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Batzelladine alkaloids from the caribbean sponge *Monanchora unguifera* and the significant activities against HIV-1 and AIDS opportunistic infectious pathogens

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Abstract—Five new polycyclic guanidine alkaloids, 16β-hydroxycrambescidin 359 (**1**), batzelladines K, L, M, and N (**2**–**5**), along with the previously reported ptilomycalin A (**6**), crambescidine 800 (**7**), batzelladine C (**8**), and dehydrobatzelladine C (**9**), were isolated from the Jamaican sponge *Monanchora unguifera*. Their structures were assigned on the basis of detailed analysis of 1D and 2D NMR, and mass spectral data. Their activities against cancer cell lines, protozoa, HIV-1 and AIDS opportunistic infectious pathogens (AIDS-OIs) including Mtb were evaluated.

1. Introduction

Marine organisms have become an important source of novel and biologically active secondary metabolites.[1] The discovery of ptilomycalin A,[2] a unique pentacyclic guanidine alkaloid with noteworthy antiviral and antifungal activities generated considerable interest in evaluating marine invertebrates for new guanidine alkaloids followed by their total synthesis. A number of polycyclic guanidine alkaloids have been reported and include: crambescidins 800, 816, 830, and 844,[3] 13,14,15-isocrambescidin 800,[4] crambidine,[5] batzelladines A–J,[6,7] crambescidin 359, dehydrobatzelladine C, crambescidin 431,[8] and crambescidin 657[9] from sponges identified as belonging to the genera *Batzella* and *Monanchora*, and ptilomycalin A, crambescidin 800, celeromycalin, and fromiamycalin from starfishes.[10] The isolation of these compounds from such taxonomically different species raises the question of whether the compounds are of microbial origin or perhaps metabolites, which are merely sequestered by the starfish from their sponge diet. Ptilomycalin A, crambescidin 657 and its methyl ester, crambescidin 800,[11] crambescidin 359,[12] batzelladines A,[13] D,[14] E,[15] and F,[16] and dehydrobatzelladine C[17] have all been prepared through total synthesis and have lead to some revisions of configuration.

Diverse biological activities have been reported for these polycyclic guanidines including cytotoxicity,[3–5] antifungal, antimicrobial, and antiviral activities.[2,3] An interesting and highly significant aspect of polycyclic guanidine alkaloids is their ability to disrupt protein–protein interactions including HIV-1 gp120-human CD4,[6,18,19] $p56^{lck}$-CD4,[7]

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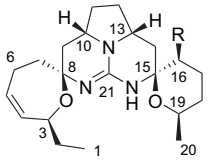
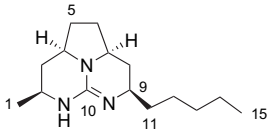
HIV-1 Nef[20] with p53, actin, and p56^{lck}. Protein–protein association events may be recognized in all aspects of cell biochemistry and small molecules that influence protein–protein association provided new biological tools and potential therapeutic agents.[21] The process of HIV-1 infection is initiated by attachment of HIV-1 to cells through a high affinity interaction between viral envelope gp120 and CD4 receptor on the surface of a T cell. Blocking the binding of gp120 to CD4 inhibits the entry of HIV-1 into the cell and thus inhibits HIV-1 replication. To date, there is no small molecular HIV entry inhibitor available on market. Batzelladines A–E are the first natural products of small molecular weight that have been shown to inhibit gp120–CD4 interaction.[6] Crambescidins 800 and 826, fromiamycalin, and synthetic batzelladine analogs were reported to inhibit HIV-1 envelope-mediated fusion.[18,31]

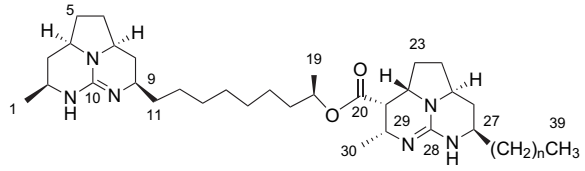
As part of our continuing efforts to identify biologically active marine natural products for development as anti-HIV-1 and anti-infective agents,[22–25] we evaluated the highly active ethanol extract of the marine sponge *Monanchora unguifera* (de Laubenfels, 1953) (Order Poecilosclerida: Family Crambeidae) with the guidance of bioassays and obtained five new polycyclic guanidine alkaloids [16β-hydroxycrambescidin 359 (**1**) and batzelladines K, L, M, and N (**2**–**5**)] and four known compounds. In this paper we describe the isolation, structure elucidation, and biological activity of these nine compounds.



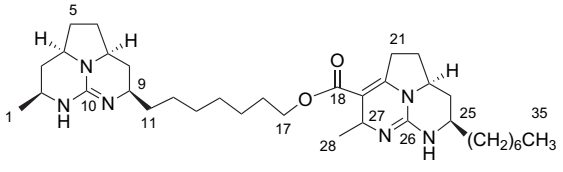
16β–Hydroxycrambescidin 359 (**1**) R=OH
Crambescidin 359 (**10**) R=H



Batzelladine K (**2**)



Batzelladine L (**3**) n=8
Batzelladine F (**11**) n=6



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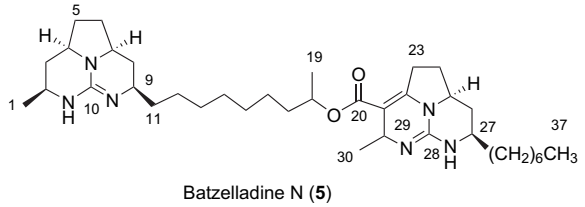
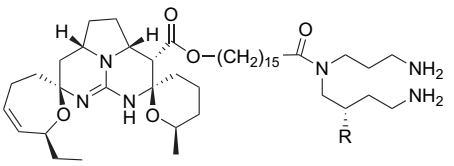
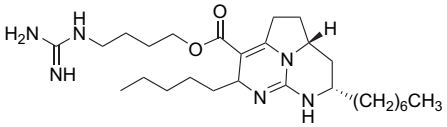


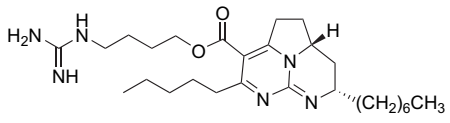
Batzelladine N (**5**)



Ptilomycalin A (**6**): R=H
Crambescidin 800 (**7**): R=OH



Batzelladine C (**8**)



Dehydrobatzelladine C (**9**)

2. Results and discussion

The sponge *M. unguifera* was collected from Discovery Bay, Jamaica in November 2002 and kept frozen until extracted. The sponge was freeze-dried and extracted with MeOH. The MeOH extract of the sponge and its hexane, $CHCl_3$, and EtOH soluble portions showed strong antimicrobial activity. Compounds **1**–**9** were isolated from the $CHCl_3$ soluble portion by silica gel column chromatography followed by Sephadex LH-20 column chromatography or reverse phase HPLC. The known compounds **6**–**9** were identified as ptilomycalin A, crambescidin 800, batzelladine C, and dehydrobatzelladine C by ESIMS and NMR spectra and by comparison with the data from the literature.[3,6,8]

