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POLYCYCLIC GUANIDINE ALKALOIDS FROM THE MARINE SPONGE *CRAMBE CRAMBE* AND Ca^{++} CHANNEL BLOCKER ACTIVITY OF CRAMBESCIDIN 816

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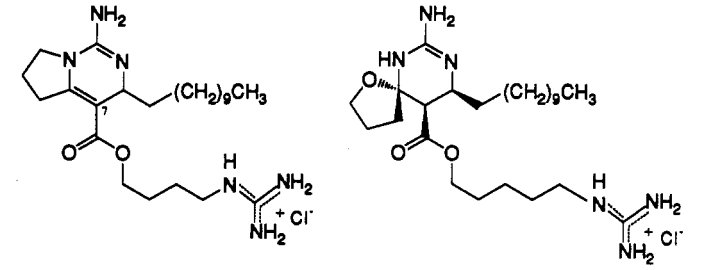
ABSTRACT.—Four pentacyclic guanidine derivatives (crambescidin 800 [**5**], crambescidin 816 [**6**], isocrambescidin 800 [**9**], and crambidine [**10**]) related to ptilomycalin A [**11**] have been isolated from the Mediterranean sponge *Crambe crambe*. Isocrambescidin 800 and crambidine are new derivatives, the structures of which have been determined on the basis of their spectral properties. The absolute configuration of crambescidin 816 at the stereogenic center C-43 has been determined by applying Mosher's method. Pharmacological and biological activities of the *Crambe crambe* alkaloids are reported. In particular, crambescidin 816 was found to have a potent Ca^{++} antagonist effect and to inhibit the acetylcholine-induced contraction of guinea pig ileum at very low concentrations.

Crambe crambe Schmidt (Poecilosclerida, Crambidae) is a bright red encrusting sponge very common between −1 and −20 m along the rocky coasts of the Mediterranean. Generally devoid of epizoites, it induces necrosis of the tissues of other sponges when they are kept in contact (1). The MeOH extract of *C. crambe* is highly ichthyotoxic and inhibits the reaggregation of cells of the fresh water sponge *Ephydatia fluviatilis* (2). It exhibits also potent antibacterial and antifungal activities (3,4).

In previous papers we reported the isolation of four bisguanidine alkaloids from three different samples of *C. crambe*: crambines A [**1**], B [**2**], C1 [**3**], and C2 [**4**], having an unprecedented skeleton (5,6). [According to the results obtained recently by Snider and Shi (12), the configuration at C-8 for crambine B has been revised as represented in **2**]. Independently, a family of complex pentacyclic guanidines linked by a linear ω-hydroxy fatty acid to a hydroxyspermidine moiety (crambescidins 800 [**5**], 816 [**6**], 830 [**7**], and 844 [**8**]) have been obtained by Jares-Erijman *et al.* (7) from samples of the same sponge. The structures and relative configurations of the latter are closely related to those of ptilomycalin A [**11**] isolated by Kashman *et al.* (8) from the Caribbean sponge *Ptilocaulis spiculifer* and from a red *Hemimycale* sp. of the Red Sea. Herein we describe the isolation of further components from the toxic extract of samples of *C. crambe* collected near Banyuls and Favignana.

As reported in our previous papers, the *n*-BuOH-soluble material of the MeOH extract of the Banyuls sample was submitted to two successive chromatographies on Sephadex LH-20, leading to five main fractions (5,6). From one of these fractions, we isolated the crambines A, B, and C1. The first-eluting fraction, which contained about one third of the weight of the *n*-BuOH extract, was found to be a complex mixture of ninhydrin-positive polar compounds that were acetylated before separation. This led to the isolation of four peracetyl derivatives **5a**, **6a**, **9a**, and **10a**.

