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CRAMBINES C1 AND C2: TWO FURTHER ICHTHYOTOXIC GUANIDINE ALKALOIDS FROM THE SPONGE *CRAMBE CRAMBE*

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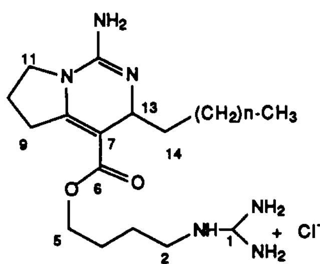
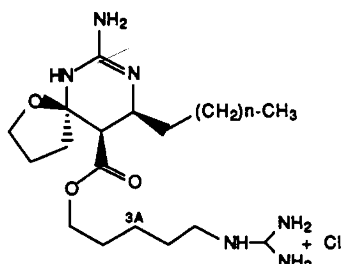
ABSTRACT.—Crambines C1 [**3**] and C2 [**4**] are two new ichthyotoxic guanidine alkaloids isolated from the Mediterranean sponge *Crambe crambe*. Their structures have been determined on the basis of their spectral properties. Biogenetically they are closely related to crambines A [**1**] and B [**2**], which had been isolated previously from the same sponge.

Recently we reported the isolation and structure determination of two ichthyotoxic guanidine compounds, crambines A [**1**] and B [**2**], from a specimen of the encrusting Mediterranean sponge *Crambe crambe* Schmidt (Poecilosclerida; Crambidae) collected near Banyuls, France (1). The toxic *n*-BuOH extract from which these two compounds were isolated is a complex mixture. After repetitive chromatographies on Sephadex LH 20, Si gel, and polyvinylpolypyrrolidone, a further toxic Sakaguchi-positive fraction homogeneous by tlc could be isolated. This fraction contained a new guanidine alkaloid, crambine C1 [**3**], accompanied by a series of homologues of **3** where n = 8, 10, and 11. The same compound was also isolated as a minor component from specimens of *C. crambe* collected off Favignana, Italy together with crambine A (major), crambine B, and a further minor compound, crambine C2 [**4**]. Crambine C1 was also isolated from a specimen of *C. crambe* collected near Nice, France, but in this last sample, crambine C1 was, together with crambine A, one of the major compounds of the toxic extract.

Crambine C1 [**3**] displayed an $[M+H]^+$ ion at *m/z* 481.390 in positive hrfabms, which corresponds to the molecular formula $C_{25}H_{48}N_6O_3$ for the corresponding free base. This molecular formula was further confirmed by negative fabms which exhibits peaks at *m/z* 479 $[M-H]^-$ and 515 $[M-H+HCl]^-$. These fab mass spectra also showed peaks



1 (n = 9)



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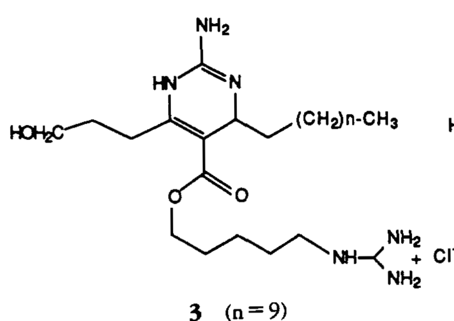

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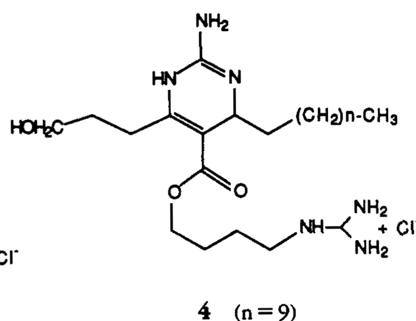
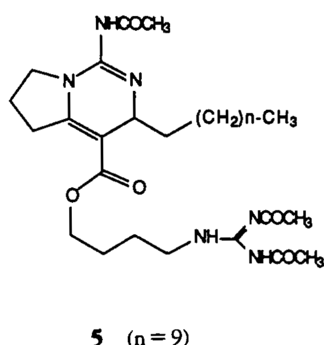
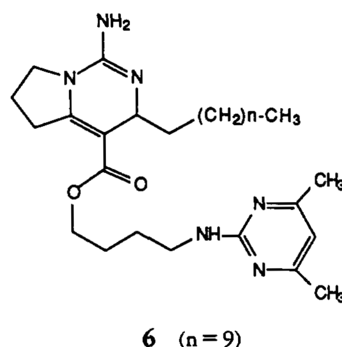


4 (n=9)



5 (n=9)



6 (n=9)

attributable to homologues (**3**, n=8, 10, 11) of crambine C1, the relative intensities of which depended upon the origin of the sample (Table 1). In spite of numerous trials, we have been unable to purify compound **3** completely.