16β-Hydroxycrambescidin 359 (**1**), $[\alpha]_D$ −3.0, exhibited a pseudomolecular ion at *m/z* 376 $[M+H]^+$ in the ESI mass spectrum. Its molecular formula was determined as $C_{21}H_{33}N_3O_3$ ($[M+H]^+$ *m/z* 376.2761 in positive HRESIMS). The assignments of the ^1H and ^{13}C NMR spectra of **1** are presented in Table 1 based upon the detailed analysis of the 1D and 2D NMR spectra (COSY, HMQC, and HMBC). The data exhibited characteristics for a guanidine group (δ 148.2) as well as for disubstituted double bonds (δ_C 133.1 and 130.2; δ_H 5.52 and 5.73). Comparison of these data with those of crambescidin 359[8] clearly indicated that compound **1** and crambescidin 359 (**10**) had the same pentacyclic guanidine moiety. The difference in the ^{13}C NMR spectra between the two compounds revealed the replacement of the CH_2 signal at δ_C 33.7 of crambescidin 359 (**10**) by a hydroxymethine carbon at δ 66.3. The position of the hydroxyl group was assigned by the HMBC experiment, which indicated the proton at δ 3.56 of the hydroxymethine carbon, correlated with the carbons at δ 35.3 (C-14) and 25.9 (C-17, C-18). The carbon signal at δ 66.3, in turn, correlated with H-14, H-17, and H-18. Thus the hydroxyl group could unambiguously be assigned at C-16. More evidences from ^1H–^1H correlations between H-16/H-17β, H-17β/H-18β, H-18β/H-19 supported this assignment. The relative configuration of the 16-hydroxy group was assigned to be β based

Table 1. ^{1}H and ^{13}C NMR data of 16β-hydroxycrambescidin 359 (**1**) and crambescidin 359 (**10**)

Position	Compound **1**			Crambescidin 359 (**10**)	
	^{1}H (CD_3OD, *J* in Hz)	^{13}C	NOE correlation	^{1}H($CDCl_3$, *J* in Hz)	^{13}C
1	0.85 (t, 4.0)	9.71 q	H-2α, H-2β, H-3, H-10, H-19	0.81 (t, 7.3)	10.2
2	1.57 (ddd, 13.0, 7.0, 3.5) β	29.0 t	H-1, H-2α, H-3, H-4	1.50 (m)	29.1
	1.48 (m) α		H-1, H-2β, H-3, H-4	1.42 (m)	
3	4.38 (br d, 10.0)	71.0 d	H-1, H-2α, H-2β, H-4, H-6α	4.49 (m)	70.9
4	5.52 (dt, 11.0, 2.0)	133.1 d	H-2α, H-2β, H-3, H-5	5.46 (br d, 11)	133.7
5	5.73 (ddd, 11.0, 5.0, 2.5)	130.2 d	H-4, H-6α, H-6β	5.64 (br dd, 11.8)	129.7
6	2.38 (m) α	23.2 t	H-3, H-5	2.30 (m)	23.5
	2.17 (m) β		H-5, H-7α, H-7β	2.18 (m)	
7	2.34 (m) α	36.9 t	H-6β, H-7β, H-9β	2.48 (m)	37.0
	1.99 (dd, 14.0, 6.0) β		H-6β, H-7α, H-9α	1.88 (m)	
8		84.1 s			83.5
9	2.62 (dd, 13.0, 6.0) β	36.5 t	H-7α, H-9α, H-10	2.53 (dd, 13.5)	37.3
	1.52 (m) α		H-7β, H-9β, H-11α	1.28 (dd, 13.5)	
10	4.06 (m)	53.9 d	H-1, H-9β, H-11β	3.98 (m)	53.0
11	2.36 (m) β	29.6 t	H-10, H-11α	2.27 (m)	30.0
	1.79 (m) α		H-9α, H-11β	1.65 (m)	
12	2.36 (m) β	29.7 t	H-12α, H-13	2.27 (m)	23.5
	1.81 (m) α		H-14α, H-12β	2.16 (m)	
13	3.99 (m)	52.2 d	H-12β, H-14β	3.98 (m)	51.4
14	2.56 (dd, 13.5, 4.5) β	35.3 t	H-13, H-12α, H-14α	2.16 (m)	39.7
14	1.45 (m) α		H-14β, H-16, H-12α	1.42 (m)	
15		82.9 s			80.1
16	3.56 (dd, 2.5, 2.5)	66.3 d	H-14α, H-17α, H-17β	1.75 (m)	33.7
				1.57 (m)	
17	2.10 (ddd, 14.0, 4.5, 3.0) α	25.9 t	H-16, H-17β, H-18α H-19	2.27 (m)	18.5
	1.89 (ddd, 14.0, 6.5, 3.0) β		H-16, H-17α, H-18α, H-18β	1.75 (m)	
18	1.70 (ddd, 13.5, 5.0, 2.0) β	25.9 t	H-17β, H-18α, H-20	1.65 (m)	32.3
	1.50 (m) α		H-17α, H-17β, H-18β, H-19, H-20	1.16 (m)	
19	3.80 (ddq, 11.5, 6.0, 2.0)	66.9 d	H-1, H-17α, H-18α, H-20	3.84 (m)	67.0
20	1.12 (d, 6.0)	20.5 q	H-18α, H-18β, H-19	1.03 (d, 7)	21.5
21		148.2 s			148.4

^{1}H at 400 MHz, ^{13}C at 100 MHz, residual solvent signal as internal standard.

on the 2D NOESY, coupling constants, and molecular modeling. The small coupling constant of H-16 (dd, 2.5, 2.5) and the NOE correlation with both H-17α and H-17β indicated that H-16 is equatorial to the pyran ring and 16-OH is axial. The H-14α, β, and H-12α, β were assigned based on NOE and coupling constant data (see Table 1). Since the multiring system, especially the two spiro-rings, could exist in different conformations, a conformational analysis was essential for the determination of the configurations of the 16-hydroxyl group. The conformations with minimized energy of both 16-α and 16-β epimers (Fig. 1) were obtained using Chem3DUltra 10 engaged MM2 force field. This conformation is the same as the lowest conformation obtained using Macromodel version 5.5.[11a,b] All of the distances of the NOE correlated protons in the minimized energy conformation of 16-β hydroxyl epimer (Fig. 1a) were less than 2.9 Å, including the correlation across the guanidine ring between H-1 and H-19 of the two spiro-rings. In this conformer, the distances between H-16 and H-14α, H-17α, and β are 2.56, 2.49, and 2.44 Å, respectively, corresponding to the NOE correlations between H-16 and H-14α, H-17α, and β. In the minimized energy conformation of 16-α hydroxyl epimer (Fig. 1b), the distances between the NOE correlated protons (e.g., H-16 with H-14α and H-17α) are much larger than the distances between the protons without NOE correlations (e.g., H-16 with H-14β and H-18β). From this model it is clear that H-16 is α oriented and the 16-hydroxyl group must be β. The relative configuration of the rest of the molecule was determined to be the same as crambescidin 359 (**10**) by careful comparison of the ^{1}H, ^{13}C, and NOESY NMR data.[8] The absolute configuration of the pentacyclic guanidine moieties of crambescidins and ptilomycalin A was defined using oxidative degradation of crambescidin 816,[4] and rigorously confirmed by total synthesis.[11,26] The absolute configuration was not defined for crambescidin 359, but drawn as the opposite to the others.[8] 16β-Hydroxycrambescidin 359 (**1**) is drawn following the same absolute configuration as this family but no data are available to confirm this assignment.