The molecular formula $C_{51}H_{87}N_6O_9$ of the peracetylated derivative **5a**, assigned by



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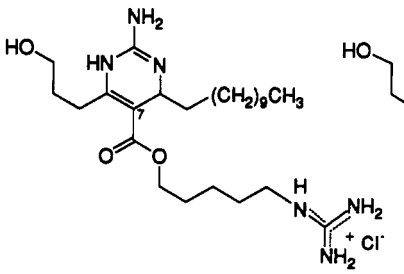

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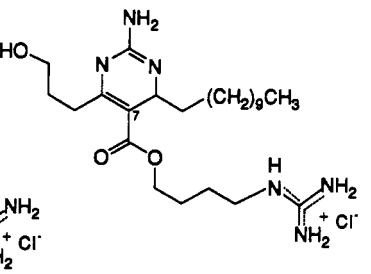

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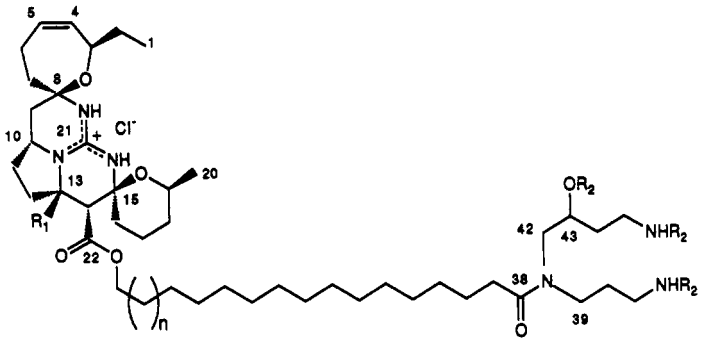
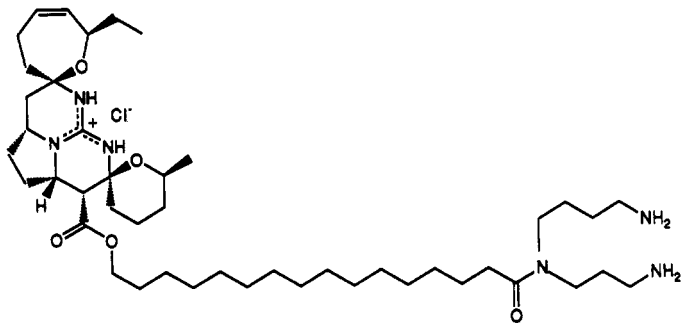
positive ion hrfabms, was identical to that of 43-*O*-acetyl-41,45-*N,N'*-diacetylcrambescidin 800, suggesting that compound **5** is crambescidin 800 (7). This was confirmed by extensive 1D and 2D nmr experiments (DQF COSY and HMQC) in $CDCl_3$. All the assignments proposed by Jares-Erijman *et al.* (7) for the ^{1}H-nmr spectrum of 43-*O*-acetyl-41,45-*N,N'*-diacetylcrambescidin 800 in CD_3OD were confirmed, except that the assignment of H-10 and H-13 should be reversed in Jares-Erijman *et al.* (7).

The tlc behavior and the ^{1}H-nmr spectrum of **6a** were identical to those of the derivative prepared by acetylation of a compound, $[\alpha]_D$ −16.5° (MeOH, c=0.4), isolated from a sample of *C. crambe* collected off Favignana. The ^{1}H- and ^{13}C-nmr data of the latter were entirely compatible with those reported for crambescidin 816 [**6**] (7). Although all the nmr assignments proposed by Jares-Erijman *et al.* (7) for crambescidin 816 could be confirmed by a series of 2D nmr experiments at 500 and 600 MHz (COSY, H,C-COSY; HOHAHA; HMBC; HMQC), several supplementary peaks of lower intensities were observed in our spectra (see Experimental) that were not mentioned by the Urbana group. These peaks resulted from the existence of a mixture of rotational isomers (about 4:1) at the C-38 amide bond. A similar observation was reported by Kashman *et al.* (8) for ptilomycalin A.

We have now established the absolute configuration of crambescidin 816 at the stereogenic center C-43 that remained undetermined. For this purpose we have applied Mosher's method for the determination of absolute configuration of secondary carbinols (9). This method is based on derivatization with (*R*)- and (*S*)-2-methoxy-2-trifluoromethyl-2-phenylacetic acid (MTPA). Comparison of the ^{1}H-nmr spectra of the (*R*)- and (*S*)-MTPA esters, and in particular of the chemical shifts of the C-42 and C-44 methylene protons, allowed us to assign the *S* configuration to C-43 in crambescidin 816 [**6**] (Table 1). Analysis of the ^{1}H-nmr spectra of the two diastereoisomeric 43-MTPA esters was performed by Homonuclear Decoupling Difference experiments, which allowed simplification of the very complex regions of interest and clear identification of the C-42 and C-44 methylene proton signals of each conformer in both derivatives by successive



