.1 g) which contained active compounds of almost identical R_f values and appeared homogeneous on silica gel TLC using MeOH:CH_2Cl_2:H_2O:HCOOH (20:80:2.5:3.5) represented a very difficult resolution problem. They were finally separated by repeated SiO_2 gel column chromatography and PTLC using Me_2CO:MeOH:CH_2Cl_2:H_2O:HCOOH (30:20:50:2.5:3.5) to get batzelladine A (**1**, 1.37 g), B (**2**, 1.42 g), C (**3**, 0.11 g), D (**4**, 0.211 g), and E (**5**, 0.076 g) and crambescin A (**8**, 0.43 g). The polar fractions (9.11 g) had ptilomycalin A as the most abundant component. Its final purification was achieved using a (150 × 1.5 cm) SiO_2 gel column eluted with MeOH:CH_2Cl_2:H_2O:HCOOH (25:75:2.5:3.5) to yield ptilomycalin A (**6**, 7.71 g), crambescidin 800 (**9**, 0.092 g), and crambescidin 816 (**10**, 0.037 g).

Batzelladine A (1): colorless gum which yielded a white powder after lyophilization; $[\alpha]^{25}_D$ +8.9° (*c* 2.3); UV (MeOH) λ_{max} 205 (ϵ 20 098) and 288 nm (ϵ 6824); IR (KBr) ν_{max} 3600–3100 br, 3100–2800, 1733, 1696 and 1684, 1653, 1646, 1635, 1560, 1345 1089 cm^{-1}; positive FABMS *m/z* 768 (M + H), 655 (M + H − $C_5H_{11}N_3$, $C_{37}H_{63}N_6O_4$, HRFABMS 655.4921), 474 ($C_{29}H_{52}N_3O_2$), 350 ($C_{20}H_{36}N_3O_2$), 304, 290, 136, 114, 55; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; HRFABMS calcd M_r for $C_{42}H_{74}N_9O_4$ 768.5863 (M + H)$^+$, found M_r 768.5839.

Batzelladine B (2): colorless, amorphous solid, $[\alpha]^{25}_D$ +44.3° (*c* 3.7); UV (MeOH) λ_{max} 206 (ϵ 16 582), 289 (ϵ 9298), 340 (ϵ 4375); IR (KBr) ν_{max} 3600–3100, 3100–2800, 1691, 1686, 1632, 1618, 1347 1268, 1194, 1109, 1080 cm^{-1}; positive FABMS *m/z* 738 (M + H), 318.2188 ($C_{18}H_{28}N_3O_2$), 302.2218 ($C_{18}H_{28}N_3O$), 274.2280 ($C_{17}H_{28}N_3$); ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2.; HRFABMS calcd M_r for $C_{40}H_{68}N_9O_4$ 738.5394 (M + H)$^+$, found M_r 738.5356.

Batzelladine C (3): white, amorphous powder, $[\alpha]^{25}_D$ −3.7° (*c* 2.4); UV (MeOH) λ_{max} 211 (ϵ 8078), 230 (ϵ 7828), 298 (ϵ 6936) nm; IR (KBr) ν_{max} 3600–3100, 3100–2800, 1698, 1683, 1649, 1632, 1344, 1218, 1199, 1088 cm^{-1}; positive FABMS *m/z* 489 (M + H), 375 (M + H − $C_5H_{12}N_3$, $C_{22}H_{37}N_6O_2$, HRFABMS, 375.2877); ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; HRFABMS calcd M_r for $C_{27}H_{49}N_6O_2$ 489.3917 (M + H)$^+$, found M_r 489.3903.

Batzelladine D (4): colorless, amorphous powder, $[\alpha]^{25}_D$ −1.2° (*c* 0.9); UV (MeOH) λ_{max} 205 (ϵ 9170), 298 nm (ϵ 2379); IR (KBr) ν_{max} 3600–3100, 3100–2800, 1731, 1655, 1638, 1330, 1220 cm^{-1}; positive FABMS *m/z* 463 (M + H), 350 (M − $C_5H_{12}N_3$, $C_{20}H_{35}N_3O_2$, HRFABMS 350.2821), 114.1014 ($C_5H_{12}N_3$); ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; HRFABMS calcd M_r for $C_{25}H_{47}N_6O_2$ M_r (M + H)$^+$, found M_r 463.3740.

Batzelladine E (5): colorless gum, $[\alpha]^{25}_D$ +87.1° (*c* 1.9); UV (MeOH) λ_{max} 207 (ϵ 7954), 227 (ϵ 7022), 291 (ϵ 3511), 345 (ϵ 2876) nm; IR (KBr) ν_{max} 3416, 3100–3000, 1683, 1631, 1620, 1457, 1270, 1074 cm^{-1}; positive FABMS *m/z* 487 (M + H), 374 (M + H − $C_5H_{11}N_3$, $C_{22}H_{36}N_3O_2$, HRFABMS, 374.2814), 114.1014 ($C_5H_{12}N_3$); ^{1}H NMR see Table 1; ^{13}C NMR see Table 2; HRFABMS calcd M_r for $C_{27}H_{47}N_6O2$ 487.3760 (M + H)$^+$, found M_r 487. 3728.