Batzelladine K (**2**) was isolated as a colorless gum, $[\alpha]_D$ +6.4, and displayed a pseudomolecular ion at *m/z* 250 $[M+H]^+$ in the ESI mass spectrum, which corresponded to a molecular formula of $C_{15}H_{28}N_3$ ($[M+H]^+$ at *m/z* 250.2322 in HRESIMS). ^{1}H and ^{13}C NMR spectra indicated that compound **2** belongs to the batzelladine family of compounds. The ^{1}H NMR spectrum of batzelladine K (**2**) included four methine multiplets in the region between δ 3.75 and 3.43. The ^{13}C NMR spectrum of **2** revealed a guanidine carbon at δ 151.2 and four methine signals between δ 57.6 and 47.4. All data indicated that there was one tricyclic guanidine unit in **2**. The ^{1}H and ^{13}C NMR signals assigned to the tricyclic guanidine were identical to corresponding signals for the left-hand tricyclic moiety in batzelladine F.[7a] In the upfield region of the ^{1}H NMR spectrum, there were four methylene signals and two methyl signals in addition to the signals mentioned above. In the HMBC spectrum, the methyl proton signal at δ 1.27 (d, H-1)

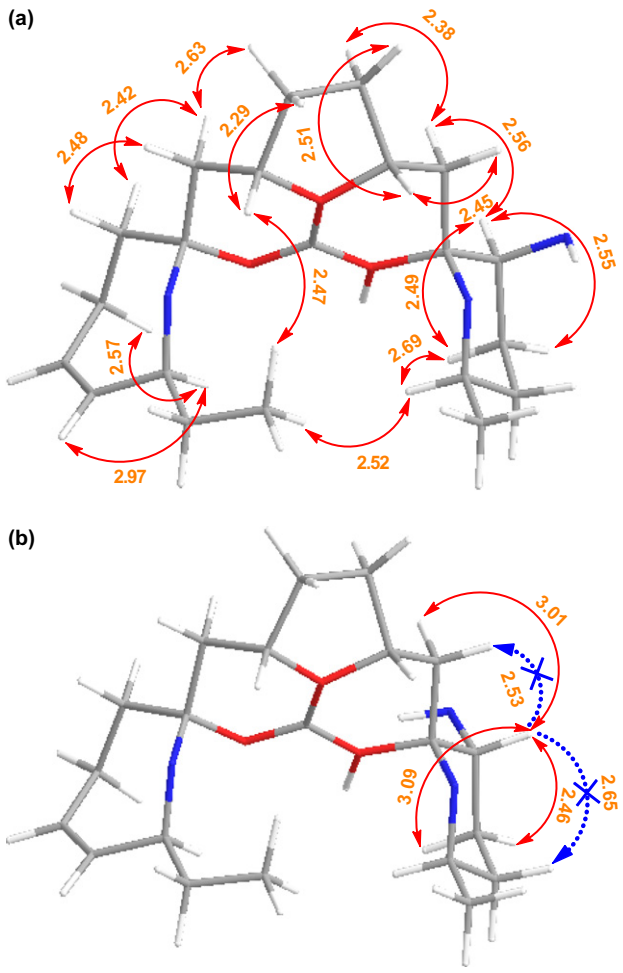
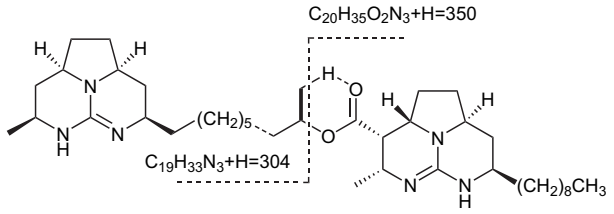


Figure 1. Conformation with minimized energy of **1**, (a) 16β-hydroxyl epimer; (b) 16-α-hydroxyl epimer. Red double headed arrows indicate the distances between NOE correlated protons in Å. Blue single headed arrows indicate the distances between protons without NOE correlation. From this data 16β-hydroxyl is clearly supported by the NOESY results.

correlated with the carbons at δ 47.4 (C-2) and 36.9 (C-3), while the proton signal at δ 3.43 (m, H-9) correlated with the carbons at δ 57.4 (C-7), 34.9 (C-8), 35.9 (C-11), and 26.0 (C-12). The methyl group at δ 1.27 was clearly located at C-2 of the tricyclic guanidine and the five-carbon aliphatic side chain attached at C-9. The NOESY experiment revealed the correlation between the protons at δ 3.55 (H-2) and 3.74 (H-4), 3.74 (H-7) and 3.43 (H-9), respectively, which showed that H-2 and H-4 were in the same face as well as H-7 and H-9. Comparison of the ^{1}H and ^{13}C NMR data of **2** with synthetic *syn*-(2aα,4β,7β,8aα)-2,2a,3,4,5,7,8,8a-octahydro-4-methyl-7-nomyl-1*H*-5,6,8a-triazacenaphthylene tetrafluoroborate and its *anti*-isomer suggested that **2** was the *syn*-tricyclic guanidine.[27]

Batzelladine L (**3**) was isolated as a colorless gum, $[\alpha]_D$ +5 and showed a weak $[M+H]^+$ peak at *m/z* 653 and a strong $[M+2H]^{++}$ peak at *m/z* 327 in the ESI mass spectrum. The molecular formula was determined to be $C_{39}H_{68}N_6O_2$ by the HRESIMS. The ^{1}H NMR spectrum of **3** showed an oxygenated methine multiplet at δ 4.97 and nine overlapping methine multiplets in the region between δ 3.96 and 3.08 (Table 2). The ^{13}C NMR spectrum revealed an ester carbonyl carbon at δ 170.4, two guanidine carbons at δ 151.5 and 151.1, an oxygenated methine signal at δ 73.4 and nine methylene signals between 57.8 and 45.4. The ^{1}H and ^{13}C NMR data (Tables 2 and 3) were identical to those of batzelladine F[7a]. The difference between batzelladines L and F was that the former had two additional CH_2 units in its molecular formula than batzelladine F. As a result, the length of the aliphatic central chain, which connected the two tricyclic guanidine units or the side chain had to be different from those of batzelladine F.[7a] A secondary mass spectrum (MS/MS) was engaged to determine the length of the chains using a Fourier transform mass spectrometer. The precursor peak $[M+2H]^{++}$ at *m/z* 327 gave two fragment peaks at 304.2310 ($C_{19}H_{34}N_3$) and 350.2304 ($C_{20}H_{36}N_3O_2$), which corresponded to the fragmentation of the ester bond through the McLafferty rearrangement. Therefore the length of the C_9 side chain could be established. The configuration of batzelladine L (**3**) was determined to be the same as batzelladine F (**11**) by comparison of its ^{1}H, ^{13}C, and NOESY NMR data with those of batzelladine F and its synthetic analogs.[16,27] The relative configuration of batzelladine F was first defined as *anti* for both tricyclic guanidine angular hydrogens,[7a] and revised to *syn* for the left-hand tricyclic guanidine by a model syntheses without providing the relative configurations of two tricyclic guanidine rings.[27] The absolute configuration including that of C18 in the central chain was determined by an enantioselective total synthesis of batzelladine F (**11**).[16]