5	R_1=H, R_2=H, n=1
5a	R_1=H, R_2=Ac, n=1
6	R_1=OH, R_2=H, n=1
6a	R_1=OH, R_2=Ac, n=1
7	R_1=OH, R_2=H, n=2
8	R_1=OH, R_2=H, n=3



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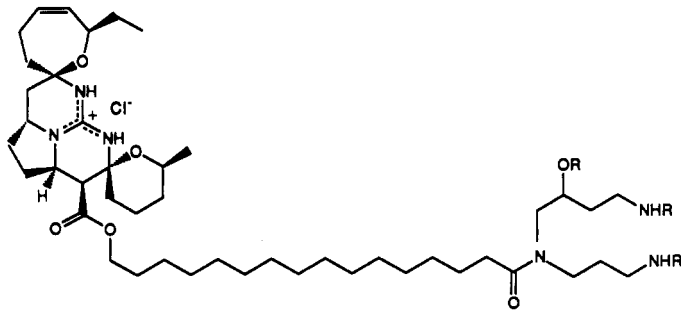
irradiation of the H-43 signals. In the ^{1}H-nmr spectrum of the (*R*)-MPTA derivative, the C-42 methylene proton signals of both conformers were upfield relative to the corresponding ones in the ^{1}H-nmr spectrum of the (*S*)-MTPA derivative. A reverse behavior was observed for the C-44 methylene proton signals. According to Mosher's configurational correlation models, such a behavior is expected for the (*S*)-43 stereoisomer (9).

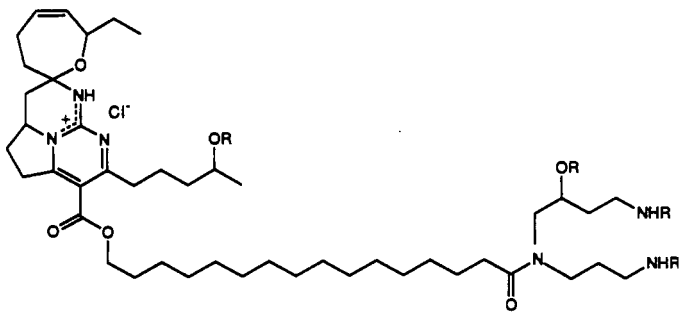
The molecular formula $C_{51}H_{86}N_6O_9$ of derivative **9a** was determined by positive ion hrfabms, $[MH]^+$ 927.6521 (calcd 927.6534). Derivative **9a** is thus an isomer of **5a**.

TABLE 1. Chemical Shifts of H-42, H-43, and H-44 of the (*R*)- and (*S*)-MTPA Esters of Crambescidin 816 (δ, $CDCl_3$, 500 MHz).

Compound	Proton				
	H-42α	H-42β	H-43	H-44α	H-44β
(*R*)-MTPA ester					
major conformer	3.59	3.06	5.22	1.88	1.84
minor conformer	3.47	3.29	5.08	1.91	1.88
(*S*)-MTPA ester					
major conformer	3.62	3.40	5.32	1.86	1.76
minor conformer	3.52	3.33	5.28	1.87	1.79



9 R=H
9a R=Ac



10 R=H
10a R=Ac

Extensive 1D and 2D nmr experiments (DQF COSY; H,C-COSY; HMBC; HMQC) indicated identical atom connectivities for both compounds, suggesting that they are indeed stereoisomers. The ^{1}H- and ^{13}C-nmr signals corresponding to the aliphatic part of the molecules were nearly identical. In contrast, significant differences were found for most of the signals attributable to the atoms of the polycyclic moiety (Table 2), indicating that the configurations of some of the asymmetric centers of this moiety may be different for the two isomers. The relative configurations at C-3, C-8, and C-10 were the same for the two derivatives as shown by the observation of nOe's (differential nOe and ROESY) between H-1 and H-10, H-3 and the NH at δ 9.9, H-3 and H-7, H-10 and H-9β, H-10 and H-11β (Figure 1). The close similarity of the chemical shifts of C-1 to C-7 also supports this conclusion. On the other hand, in contrast to ptilomycalin A (8), crambescidin 816 (7), and **5a**, no nOe was observed between H-10 and H-13, suggesting that the configuration of **9a** at C-13 is inverted compared with that of **5a**. Moreover, nOe's between H-13 and H-14, H-13 and H-20, H-14 and H-19 (Figure 1) imply that the configurations at C-14 and C-15 in **9a** should also be inverted in comparison with **5a**. Thus, we propose structure **9** and the name isocrambescidin 800 for this stereoisomer of **5**.