Methanolysis of Batzelladine A (1). In a sealed glass tube, a mixture of **1** (25 mg) dissolved in MeOH (3 mL) and 25% NaOMe in MeOH (1 mL) was heated at 65 °C for 16 h. The solvent was removed and the residue dissolved in H_2O (15 mL), neutralized with dilute CH_3COOH, and extracted with $CHCl_3$ (3 × 15 mL). The organic layer after evaporation gave a residue which was fractionated by silica gel PTLC using MeOH:CH_2Cl_2:H_2O:HCOOH (15:85:0.5:0.5) as a solvent system

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to yield **11** (5.1 mg) and **12** as an acid (11.2 mg). **11**: colorless gum, $[\alpha]^{25}_D$ +30.5 (*c* 0.3); UV (MeOH) λ_{max} 218 (ϵ 6176), 286 (ϵ 5238) nm; IR (KBr) ν_{max} 3450–3000, 2600, 1697, 1654 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4) δ 4.38 (1 H, t, J = 7.1 Hz), 3.80 (1 H, m), 3.65 (1 H, m), 3.74 (3 H, s), 3.52 (2 H, t, J = 6.9 Hz), 3.31 (1 H, m), 2.96 (1 H, m), 2.25 (1 H, m), 2.11 (1 H, m), 2.01 (1H, t, J = 10.5 Hz), 1.45–1.65 (4 H, m), 1.31 (12 H, bs); HRFABMS calcd M_r for $C_{18}H_{32}N_3O_3$ 338.2465 $(M + H)^+$, found M_r 338.2465. **12**: hygroscopic solid, $[\alpha]^D_{25}$ −28.4° (*c* 1.41); UV (MeOH) λ_{max} 213 nm (ϵ 5091); IR (KBr) ν_{max} 3500–3200, 2800, 1711 cm^{-1}; ^1H NMR (400 MHz, pyridine-d_5) δ 10.9 (1 H, brs), 3.71 (1 H, m), 3.60 (1 H, m), 3.48 (1 H, m), 3.40 (1 H, m), 2.26–2.20 (2 H, m), 1.92 (1 H, t, J = 10.1 Hz), 1.60–1.80 (2 H, m), 1.48 (2 H, br), 1.28 (H, d, J = 7.0 Hz), 1.23 (14 H, brs), 0.90 ppm (3 H, t, J = 6.9 Hz); HRFABMS calcd M_r for $C_{20}H_{36}N_3O_2$ 350.2807 $(M + H)^+$, found M_r 350.2793.

Methanolysis of Crambescin A (8). NaOMe (25%) in MeOH solution (1 mL) was added to a solution of **8** (25 mg) in MeOH (3 mL) and heated in a sealed tube at 65 °C for 16 h. The residue obtained after the workup as described above was purified by silica gel PTLC ($MeOH:CH_2Cl_2:H_2O:HCOOH$ 15:85:1:1) to yield **13** as a colorless gum (15.5 mg); $[\alpha]^{25}_D$ +52.9° (*c* 0.4); UV (MeOH) λ_{max} 217 (ϵ 6234), 276 (ϵ 5724) nm; IR (KBr) ν_{max} 2800, 1694, 1655 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4) δ 4.38 (1 H, t, J = 7.1 Hz), 3.80 (1 H, m), 3.65 (1 H, m), 3.74 (3 H, s), 3.31 (1 H, m), 2.96 (1 H, m), 2.11–2.25 (2 H, m), 1.45–1.65 (4 H, m), 1.31 (12 H), 0.90 ppm (3 H, t, J = 7.0 Hz); HRFABMS calcd M_r for $C_{18}H_{31}N_3O_2$ 322.2494 $(M + H)^+$, found M_r 322.2416.