Batzelladine M (**4**) was isolated as a colorless gum, $[\alpha]_D$ +41.1 and revealed a weak $[M+H]^+$ peak at *m/z* 595 and a strong $[M+2H]^{++}$ peak at *m/z* 298 in the ESI mass spectrum, which corresponded to a molecular formula of $C_{35}H_{58}N_6O_2$, indicating 10 degrees of unsaturation. Batzelladine M had four methylene units less and one degree of unsaturation more than batzelladine L. The ^{1}H and ^{13}C NMR signals of **4** were assigned in Tables 2 and 3 by using COSY, HMQC, and HMBC experiments. The ^{1}H NMR spectrum of **4** showed three methyl signals at δ 1.36 (d), 1.26 (d), and 0.90 (t) and seven overlapping methine multiplets between δ 4.6 and 3.4 instead of the nine observed for **3**. The ^{13}C NMR spectrum of **4** revealed five quaternary signals at δ 166.4, 151.2, 149.5, 149.3, and 103.1, an oxygenated methylene signal at δ 65.8, and seven methine signals between δ 47.3 and 58.7, which indicated that **4** contained one more double bond than **3** and is located at the guanidine ring. The ^{1}H and ^{13}C NMR signals assigned to the left-hand tricyclic ring system were identical to the corresponding signals for batzelladine L (**3**), while those signals assigned to the right-hand tricyclic ring system were identical to the corresponding signals for batzelladine C (**8**)[5]. In the HMBC experiment, the methyl signal at δ 1.36 (d, 5.2 Hz, H_3-28) was correlated with C-27 (48.6) and C-19 (103.1). Thus the double bond was positioned between C-19 and C-20.

Table 2. ^{1}H NMR data of batzelladine K–N (**2**–**5**) (400 MHz, CD_3OD, *J* in Hz)

Position	Batzelladine K (**2**)	Batzelladine L (**3**)	Batzelladine M (**4**)	Batzelladine N (**5**)
1	1.27 (d, 3H, 6.0)	1.26 (d, 3H, 6.4)	1.26 (d, 3H, 6.4)	1.26 (d, 3H, 6.4)
2	3.55 (m)	3.54 (m)	3.54 (m)	3.54 (m)
3	2.33 (m), 1.24 (m)	2.22 (m), 1.25 (m)	2.22 (m), 1.24 (m)	2.23 (m), 1.24 (m)
4	3.74 (m)	3.74 (m)	3.73 (m)	3.72 (m)
5	2.24 (m), 1.69 (m)	2.22 (m), 1.67 (m)	2.22 (m), 1.68 (m)	2.23 (m), 1.67 (m)
6	2.24 (m), 1.69 (m)	2.22 (m), 1.67 (m)	2.23 (m), 1.68 (m)	2.23 (m), 1.67 (m)
7	3.74 (m)	3.74 (m)	3.73 (m)	3.72 (m)
8	2.28 (m), 1.25 (m)	2.24 (m), 1.23 (m)	2.23 (m), 1.23 (m)	2.27 (m), 1.24 (m)
9	3.43 (m)	3.41 (m)	3.41 (m)	3.41 (m)
11	1.62 (m), 1.55 (m)	1.60 (m), 1.48 (m)	1.60 (m), 1.50 (m)	1.60 (m), 1.50 (m)
12	1.40 (m, 2H)	1.33 (m)	1.35 (m, 2H)	1.34 (m, 2H)
13	1.35 (m, 2H)	1.33 (m)	1.35 (m, 2H)	1.34 (m, 2H)
14	1.35 (m, 2H)	1.33 (m)	1.35 (m, 2H)	1.34 (m, 2H)
15	0.93 (t, 3H, 7.0)	1.33 (m)	1.35 (m, 2H)	1.34 (m, 2H)
16		1.33 (m)	1.74 (m, 2H)	1.34 (m, 2H)
17		1.60 (m), 1.54 (m)	4.15 (m, 2H)	1.60 (m), 1.54 (m)
18		4.97 (m)		4.99 (m)
19		1.24 (d, 3H, 6.4)		1.24 (d, 3H, 6.4)
21		3.08 (t, 4.0)	3.34 (m), 2.82 (m)	
22		3.96 (m)	2.33 (m), 1.78 (m)	
23		2.23 (m), 1.62 (m)	3.59 (m)	3.36 (m), 2.82 (m)
24		2.20 (m), 1.64 (m)	2.36 (m), 1.49 (m)	2.34 (m), 1.76 (m)
25		3.54 (m)	3.86 (m)	3.59 (m)
26		2.34 (m), 1.42 (m)		2.40 (m), 1.48 (m)
27		3.54 (m)	4.51 (m)	3.85 (m)
28			1.36 (d, 3H, 5.2)	
29		3.84 (m)	1.73 (m), 1.56 (m)	4.50 (m)
30		1.28 (d, 3H, 6.4)	1.35 (m, 2H)	1.36 (3H)
31		1.64 (m), 1.54 (m)	1.35 (m, 2H)	1.74 (m), 1.57 (m)
32		1.33 (m)	1.35 (m, 2H)	1.34 (m)
33		1.33 (m)	1.35 (m, 2H)	1.34 (m)
34		1.33 (m)	1.35 (m, 2H)	1.34 (m)
35		1.33 (m)	0.90 (t, 3H, 7.0)	1.34 (m)
36		1.33 (m, 2H)		1.34 (m)
37		1.33 (m, 2H)		0.90 (t, 3H, 7.0)
38		1.33 (m, 2H)		
39		0.90 (t, 3H, 7.0)		

The ester carbonyl carbon exhibited a 4 ppm upfield shift due to the conjugation with the double bond.[25] The correlation between the oxygenated methylene signal at δ 4.15 and the ester carbonyl at δ 166.4 indicated that the central chain joining the two tricyclic portions was not substituted.