The acetylated derivative **10a** was assigned the molecular formula $C_{53}H_{86}N_6O_{10}$ by positive ion hrfabms, $[MH]^+$ 967.6415 (calcd 967.6483), indicating 14 degrees of unsaturation, that is, two more than for **6a**. The uv spectrum displays maxima at 254 (22,000), 299 (5300), 315 (3100), and 325 nm (2500), suggesting the presence of a heteroaromatic chromophore. Structure **10a** is consistent with these data. It corresponds to 43-*O*-acetyl-41,45-*N,N'*-diacetylcrambescidin 816 [**6a**] having lost a molecule of H_2O and in which the tetrahydropyrane ring is open. The nmr data fully support this hypothesis. Indeed, all the signals attributable to the tetrahydrooxepine and the

TABLE 2. Comparison of the Nmr Data of **5a** and **9a** (δ, $CDCL_3$, 600 and 150 MHz).

Location	Compound			
	5a		**9a**	
	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H
1	10.1	0.83 (t, 7.2)	10.2	0.99 (t, 7.3)
2	29.8[a]	1.48 (m), 1.52 (m)	29.1	1.52 (m)
3	71.0	4.49 (m)	70.7	4.53 (m)
4	133.6	5.49 (dt, 10.5, 2.5)	133.5	5.49 (dt, 11, 2.4)
5	129.8	5.67 (ddt, 10.5, 7.2, 2.5)	129.5	5.65 (ddt, 11, 7, 2.5)
6α	23.5[a]	2.16 (m)	24.0[a]	2.18 (m)
6β		2.34 (m)		2.30 (m)
7α	37.0[b]	2.54 (m)	37.3	2.58 (m)
7β		1.92 (m)		1.84 (m)
8	83.7		85.1	
9α	36.9[b]	1.40 (t, 11.7)	37.3	1.40 (t, 12.6)
9β		2.56 (dd, 11.7, 3.6)		2.55 (dd, 12.6, 2.2)
10	53.9	4.02 (m)	52.9	3.92 (ddt, 12.6, 5.3, 2.2)
11α	30.6	1.65 (m)	32.5	1.62 (m)
11β		2.27 (m)		2.22 (m)
12α	27.0[a]	1.80 (m)	29.5	1.75 (m)
12β		2.27 (m)		2.20 (m)
13	51.9	4.30 (ddd, 10, 4.8, 4.9)	52.9	4.02 (m)
14	48.9	2.94 (d, 4.8)	52.1	3.47 (d, 3.6)
15	80.7		83.1	
19	67.2	3.93 (m)	69.2[b]	3.71 (m)
20	21.4	1.05 (d, 6.5)	21.0	1.19 (d, 6)
21	148.8		149.1	
22	168.8		167.8	
23	65.5	4.12 (m), 4.07 (m)	65.3	4.02 (m), 4.16 (m)
24	28.5	1.62 (m)		1.60 (m)
26–36	[d]	1.28 (m)	[d]	1.28 (m)
38	174.5		174.5	
39	42.6	3.30 (m), 3.60 (m)	42.6	3.25 (m), 3.60 (m)
40	26.5	1.65 (m)	26.5	1.66 (m)
41	36.5[c]	3.10 (m)	35.5[c]	3.10 (m)
42	50.5	3.30 (m), 3.54 (m)	51.0	3.25 (m), 3.60 (m)
43	70.8	5.10 (m)	69.1[b]	5.10 (m)
44	32.0	1.70 (m)	32.0	1.70 (m)
45	36.0[c]	3.10 (m)	35.9[c]	3.10 (m)

[a]Tentative assigments.
[b, c]Assignments may be interchanged.
[d]Several signals between 28.4 and 32.0.