Thus, crambine C1 is an isomer of crambine B. Analysis of its ^{1}H-nmr spectra (Table 2) clearly shows the presence of three separate spin systems as for crambines A and B (1): an alkyl chain with a terminal methyl group and a methine (H-13, δ_H 4.42) at the other end, similar to that present in **1**; five methylenes in an open chain at one end linked to an oxygen atom (H_2-5, δ_H 4.18, δ_C 66.1) and to a nitrogen atom at the other one (H_2-2, δ_H 3.18, δ_C 42.8),

TABLE 1. Fabms Data of Crambine C1 [**3**].[a]

Origin of the sample	Positive mode			
	m/z 467[b]	*m/z* 481[c]	*m/z* 495[d]	*m/z* 509
Banyuls	30	100	60	10
Nice	8	100	5	<1
	Negative mode			
	m/z 465	*m/z* 479	*m/z* 493	*m/z* 507
Banyuls	35	100	30	10
Nice	10	100	<1	<1

[a]Relative intensities (%) of the different $[M+H]^+$ ions.
[b]Hr fabms 467.375 (calcd for $C_{24}H_{47}N_6O_3$, 467.371).
[c]Hr fabms 481.390 (calcd for $C_{25}H_{49}N_6O_3$, 481.387).
[d]Hr fabms 495.407 (calcd for $C_{26}H_{51}N_6O_3$, 495.403).

TABLE 2. ^1H- and ^{13}C-nmr Data of Crambine C1 [3] (δ, TMS, CD_3OD, 600 MHz).

Position	δ $^{13}C^a$	δ ^1H (*J*, Hz)	^1H-^1H COSY observed correlations	^1H-^{13}C COSY observed correlations
C-1	159.0	—		
CH_2-2	42.8	3.18 t (7;7)	H-3	+
CH_2-3	30.0[b]	1.58 m	H-2, H-3A	
CH_2-3A	27.4[b]	1.39 m	H-3, H-4	
CH_2-4	30.1[b]	1.69 m	H-3A, H-5	
CH_2-5	66.1	4.18 m	H-4	+
C-6	166.0	—		
C-7	106.4	—		
C-8	149.2[c]	—		
CH_2-9	29.1	2.82 m	H-10	+
CH_2-10	32.8	1.81 m	H-9, H-11	+
CH_2-11	62.4	3.61 t (6.4)	H-10	+
C-12	153.8[c]			
CH-13	51.4	4.42 dd (5;7)	H-14	+
CH_2-14	37.3	1.58 m	H-13, H-15	+
CH_2-15	25.4	1.39 m	H-14	
CH_2-16	27.9[b]	1.29 m		
CH_2-17	32.3[b]	1.29 m		
CH_2-18	30.8[b]	1.29 m		
CH_2-19	30.6[b]	1.29 m		
CH_2-20	30.5[b]	1.29 m		
CH_2-21	30.5[b]	1.29 m		
CH_2-22	33.3[b]	1.29 m		
CH_2-23	23.9	1.29 m	H-24	+
Me-24	14.7	0.9 t (6.5)	H-23	+

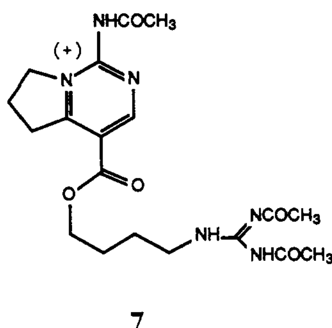
[a]Assignments were made by DEPT and by comparison with crambines A and B.
[b,c]Assignments may be interchanged.

similar to the spin system found in **2**; and three adjacent methylenes in another open chain (H_2-9 to H_2-11).

All these data, together with its ^{13}C-nmr spectra in comparison with those of **1** and **2**, are in complete agreement with structure **3** for crambine C1. In order to confirm this structure, several attempts were made to derivatize the new compound. Crambine C1 was treated with pyridine/Ac_2O or with 2,4-pentanedione/pyridine/Na_2CO_3. This led in each case to complex mixtures from which it was impossible to isolate a well characterized derivative. Submitted to the same conditions, crambine B also led to untractable mixtures. In contrast, crambine A furnished the triacetate **5**, the ^1H-nmr spectrum of which showed, next to the expected signals for the crambine skeleton, characteristic singlets at δ 2.17 (3H) and 2.12 (6H) attributable to three acetamide groups. Moreover, the 4,6-dimethylpyrimidine derivative **6** of crambine A could also be prepared.