The length of the central chain and the side chain was established by using the MS/MS fragmentation pattern. Fragments arising from the left-hand and right-hand ring systems were observed at *m/z* 276 and 320, respectively, in the secondary ion mass spectrum of the precursor $[M+2H]^{++}$ at *m/z* 298. Thus **4** had seven carbons in the central chain connecting the left-hand tricyclic ring to the ester oxygen and also seven carbons in the side chain. The relative configuration of all chiral centers except C-27 could be established by comparison of the ^{1}H, ^{13}C, and NOESY data with batzelladine F and related compounds.

Batzelladine N (**5**) was isolated as a colorless gum, $[\alpha]_D$ +8.0, and revealed a pseudomolecular ion at *m/z* 623 $[M+H]^{+}$ and 312 $[M+2H]^{++}$ in the ESI mass spectrum, which corresponded to a molecular formula of $C_{37}H_{62}N_6O_2$. Batzelladine N had two methylene units more than batzelladine M (**4**). Comparison of the NMR data of **5** (Tables 2 and 3) with **3** and **4**, clearly indicated that **5** had the same left-hand tricyclic guanidine unit including the central chain as **3** did and the same right-hand guanidine cyclic system as **4** did. Similarly, the secondary mass spectrum of $[M+2H]^{++}$ at *m/z* 312 gave two fragments at *m/z* 304 and 320 indicating that the two additional methylene groups in **5** were located at the central chain and the length of the side chain was C_7. All of the NMR data including NOESY indicated that batzelladine N had the same relative configuration as batzelladines L and M with the configuration of C-18 and C-29 under investigation.

Each pure compound except compound **2** was evaluated for activity against cancer cell lines, protozoa, HIV-1, and AIDS opportunistic infectious pathogens (AIDS-OIs) including Mtb. Batzelladines L (**3**), M (**4**), and C (**8**), ptilomycalin A (**6**), crambescidine 800 (**7**), and dehydrobatzelladine C (**9**) showed strong activities against AIDS-OIs *Candida albicans*, *Cryptococcus neoformans*, *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRS), *Pseudomonas aeruginosa*, *Mycobacterium intracellulare*, and *Aspergillus fumigatus* with IC_{50} values from 0.15 to 15 μg/mL (Table 4). Most promising are ptilomycalin A (**6**) and crambescidine 800 (**7**), exhibiting selective activities against *C. albicans*, *C. neoformans*, *S. aureus*, and MRS with IC_{50} values of 0.10–0.35 μg/mL, which are comparable with the positive control amphotericin B and ciprofloxacin. Both **6** and **7** were less active against *P. aeruginosa* and *A. fumigatus* with IC_{50} values of 0.95–1.25 μg/mL and had marginal activity against *M. intracellulare*. Batzelladines L (**3**) and

Table 3. ^{13}C NMR data of batzelladine K–N (**2–5**) in CD_3OD (100 MHz)

Position	Batzelladine K (**2**)	Batzelladine L (**3**)	Batzelladine M (**4**)	Batzelladine N (**5**)
1	20.8	20.8	20.8	20.9
2	47.4	47.3	47.3	47.3
3	36.9	37.0	36.9	36.9
4	57.6	57.5	57.6	57.6
5	31.2	31.1	31.2	31.2
6	31.1	31.1	31.2	31.0
7	57.4	57.4	57.5	57.5
8	34.9	34.8	34.9	34.9
9	51.7	51.6	51.7	51.7
10	151.2	151.1	151.2	150.7
11	35.9	35.9	35.9	35.9
12	26.0	26.2	26.3	26.3
13	32.9	30.5	30.6	30.6
14	23.7	30.5	30.4	30.6
15	14.4	30.5	27.2	30.5
16		26.5	29.9	26.6
17		36.8	65.8	37.1
18		73.4	166.4	72.9
19		20.5	103.1	20.5
20		170.4	149.5	166.2
21		45.6	33.0	103.2
22		57.9	31.5	149.5
23		29.2	58.7	33.0
24		31.4	33.2	31.6
25		57.3	53.6	58.7
26		34.2	149.3	33.6
27		53.2	48.6	53.6
28		151.5	24.6	149.2
29		49.9	36.0	48.6
30		18.6	26.1	24.7
31		37.0	30.3	36.0
32		26.2	30.3	26.1
33		30.5	33.0	30.3
34		30.5	23.8	30.3
35		30.5	14.5	33.0
36		30.5		23.8
37		33.0		14.6
38		23.7		
39		14.5		

C (**8**) were also active against *C. albicans*, *C. neoformans*, *S. aureus*, and MRS with IC_{50} values of 0.20–0.90 μg/mL, followed by dehydrobatzelladine C (**9**) and batzelladine M (**4**). *P. aeruginosa* and *A. fumigatus* were less sensitive to all the tested compounds. Among the tested compounds, batzelladine L (**3**) showed the best activity against *M. intracellulare* with an IC_{50} value of 0.25 μg/mL, which is slightly better than the positive control ciprofloxacin.

In the antituberculosis assay against *Mycobacterium tuberculosis* (Table 5), batzelladine L (**3**) showed the most potent activity with a MIC of 1.68 μg/mL followed by batzelladine N (**5**) with a MIC value of 3.18 μg/mL. Batzelladines L (**3**), M (**4**), N (**5**), and C (**8**), crambescidine 800 (**7**), dehydrobatzelladine C (**9**), ptilomycalin A (**6**), and 16β-hydroxycrambescidin 359 (**1**) showed marginal to none activity.

Table 5. Activity against tuberculosis and HIV-1

Compound	*M. tuberculosis* (Mtb)	Anti-HIV-1 in human PBM cells	
	MIC (μg/mL)	EC_{50} (μM)	EC_{90} (μM)
16β-Hydroxycrambescidin 359 (**1**)	>128	NT	NT
Ptilomycalin A (**6**)	>128	0.011	0.046
Batzelladine L (**3**)	1.68	1.6	7.1
Batzelladine M (**4**)	28.5	7.7	24
Batzelladine C (**8**)	34.7	7.7	25.6
Dehydrobatzelladine C (**9**)	37.7	5.5	21.8
Crambescidine 800 (**7**)	46.5	0.04	0.12
Batzelladine N (**5**)	3.18	2.4	9.0
Rifampicin	0.08		
AZT		0.0048	0.032

Ptilomycalin A (**6**) and crambescidine 800 (**7**) exhibited potent activities against human HIV-1 virus with EC_{50}/EC_{90} values of 0.011/0.046 and 0.04/0.12 μM, respectively, while batzelladines L (**3**), M (**4**), C (**8**), and N (**5**) and dehydrobatzelladine C (**9**) were less active with EC_{50} values of 1.6–7.7 μM and EC_{90} values of 7.1–25.6 μM.

Batzelladines L (**3**), M (**4**), and C (**8**), ptilomycalin A (**6**), crambescidine 800 (**7**), and dehydrobatzelladine C (**9**) were evaluated against protozoa (Table 6). Each metabolite exhibited antimalarial activity against *Plasmodium falciparum* D6 clone and W2 clone with IC_{50} values ranging from 73–270 ng/mL, which is within about an order magnitude of the controls artemisinin and chloroquine. Ptilomycalin A (**6**) and crambescidine 800 (**7**) exhibited toxicity to the normal Vero cell line (monkey kidney cell), while the remaining compounds did not show cytotoxicity at the highest tested concentration of 4.7 μg/mL. Batzelladine L (**3**) showed comparable activity with pentamidine and amphotericin B against leishmania, with an IC_{50} value of 1.9 μg/mL.