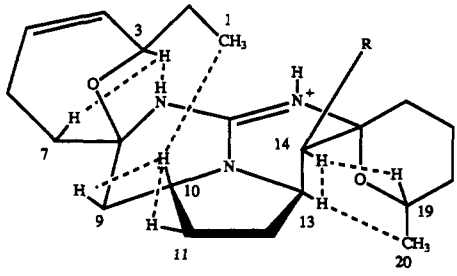


FIGURE 1. NOe's observed for peracetylisocrambescidin 800 [**9a**].

hydroxyspermidine subunits, characteristic of the crambescidins, are present. In addition, extensive 2D nmr experiments (DQF COSY, HMQC, HMBC) led to the assignments presented in Table 3. Noteworthy were the long range correlations between C-13 (δ 164.5) and the H-11 at δ 2.69 and the H-12 at δ 3.43. The presence of a Δ^{13} double bond was also substantiated by the value of the geminal coupling constant (J=19 Hz) seen for one of the C-12 methylene protons. The quaternary carbon signal at δ 112.8 was assigned to C-14 since its chemical shift is comparable to that of the corresponding carbon atom (C-7) of the crambines A [**1**] and C1 [**3**] (5,6). Thus, we propose structure **10** for the underivatized compound for which the name crambidine is coined.

TABLE 3. Nmr Data of Peracetylcrambidine [**10a**] (δ, $CDCl_3$, 600 and 150 MHz).

Location	δ ^{13}C	δ ^{1}H
1	10.0	0.76 (t, 7.3)
2	28.8[d]	1.47 (m)
3	71.5	4.92 (m)
4	132.7	5.50 (bd, 11.3)
5	130.0	5.68 (m)
6α	24.1	2.22 (m)
6β		2.35 (m)
7α	35.5[e]	3.21 (m)
7β		1.98 (m)
8	86.0	
9α	34.7	1.72 (m)
9β		2.82 (dt, 13.3, 4.1)[b]
10	59.2	4.78 (m)
11α	29.0[d]	2.22 (m)
11β		2.69 (m)
12α	33.1[f]	3.40 (m)
12β		3.65 (bdd, 19.0, 8.2)
13	164.5	
14	112.8	
15	170.9 or 173.8	
16	33.0[f]	2.35 (m)[c]
17		1.55 (m)[c]
18		1.60 (m)[c]
19	70.3	4.79 (m)
20	20.1	1.17 (d, 6)
21	150.1	
22	162.9	
23	66.7	4.30 (t, 6.6)
24	28.5[d]	1.72 (m)
25–36	[a]	1.28 (m)
37	33.3[f]	2.35 (m), 2.23 (m)
38	174.2	
39	42.8	3.55 (m), 3.20 (m)
40		
41	37.7	3.12 (m)
42	50.5	3.48 (m), 3.33 (m)
43	69.2	5.12 (m)
44	32.0	1.36 (m)
45	35.9[e]	

[a]Several signals between 28.5 and 32.0.

[b]One of the coupling constants (J=4.1) is probably due to a long range coupling with a NH since it is not present in the spectrum taken in pyridine-d_5.

[c]Tentative assignments.

[d–f]Assignments may be interchanged.

Besides these four ptilomycalin-A-related compounds, several other metabolites have been isolated from the remaining fractions of the *n*-BuOH extract of the Banyuls sample, after chromatography on Sephadex LH-20. They have been identified as ptiloceramide (10), synephrine (11), thymidine, 2-desoxyadenosine, and adenine by comparison of their spectral properties with those reported in the literature.

The MeOH extract of *C. crambe* inhibits the reaggregation of cells of *E. fluviatilis* (2) and is highly toxic for the fish *Lebistes reticulatus* (<50 mg/liter) and the shrimp *Artemia salina*. The *A. salina* test was used to monitor the activity during the separation steps of the Favignana sample. Toxicity tests performed on the different isolated alkaloids (see Experimental) indicate that they are responsible at least in part for the toxicity of the sponge.

Moreover, crambescidin 816 was found to be active against HCT-16 human colon carcinoma cells (IC_{50} 0.24 μg/ml). Since it is notorious that ionic channels are frequent targets for natural toxins, the potential activity of crambescidin 816 on voltage-sensitive Ca^{++} channels was also tested. This activity was evaluated in vitro, using a neuroblastoma × glioma cell line (NG 108–15) and acetylcholine-induced contractions of isolated guinea pig ileum. The results obtained with the NG 108–15 cells indicated that crambescidin 816 exerts a potent Ca^{++} antagonist activity (IC_{50} 1.5 10^{-4} μM), that is much more important than that of nifedipine (IC_{50} 1.2 μM), a well known selective blocker of L-type Ca^{++} channels. Furthermore, crambescidin 816 was able to inhibit the acetylcholine-induced contraction of guinea pig ileum at very low concentrations. It is worth mentioning that, contrary to ω- conotoxin, crambescidin 816 operates through a reversible blockage of Ca^{++} channels.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Uv spectra were performed on a Philips PU 8700 UV–VIS spectrometer and ir spectra on a Bruker IFS25 Fourier transform spectrometer. Fabms measurements were performed on a VG 70S mass spectrometer and eims on a VG Micromass 7070F. ^{1}H-nmr spectra were recorded at 600 MHz on a Varian UNITY 600. ^{13}C-nmr spectra were taken on the same instrument at 150.87 MHz. Voucher specimens of *C. crambe* are deposited in the laboratories of the different authors.