Interestingly, the eims of triacetate **5** and pyrimidine derivative **6** were indicative of the fact that crambine A differs from its respective homologues by the length of the alkyl chain at C-13. Indeed, for **5** and **6** the peak of the mass spectrum attributable to cation **7** (*m/z* 419) or **8** (*m/z* 357), respectively, is devoid of any homologous peak, contrary to the $[M + H]^+$ ion.

Crambine C2 [**4**] differs from crambine C1 only by the length of the methylene chain linked to the ester group. Indeed, analysis of its ^1H-nmr spectrum shows the presence of the alkyl chain linked to the C-13 methine and of the hydroxypropyl moiety C-9 through



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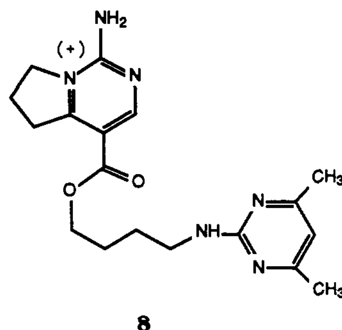

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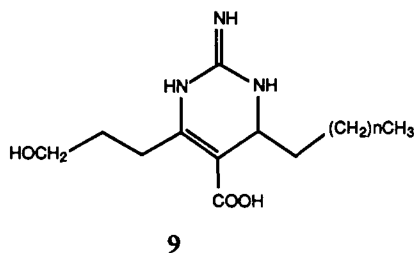

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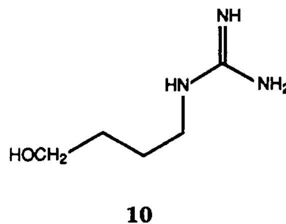

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C-11 as in **3**, but the open chain linked to the ester group is now made up of four methylene groups as in crambine A [**1**], as is clearly shown by the spin system from H_2-2 to H_2-5, identical to that observed in the ^{1}H-nmr spectrum of **1** (1).

Crambines C1 and C2 are further members of a series of unique guanidine derivatives isolated from the sponge *C. crambe*. Biogenetically, the crambines may be regarded as deriving from the hypothetical intermediate **9**, possibly formed by condensation of a fatty acid with alcohol **10**. The latter is a reduced form of γ-guanidinobutyric acid, a degradation product of arginine that is encountered in many marine invertebrates (2). Esterification of **9** by a second molecule of γ-guanidinobutanol could lead to crambine C2. Further cyclization leading to the formation of the pyrrolidine ring could explain the formation of crambine A. On the other hand, crambines C1 and B could arise from the esterification of **9** by δ-guanidinopentanol.

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 250 MHz on a Bruker WM250 or at 600 MHz on a Varian VXR 600. ^{13}C-nmr spectra were taken on the same instruments at 62.5 MHz or 150.87 MHz. The voucher specimens of *C. crambe* are deposited in the laboratories of the different authors.

ISOLATION PROCEDURE.—Specimens of *C. crambe* (80 g dry wt), collected off Banyuls, France and stored in MeOH, were exhaustively extracted with CH_2Cl_2-MeOH (1:1). After evaporation of CH_2Cl_2, the residual MeOH/H_2O solution was successively extracted with hexane and CCl_4. After evaporation of MeOH under reduced pressure, the resulting aqueous solution was extracted with *n*-BuOH. Most of the ichthyotoxicity present in the initial MeOH extract was found again in the *n*-BuOH extract (4.1 g). The isolation of crambines A, B, and C1 from this fraction was performed as follows, each separation step being monitored by tlc [Si gel, CH_2Cl_2-MeOH-HOAc (8:2:0.1), vanillin/H_2SO_4]. The fraction was subjected to two chromatographies on Sephadex LH 20 [eluents (1) MeOH, (2) CH_2Cl_2-MeOH (3:2)], a dccc [$CHCl_3$–MeOH-*n*-BuOH–H_2O (10:10:1:6)], and a flash chromatography on Si gel [CH_2Cl_2-MeOH (9:1 to 7:3)+0.5% CF_3COOH].

The fraction containing crambine C1 was then chromatographed on polyvinylpolypyrrolidone

(MeOH) and subsequently on Sephadex LH 20 (MeOH).

This led to crambine C1 (40 mg) contaminated by three homologues (**3**, n = 8, 10, 11) as observed by ms. The physical properties of crambine C1 were taken on this sample.

Crambine C1 [**3**].—Glassy solid: $[\alpha]_{404}$ + 13.7° (MeOH, *c* = 0.8); cd (MeOH, *c* = 0.024) $[\theta]_{250}$ +2305, $[\theta]_{277}$ +400, $[\theta]_{280}$ +560, $[\theta]_{290}$ 0; uv (MeOH) λ max 207 (ε 4600), 279 (ε 2200) nm; ir (film) 3300–3100, 1695–1650 cm^{-1}; ^1H and ^{13}C nmr see Table 2.