Batzelladines L (**3**), M (**4**), C (**8**), and N (**5**), ptilomycalin A (**6**), crambescidine 800 (**7**), and dehydrobatzelladine C (**9**) were tested against 11 different cancer cell lines including prostate DU-145, ovary IGROV, breast SK-BR3, melanoma SK-MEL-28, NSCL A549, leukemia L-562, pancreas

Table 4. Antimicrobial activity

Compound	*C. albicans* IC_{50}/MIC	*C. neoformans* IC_{50}/MIC	*S. aureus* IC_{50}/MIC	MRS IC_{50}/MIC	*P. aeruginosa* IC_{50}/MIC	*M. intracellulare* IC_{50}/MIC	*A. fumigatus* A.C.
Ptilomycalin A (**6**)	0.15/1.25	0.10/1.25	0.25/0.63	0.30/0.63	1.0/2.5	10.0/20.0	1.25
Batzelladine L (**3**)	0.40/5.0	0.55/5.0	0.35/1.25	0.40/0.63	3.5/20.0	0.25/0.31	2.5
Batzelladine M (**4**)	6.0/10.0	8.0/10.0	3.0/5.0	5.0/10.0	NA	3.5/10.0	NA
Batzelladine C (**8**)	0.90/2.5	0.40/2.5	0.20/0.63	0.30/1.25	10.0/20.0	0.90/2.5	5.0
Dehydrobatzelladine C (**9**)	1.0/2.5	0.60/5.0	0.40/0.63	0.70/1.25	NA	1.0/2.5	20.0
Crambescidine 800 (**7**)	0.15/1.25	0.10/0.63	0.20/1.25	0.35/0.63	0.95/2.5	15.0/20.0	1.25
16β-Hydroxycrambescidin 359 (**1**)	NA	NA	NA	NA	NA	NA	NA
Amphotericin B	0.20/0.63	0.75/1.25	NT	NT	NT	NT	NT
Ciprofloxacin	NT	NT	0.15/0.31	0.15/0.31	0.07/0.31	0.35/1.25	0.63

IC_{50}/MIC in μg/mL; MRS=methicillin-resistant *S. aureus*; A.C. (active concentration) is the lowest concentration that significantly inhibits growth; NA=not active. IC_{50} values >15 μg/mL are considered inactive; NT=not tested.

Table 6. Antiprotozoal activity

Compound	*P. falciparum* D6 clone		*P. falciparum* W2 clone		*L. donovani*		Cytotoxicity (Vero)
	IC_{50} (ng/mL)	S.I.	IC_{50} (ng/mL)	S.I.	IC_{50} (μg/mL)	IC_{90} (μg/mL)	TC_{50} (μg/mL)
16β-Hydroxycrambescidin 359 (**1**)	3800	>1.3	NA		NA	NA	NC
Ptilomycalin A (**6**)	120	<4.4	110	>43.3	5.9	10	<0.53
Batzelladine L (**3**)	73	>65.2	96	49.6	1.9	10	NC
Batzelladine M (**4**)	210	>22.7	270	>17.6	8.5	>10	NC
Batzelladine C (**8**)	90	>52.9	110	>43.3	5.5	10	NC
Dehydrobatzelladine C (**9**)	73	>65.2	130	>36.6	5.7	10	NC
Crambescidine 800 (**7**)	110	<4.8	130	>36.6	6.8	>10	<0.53
Chloroquine	15.5		80				
Artemisinin	6.5		4.1				
Petamidine					1.7	7	
Amphotericin B					1.8	2.5	

NC=no cytotoxicity, values >4.7 μg/mL are considered not cytotoxic. S.I. (selectivity index)=TC_{50} (Vero cell)/IC_{50}.

PANCL, colon HT-29, LOVO, LOVO-DOX, and cervix HeLa (Table 7). Ptilomycalin A (**6**) and crambescidine 800 (**7**) showed significant growth inhibition of 11 cell lines with GI_{50} values of 0.04–0.19 μg/mL. Batzelladine L (**3**) exhibited good activity against DU-145, IGROV, SK-BR3, leukemia L-562, PANCL, HeLa, SK-MEL-28, A549, HT-29, LOVO, and LOVO-DOX cell lines with GI_{50} values of 0.23–4.96 μg/mL. Batzelladines M (**4**), C (**8**), and N (**5**), ptilomycalin A (**6**), crambescidine 800 (**7**), and dehydrobatzelladine C (**9**) were active against 11 different cancer cell lines.

In conclusion, both batzelladine (tricyclic guanidine ring) and ptilomycalin (pentacyclic guanidine ring) type of polyguanidine alkaloids exhibited very potent activity against cancer, protozoa, HIV-1, and AIDS opportunistic infectious pathogens including Mtb. Ptilomycalin A (**6**) and crambescidine 800 (**7**) both with the pentacyclic guanidine ring system showed the most potent activities against HIV-1, *C. albicans*, *C. neoformans*, *S. aureus*, MRS, and 11 cancer cell lines, however, they were not active against *M. intracellulare* and *M. tuberculosis*. Ptilomycalin A (**6**) and crambescidine 800 (**7**) showed similar activities in all assays, suggesting that the hydroxyl group at C-43 in crambescidine 800 (**7**) did not affect the bioactivity. Surprisingly, 16β-hydroxycrambescidin 359 did not show antimicrobial or anti-HIV activity, suggesting that the 16-hydroxyl group diminished the activity. The tricyclic polyguanidine alkaloids, **3**, **4**, **8**, and **9** are less active against *C. albicans*, *C. neoformans*, *S. aureus*, and MRS than ptilomycalin A (**6**) and crambescidine 800 (**7**), but are more active against *M. intracellulare* and *M. tuberculosis*. The tested tricyclic polyguanidine alkaloids are less potent against HIV-1 and cancer than the pentacyclic polyguanidine alkaloids while they are slightly more active against the malaria parasite *P. falciparum*. Despite the variation of the activity observed amongst the tricyclic polyguanidine alkaloids in all of the assays, this variation does not seem to be related to the number of tricyclic guanidine rings. Ptilomycalin A (**6**), crambescidine 800 (**7**), and batzelladine L (**3**) are clearly exciting leads against HIV-1, AIDS-OIs, cancer, and protozoa providing significant opportunities for SAR and lead optimization studies.