Methanolysis of Batzelladine B (2). A mixture of 25% NaOMe in MeOH solution (1.5 mL) and **2** (30 mg) in MeOH (3 mL) was heated in a sealed tube in a similar way as described for **1** to yield a residue, which after silica gel PTLC using $MeOH:CH_2Cl_2:H_2O$ (10:90:1) afforded a UV active fragment **11** (5.9 mg) identical to that obtained from **1** and another fragment, **14** (11.2 mg), as a methyl ester, as pale yellow gum, $[\alpha]^{25}_D$ +121.1° (*c* 0.93); UV (MeOH) λ_{max} 226 (ϵ 8876), 288 (ϵ 5193), 341 (ϵ 2131) nm; IR (KBr) ν_{max} 3300, 3100–2800, 1690, 1686, 1635, 1619 cm^{-1}; HRFABMS calcd M_r for $C_{19}H_{32}N_3O_2$ 334.2494 $(M + H)^+$, found M_r 334.2409; ^1H NMR (400 MHz, MeOH-d_4) δ 4.48 (1 H, dd, J = 4, 6.0 Hz), 3.78 (1 H, m), 3.72 (3 H, s), 3.50 (1 H, m), 2.39–2.55 (2 H, m), 2.30 (3 H, s), 2.0–2.20 (2 H, m), 1.50–1.80 (m), 1.25–1.48 (brs), 0.90 ppm (3 H, t, J = 6.8 Hz).

Methanolysis of Batzelladine C (3). NaOMe (25%) in MeOH solution (0.8 mL) was added to a solution of **3** (15 mg) in MeOH (2.5 mL) and the reaction mixture heated in a sealed tube at 65 °C for 16 h. The residue obtained after the workup was purified by silica gel PTLC using $MeOH:CH_2Cl_2:H_2O$ (10:90:1) as solvent system to give methyl ester **16** (8.9 mg) as an oil: $[\alpha]^{25}_D$ −4.2° (*c* 0.93); UV (MeOH) λ_{max} 231 (ϵ 9014), 297 (ϵ 7364) nm; IR (KBr) ν_{max} 3600–3000, 3100–2800, 1701, 1688, 1652, 1345, 1219, 1200, 1092 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4) δ 4.48 (1 H, dd, J = 3.5, 6.0 Hz), 3.86 (1 H, m), 3.75 (3 H, s), 3.62 (1 H, m), 3.37 (1 H, m), 2.82 (1 H, m), 2.35–2.45 (2 H, m), 1.60–1.80 (4 H, m), 1.49 (1 H, m), 1.30 (brs), 0.90 (3 H, t, J = 6.9 Hz), 0.89 ppm (3 H, t, J = 7.0 Hz); HRFABMS calcd M_r for $C_{23}H_{40}N_3O_2$ 390.3120 $(M+H)^+$, found M_r 390.3142.

Methanolysis of Batzelladine D (4). NaOMe (25%) in MeOH solution (2 mL) was added to a solution of **4** (25 mg) in MeOH (5 mL) and the reaction mixture heated in a sealed tube at 65 °C for 16 h. The residue obtained after the workup was purified by silica gel PTLC using $MeOH:CH_2Cl_2:H_2O:HCOOH$ (10:90:0.5:0.5) as solvent system to afford a hygroscopic solid (15.5 mg) as identified by direct comparison with the sample **12** obtained from the hydrolysis of **1**.

Methanolysis of Batzelladine E (5). NaOMe (25%) in MeOH solution (1.5 mL) was added to a solution of **5** (10 mg) in MeOH (3 mL) and the reaction mixture heated at 65 °C in a sealed tube for 16 h. Residue obtained after the workup was purified by Si gel ptlc using $MeOH:CH_2Cl_2:H_2O$ (10:90:1) solvent system to yield **15** as colorless oil (5.5 mg), $[\alpha]^{25}_D$ +98.5° (*c* 0.27); UV (MeOH) λ_{max} 213 (ϵ 8498), 224 (ϵ 8893), 290 (ϵ 4902), 343 (ϵ 1669) nm; IR (KBr) 3420, 3100–3000, 1688, 1637, 1622, 1459, 1279 cm^{-1}; ^1H NMR (400 MHz, MeOH-d_4) δ 5.35–5.46 (2H, m), 4.48 (1H, dd, J = 3.8, 5.8 Hz), 3.74 (1H, m), 3.70 (s, 3H), 3.47 (1H, m), 2.30–2.75 (10H, m), 1.25–1.80 (15 H, m), 0.91 (6H, t, J = 7.3 Hz); HRFABMS calcd M_r for $C_{23}H_{38}N_3O_2$ 388.2964 $(M + H)^+$, found M_r 388.2975.

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Supplementary Material Available: Supplementary material including ^1H, ^1H–^1H COSY, ^{13}C-GASPE, HMQC, and HMBC NMR spectra for batzelladine A (**1**); ^1H, ^1H–^1H COSY, ^{13}C-GASPE, HMQC, and HMBC NMR spectra for batzelladine B (**2**); ^1H, ^1H–^1H COSY, HMQC, HMQC-TOCSY, HMBC, TOCSY-200 ms, and ^{13}C-GASPE NMR spectra for batzelladine C (**3**); ^1H and ^{13}C-GASPE NMR spectra for batzelladine D (**4**); ^1H, ^1H–^1H COSY, ^{13}C-GASPE, HMQC, and HMBC NMR spectra for batzelladine E (**5**) (47 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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