ISOLATION PROCEDURE.—The *n*-BuOH extract, obtained as reported previously (6), was subjected to two successive chromatographies on Sephadex LH-20 [eluents MeOH and CH_2Cl_2–MeOH (3:2)]. This furnished five main fractions. The first-eluting fraction was found by tlc to be a complex mixture of ninhydrin-positive compounds. It was submitted to the following separation steps, each step being monitored by tlc:dccc [*n*-BuOH–Me_2CO–H_2O (45:15:75) descending mode], chromatography on Sephadex LH20 (MeOH), and chromatography on BIOGEL P2 [H_2O-EtOH (8:2)]. The fractions containing ninhydrin-positive compounds were acetylated by pyridine-Ac_2O (1:1) overnight at room temperature. The acetylated fractions were then flash-chromatographed on two successive Si gel columns [eluents: first, increasing amounts of MeOH in CH_2Cl_2; second, decreasing amounts of Et_2O in $CHCl_3$-MeOH-H_2O-iPrOH (8:8:8:1, organic layer)]. This led to peracetylcrambescidin 816 (23 mg), peracetylcrambescidin 800 (5 mg), peracetylisocrambescidin 800 (16 mg), and peracetylcrambidine (12 mg).

Crambescidin 816 was also isolated from the *n*-BuOH extract of specimens of *C. crambe* collected off Favignana in June 1989. Isolation was guided by performing *A. salina* assay on crude extracts and chromatographic fractions. The encrusting animals (528 g), scraped from the rocky substratum, were cut into small pieces and extracted three times with Me_2CO at room temperature. The extract was evaporated in vacuo, and the aqueous residue was partitioned between *n*-BuOH and Et_2O. The bioactive *n*-BuOH fraction (4 g) was submitted to cc on Sephadex LH-20 (300 g, 80×4 cm) with MeOH, leading to three main fractions containing bioactive and Dragendorff-positive compounds. The first two eluting fractions were subjected to dccc [*n*-BuOH–Me_2CO–H_2O (45:15:75), descending mode). Fractions of 5 ml were collected and combined accordingly by tlc monitoring [*n*-BuOH–HOAc–H_2O (60:15:25), Dragendorff spray reagent] to give five main fractions that were further purified by hplc on a C-18 μ-Bondapack column with MeOH-H_2O (43:57), affording crambescidin 816 [**6**] (160 mg) and crambine A [**1**] (60 mg). The last-eluting fractions from Sephadex LH-20 submitted to the same separation steps led to crambine B [**2**] (4.5 mg), crambine C1 [**3**] (6 mg), and crambine C2 [**4**] (7 mg).

Peracetylcrambescidin 800 [**5a**].—Glassy solid: $[\alpha]_D$ −43° (*c*=0.15, $CHCl_3$); ir (film) 3292–3093, 1738, 1683–1557 cm^{-1}; hrfabms $[MH]^+$ 927.656, (calcd 927.653 for $C_{51}H_{87}N_6O_9$); ^1H and ^{13}C nmr see Table 2 and Jares-Erijman *et al.* (7).

Crambescidin 816 [**6**].—Glassy solid: $[\alpha]_D$ −16° (*c*=0.4, MeOH); ir (film) 3350–3250, 1734, 1644–1616 cm^{-1}; hrfabms $[MH]^+$ 817.619 (calcd 817.617 for $C_{45}H_{81}N_6O_7$); ^1H and ^{13}C nmr see Jares-Erijman *et al.* (7). ^1H and ^{13}C nmr of the minor conformer (δ, CD_3OD, 600 and 150 MHz) 176.4 (C-38), 3.55 (m) and 47.8 (H_2C-39), 1.95 (m) and 27.8 (H_2C-40), 3.22/3.56 (m) and 53.2 (H_2C-42). 3.98 (m) and 69.4 (HC-43).