Table 7. Cytotoxicity to tumor cell lines (μg/mL)

Compound		Prostate	Ovary	Breast	Melanoma	NSCL	Leukemia	Pancreas	Colon			Cervix
		DU-145	IGROV	SK-BR3	SK-MEL-28	A549	L-562	PANCL	HT29	LOVO	LOVO-DOX	HeLa
Ptilomycalin A (**6**)	GI_{50}	0.05	0.04	0.07	0.03	0.08	0.04	0.04	0.03	0.05	0.05	0.04
	TGI	1.22	1.74	0.54	0.11	1.23	1.33	0.99	0.19	2.14	2.04	0.22
	LC_{50}	5.21	n.d.	n.d.	0.98	9.79	9.72	0.98	5.37	9.79	8.48	3.21
Batzelladine L (**3**)	GI_{50}	0.44	0.52	0.23	0.88	1.30	n.d.	0.34	4.96	1.09	n.d.	0.38
	TGI	1.39	1.74	0.56	2.18	9.99	n.d.	1.33	n.d.	2.41	n.d.	1.16
	LC_{50}	3.78	5.01	2.10	4.95	n.d.	n.d.	4.38	n.d.	5.36	n.d.	3.58
Batzelladine M (**4**)	GI_{50}	1.77	2.28	1.12	1.18	3.80	n.d.	1.22	3.56	1.99	n.d.	1.64
	TGI	3.44	5.08	2.51	4.66	n.d.	0.00	3.58	n.d.	3.56	n.d.	3.05
	LC_{50}	6.66	n.d.	5.59	n.d.	n.d.	0.00	n.d.	n.d.	6.37	n.d.	5.68
Batzelladine C (**8**)	GI_{50}	0.68	0.81	0.66	1.45	1.40	0.62	0.55	0.65	2.06	2.25	0.70
	TGI	2.27	3.43	2.89	3.67	3.42	3.01	2.03	2.16	4.37	4.72	2.22
	LC_{50}	0.69	n.d.	n.d.	9.24	8.31	n.d.	7.14	6.70	9.29	0.99	6.50
Dehydrobatzelladine C (**9**)	GI_{50}	0.46	0.73	0.23	0.89	1.19	0.48	0.43	0.48	1.60	2.07	0.48
	TGI	1.91	4.17	1.14	3.48	3.24	2.42	1.83	1.77	3.68	4.48	1.52
	LC_{50}	7.15	n.d.	n.d.	n.d.	8.81	n.d.	8.66	7.50	8.47	9.69	5.45
Crambescidine 800 (**7**)	GI_{50}	0.19	0.05	0.16	0.04	0.11	0.02	0.04	0.04	0.08	0.08	0.05
	TGI	1.38	2.50	0.56	0.11	1.36	0.06	1.53	0.23	2.29	2.02	0.21
	LC_{50}	7.01	n.d.	n.d.	1.70	9.68	6.73	8.66	5.75	8.97	8.50	1.58
Batzelladine N (**5**)	GI_{50}	1.39	1.78	1.12	1.47	1.94	0.66	1.37	1.31	1.96	4.42	0.59
	TGI	3.12	4.97	3.84	3.41	4.29	3.27	3.50	3.11	4.16	n.d.	1.80
	LC_{50}	7.04	n.d.	n.d.	7.97	9.47	n.d.	8.97	7.35	8.85	n.d.	5.13

n.d.=not determined.

3. Experimental

3.1. General experiment procedures

UV spectra were recorded using a Hewlett-Packard 8452A diode array spectrophotometer. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. CD spectra were recorded on a JASCO J-715 spectropolarimeter. NMR spectra were recorded on Bruker Avance DRX-400 and 500 spectrometers. The ESI-FTMS was acquired on a Bruker-Magnex BioAPEX 30 es ion cyclotron Fourier transform mass spectrometer by direct injection into an electrospray interface. MS/MS analysis was carried out by the isolation of the trapped $[M+2H]^{++}$ peak in the ion cyclotron resonance (ICR) cell using selective Rf sweeps to eject all other ions. After the isolation was optimized, argon collision gas was introduced to increase the pressure in the cell from 4×10^{-10} to 5×10^{-8}. A concurrent on-resonance excitation pulse was optimized, so the two fragment ions and the precursor ion were about the same magnitude. HPLC was carried out on a Waters 510 model system, using column 1[Luna C8 (2), 5 μm, 250×21.5 mm; flow rate, 6 mL/min] and column 2 [Luna C8 (2), 15 μm, 250×100 mm; flow rate, 100 mL/min; detector wavelength, 210, 230 nm].

3.2. Sponge collection and taxonomy

The sponge was collected from several locations in Discovery Bay, Jamaica, from about 40 m depth, in November 2002. The morphology is shrub-like or bushy, and the texture is very fibrous and tough. Some specimens were encrusting. The color in life is bright red. The skeleton consists of straight subtylostyles 200–270 μm long embedded in thick spongin fibers. Microscleres typical of the species are absent. The sponge is *M. unguifera* (de Laubenfels, 1953) (Order Poecilosclerida: Family Crambeidae). A voucher specimen has been deposited in the Natural History Museum, London (BMNH 2000.7.17.3).

3.3. Extraction and isolation

Freeze-dried sponge *M. unguifera* of 1.8 kg was extracted four times with 6000 mL of MeOH in a blender. The combined extracts were concentrated in vacuo until dried. The residue (380 g) was extracted with hexane, $CHCl_3$, and EtOH, respectively. The $CHCl_3$ soluble part (155 g) was subjected to vacuum liquid chromatography using 2000 g of silica gel and eluting with $CHCl_3$, $CHCl_3$–MeOH, and MeOH to afford 61 fractions (fr. 1–61).

Fractions 10–11 (19.3 g) were combined and rechromatographed on a silica gel column eluted with a gradient of $CHCl_3$–acetone–MeOH from 95:5:0 to 80:20:30 to give 68 sub-fractions (subfr.1–68).

Sub-fractions 14–25 were combined and rechromatographed using silica gel [eluant: $CHCl_3$–acetone–MeOH–HOAC (95:3:2:0.5)], Sephadex LH-20 [$CHCl_3$–MeOH (1:1)] and reverse phase HPLC [column 1, eluting with MeOH–0.1%TFA (60:40), over 60 min], respectively, to yield 16β-hydroxycrambescidin 359 (**1**, 20.2 mg) and batzelladine K (**2**, 3.0 mg).

Sub-fractions 32–41 were combined and subjected to chromatography on a silica gel column [eluting with a gradient of $CHCl_3$–acetone–MeOH–HOAc from 95:3:2:0.5 to 75:15:15:0.5] and reverse phase HPLC [column 1, eluting with MeOH–0.1%TFA from 60:40 to 70:30] to obtain batzelladines N (**5**, 7.2 mg), M (**4**, 10.9 mg), and L (**3**, 12.0 mg).

Fractions 32–42 were combined and subjected to repeat chromatography on HPLC column 2 [eluting with MeOH–0.1%TFA from 55:45 to 70:30] and column 1 to yield batzelladine C (**8**, 38 mg) and dehydrobatzelladine C (**9**, 42 mg).

Fractions 49–53 were combined and subjected to chromatography on a Sephadex LH-20 column eluting with MeOH and silica gel column eluting with $CHCl_3$–MeOH–HCOOH–H_2O (80:20:3:2) to give ptilomycalin A (**6**, 2.8 g) and crambescidin 800 (**7**, 1.8 g).