Crambine C2 was isolated as a minor component of the toxic alkaloid mixture from specimens of *C. crambe* collected off Favignana, Italy, by preparative hplc on a μ-Bondapak C18 column [30 cm × 4.8 mm i.d., MeOH-H_2O (43:57), flow rate 5 ml·min^{-1}] of a dccc fraction containing a mixture of crambines A, C1, and C2. The same specimens of sponge also gave crambine A (ca. 100 mg) as the major compound together with minor amounts (ca. 10 mg) of crambines B and C2.

Crambine C2 [**4**].—Glassy solid (3 mg): $[\alpha]_D$ −30° (MeOH, *c* = 0.2); uv (MeOH) λ max 203 (ε 6000), 281 nm (ε 2500); fabms *m/z* $[M + H]^+$ 467; ^1H nmr (δ, CD_3OD, 250 MHz) 3.24 (t, 7 Hz, H_2-2), 1.70 (m, H_2-3), 1.80 (m, H_2-4), 4.23 (t, H_2-5), 2.82 (m, H_2-9), 1.81 (m, H_2-10), 3.62 (t, H_2-11), 4.43 (dd, 5 and 6.7 Hz, H-13), 1.58 (m, H_2-14), 1.29 (alkyl chain), 0.90 (t, 6.5 Hz, H_3-24).

Crambines A, B, and C1 were separated from the *n*-BuOH extract of specimens of *C. crambe* collected off Nice, France after two successive chromatographies on Sephadex LH 20 [eluent (1) MeOH, (2) $CHCl_2$-MeOH (4:1)] followed by flash chromatography and preparative hplc on a DIOL column [CH_2Cl_2-MeOH (9:1 to 8:2) + 2.10^{-3} M NH_4 OAc].

ACETYLATION OF CRAMBINE A.—Crambine A (2.5 mg) was treated with pyridine-Ac_2O (2:1) (0.7 ml) during 5 h in the presence of catalytic amounts of DMAP. The solution was evaporated under reduced pressure and the residue chromatographed on Si gel [CH_2Cl_2-MeOH (100:0 to 95:5)]. This yielded triacetylcrambine A [**5**] (2.1 mg), homogeneous in tlc: eims *m/z* $[M]^+$ 574 (33), base peak at *m/z* $[M - C_{11}H_{23}]^+$ 419 (100); ir (film) ν OH 3400 cm^{-1}, ν C=O 1696, 1650–1620 cm^{-1}; ^1H nmr (δ, $CDCl_3$) 4.37 (m, H-13), 4.18 (t, *J* = 6 Hz, H_2-5), 3.78 (m, H_2-11), 3.47 (m, H_2-2), 3.24 (ddd, *J* = 18.2, 8.3, 4.4 Hz; H-9), 2.98 (ddd, *J* = 18.2, 9, 9 Hz, H-9), 2.17 (s, 3H), 2.12 (s, 6H), 0.88 (t, *J* = 6 Hz), NH signals at δ 10.61 and 9.04.

PYRIMIDINE DERIVATIVE OF CRAMBINE A.—Crambine A (13.2 mg) was dissolved in pyridine (0.5 ml) and treated with 2,4-pentanedione (0.5 ml) and Na_2CO_3 (3.4 mg). The mixture was stirred for 60 h at room temperature. The solution was evaporated to dryness and the residue dissolved in CH_2Cl_2-EtOH (95:5). The resulting solution was filtered and evaporated under reduced pressure, and the residue was chromatographed on alumina [EtOAc-MeOH (100:0 to 0:100)]. This yielded derivative **6** (12 mg) homogeneous in tlc: eims $[M]^+$ *m/z* 512 (4), characteristic fragment ions at *m/z* 399 (7), $[M - C_{11}H_{23}]^+$ 357 (45), 334 (15), 180 (100); uv (MeOH) λ max 214, 240, 293 nm; ^1H nmr (δ, $CDCl_3$) 4.42 (m, H-13), 4.18 (t, *J* = 6 Hz, H_2-5), 3.84 (m, H-11), 3.70 (m, H-11), 3.45 (m, H_2-2), 3.30 (m, H-9), 2.92 (m, H-9), 0.87 (t, H_3-24). 4,6-Dimethylpyrimidine signals at δ 6.30 (s, 1H) and 2.27 (s, 6H). NH signals at δ 9.02, 8.37, and 6.61.

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