Peracetylcrambescidin 816 [**6a**].—Glassy solid: $[\alpha]_D$ −29° (*c*=0.3, $CHCl_3$); positive ion fabms $\{MH\}^+$ *m/z* 943; ^1H and ^{13}C nmr see Jares-Erijman *et al.* (7).

Peracetylisocrambescidin 800 [**9a**].—Glassy solid: $[\alpha]_D$ −44° (*c*=0.4, $CHCl_3$); hrfabms $\{MH\}^+$ 927.652 (calcd 927.653 for $C_{51}H_{87}N_6O_9$); ^1H and ^{13}C nmr see Table 2.

Peracetylcrambidine [**10a**].—Glassy solid: uv (MeOH) λ max 254 (22,000), 299 (5300), 315 (3100), 325 nm (2500); hrfabms $[MH]^+$ 967.642 (calcd 967.648 for $(C_{53}H_{87}N_6O_{10})$; ^1H and ^{13}C nmr see Table 3.

BIOLOGICAL AND PHARMACOLOGICAL TESTS.—*Cell culture.*—NG 108–15 (neuroblastoma×glioma) hybrid cells (obtained from the ATCC, Bethesda, Maryland) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Confluent cell layers were detached by treatment with 0.05% trypsin.

Ca^{++}-sensitive fura-2 fluorescence.—Cells were harvested and loaded with fura-2-acetoxymethyl ester and suspended in HEPES buffer. To determine changes of cytosolic free calcium, fluorescence was monitored on a spectrophotometer at excitation and emission wavelengths of 340 and 510 nm, 5 and 10 nm slit width, respectively. The cells (2×10^6 cells/ml) were kept in suspension at 37° with a magnetic stirrer. Depolarization was induced by adding KCl (20 mM) to the cell suspension. The effect of crambescidin 816 or nifedipine on the K^+-induced increase of cytosolic Ca^{++} ($[Ca^{++}]_i$) was established by preincubating the fura-2 loaded cells with a vehicle or with different concentrations (0.01 nM to 10 nM) of each agent for 3 min before the challenge with KCl.

Rat jejunum strip preparation.—Strips of jejunum obtained from Sprague-Dawley male rats (200–250 g), were used in a 30-ml organ bath filled with Krebs solution and aerated with O_2/CO_2 (95 and 5%, respectively). An isometric transducer with basal strain of 1 g was employed to record the smooth muscle activity. Following a stabilization of 60 min, smooth muscle contraction was induced by acetylcholine (AcCh), (10^{-6}–10^{-4} M). The agents were added to the bath 3 min before AcCh.

Effect on efflux of Ca^{++}.—Quantitative measurement of $[Ca^{++}]_i$ in fura-2 loaded cells indicated a basal level of 350±22 nM (mean±SE, *n*=20). Upon stimulation with KCl there was a rapid rise in cytosolic free calcium (C=52.4 nM). Crambescidin 816 and nifedipin added to NG 108–15 cells produced a dose-dependent inhibition of $[Ca^{++}]_i$ elevation. IC_{50} of crambescidin 816 was 0.15±0.02 nM; IC_{50} of nifedipine was 1.20±0.09 mM. IC_{50} is defined as the concentration of agent which inhibited the maximal response by 50% when vehicle was added before KCl.

Effects on AcCh-induced smooth muscle contraction.—Crambescidin 816 did not alter the spontaneous activity of the isolated rat jejunum (0.6–6.0 pM) and in a dose dependent manner reduced the response observed after addition of AcCh (10^{-6} M). This effect was not observed following the wash of the preparation. Observed percentages of inhibition of AcCh activity by crambescidin 816 were 18% at 0.6 pM, 25% at 1.2 pM, and 30% at 6.0 pM. ω-Conotoxin GVIA, a potent blocker of voltage-dependent calcium channels, markedly antagonized the activity of AcCh (40% at 0.4 pM), and this effect was still observed following the wash of the preparation.

Ephydatia fluviatilis *and* Lebistes reticulatus *tests.*—Inhibition of reaggregation of cells was observed at 2.3×10^{-3} M for crambine A, 2.0×10^{-3} M for crambine B, 2.1×10^{-2} M for crambine C1, and 1.2×10^{-3} M for crambescidin 816. The four compounds are lethal for the fish *L. reticulatus* at 2×10^{-2} mg/liter.

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