3.3.1. 16β-Hydroxycrambescidin 359 (1). Colorless crystal; HRESIMS 376.2617 $[M+H]^+$ (calcd for $C_{21}H_{34}N_3O_3$: 376.2600); $[\alpha]_D$ −3.0 (*c* 0.10, MeOH); UV max (nm) 208 (ε=6334); CD $[\theta]_{201}$=−36,967, $[\theta]_{207}$=+15,243, $[\theta]_{233}$=+3232, $[\theta]_{237}$=−7198 (*c* 1.7×E-4, MeOH); for ^{1}H and ^{13}C NMR data see Table 1.

3.3.2. Batzelladine K (2). Colorless gum; HRESIMS 250.2322 $[M+H]^+$ (calcd for $C_{15}H_{28}N_3$: 250.2283); $[\alpha]_D$ +6.4 (*c* 0.14, MeOH); UV max (nm) 208 (ε=2879); CD $[\theta]_{209}$=+1235, $[\theta]_{226}$=+930, $[\theta]_{233}$=+1525, $[\theta]_{237}$=−3602, $[\theta]_{241}$=+1899 (*c* 3.5×E-4, MeOH); for ^{1}H and ^{13}C NMR data see Tables 2 and 3.

3.3.3. Batzelladine L (3). Colorless gum; HRESIMS 653.5458 $[M+H]^+$ (calcd for $C_{39}H_{69}N_6O_2$: 653.5482), 327.2798 $[M+2H]^{++}$ (calcd for $C_{39}H_{70}N_6O_2$: 327.2780); $[\alpha]_D$ +5 (*c* 0.1, MeOH); UV max (nm) 212 (ε=18,443); CD $[\theta]_{206}$=−13,569, $[\theta]_{224}$=−11,668, $[\theta]_{229}$=−13,374, $[\theta]_{237}$=−19,046, $[\theta]_{244}$=−5046 (*c* 9.6×E-5, MeOH); for ^{1}H and ^{13}C NMR data see Tables 2 and 3.

3.3.4. Batzelladine M (4). Colorless gum; HRESIMS 298.2399 $[M+2H]^{++}$ (calcd for $C_{35}H_{60}N_6O_2$: 298.2389); $[\alpha]_D$ +41.1 (*c* 0.09, MeOH); UV max (nm) 210 (ε=14,601), 258 (ε=10,203), 298 (ε=3532); CD $[\theta]_{207}$=+29,294, $[\theta]_{214}$=+11,401, $[\theta]_{232}$=+3722, $[\theta]_{237}$=−18,763, $[\theta]_{244}$=−5922, $[\theta]_{262}$=+15,499, $[\theta]_{310}$=+4891 (*c* 9.4×E-5, MeOH); for ^{1}H and ^{13}C NMR data see Tables 2 and 3.

3.3.5. Batzelladine N (5). Colorless gum; HRESIMS 312.2546 $[M+2H]^{++}$ (calcd for $C_{37}H_{64}N_6O_2$: 312.2545); $[\alpha]_D$ +8.0 (*c* 0.10, MeOH); UV max (nm) 206 (ε=13,340), 260 (ε=4320), 296 (ε=4184); CD $[\theta]_{205}$=+31,137, $[\theta]_{225}$=+8868, $[\theta]_{232}$=+7324, $[\theta]_{237}$=−14,482, $[\theta]_{244}$=−9264 (*c* 1.0×E-4, MeOH); for ^{1}H and ^{13}C NMR data see Tables 2 and 3.

3.4. Bioassay

3.4.1. Cytotoxic assay. (a) Cell culture cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 units/mL

penicillin and streptomycin at 37 °C and 5% CO_2. (b) Determination of cell survival: cells were washed twice with PBS, fixed for 15 min in 1% glutaraldehyde solution, rinsed twice in PBS, and stained in 0.4% sulforhodamine B (SRB) solution for 30 min at room temperature. Cells were then rinsed several times with 1% acetic acid solution and air-dried. SRB was then extracted in 10 mM trizma base solution and the absorbance measured at 490 nm. Cell survival was estimated using the National Cancer Institute (NCI) algorithm. Three dose–response parameters are calculated for each experimental agent. GI_{50}, compound concentration that produces 50% inhibition on cell growth as compared to control cells. TGI, compound concentration that produces total growth inhibition as compared to control cells. LC_{50}, compound concentration that produces 50% cell death as compared to control cells.

3.4.2. Antituberculosis assay. Primary assay is conducted at 6.25 μg/mL against *M. tuberculosis* H_{37}Rv (ATCC 27294) in BACTEC 12B MEDIUM using a broth microdilution assay (the Microplate Alamar Blue Assay).[28]

3.4.3. Anti-HIV assay. Anti-HIV-1 activity was determined in PBM cells as described previously.[29] Stock solutions (20 or 40 mM) of the compounds were prepared in sterile DMSO and then diluted to the desired concentration in growth medium. Cells were infected with the prototype HIV-1_{LAV} at a multiplicity of infection of 0.1. Details on the infection of cells and assessment of antiviral effects have been previously described.[30]

3.4.4. Antiparasitic assay. Antimalarial activity of the compounds was determined in vitro on chloroquine sensitive (D6, Sierra Leone) and resistant (W2, IndoChina) strains of *P. falciparum*. The 96-well microplate assay is based on evaluation of the effect of the compounds on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity.[31] The appropriate dilutions of the compounds were prepared in DMSO and added to the cultures of *P. falciparum* (2% hematocrit, 2% parasitemia) set-up in clear flat bottomed 96-well plates. The plates were placed into the humidified chamber and flushed with a gas mixture of 90% N_2, 5% CO_2 and 5% O_2. The cultures were incubated at 37 °C for 48 h. Growth of the parasite in each well was determined by pLDH assay using Malstat® reagent. The medium and RBC controls were also set-up in each plates. The standard antimalarial agents, chloroquine and artemisinin, were used as the positive controls while DMSO was tested as the negative control.

Antileishmanial activity of the compounds was tested in vitro on a culture of *Leishmania donovani* promastigotes. In a 96-well microplate assay, compounds with appropriate dilution were added to the leishmania promastigotes culture (2×10^6 cell/mL). The plates were incubated at 26 °C for 72 h and growth of leishmania promastigotes was determined by Alamar blue assay.[32] Pentamidine and amphotericin B were used as the standard antileishmanial agents. All the analogs were simultaneously tested for cytotoxicity on Vero (monkey kidney fibroblast) cells by Neutral Red assay.[33] IC_{50} values for each compound were computed from the growth inhibition curve.

4. Supporting information available

Detailed NMR data for compounds **1**–**5** including ^1H, ^{13}C, COSY, HMQC, and HMBC and copies of the ^1H and ^{13}C NMR spectra of **1**–**3** were available free of charge via the internet at http://pubs.acs.org.

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