Articles

Polycyclic Guanidine-Containing Compounds from the Mediterranean Sponge *Crambe crambe*: The Structure of 13,14,15-Isocrambescidin 800 and the Absolute Stereochemistry of the Pentacyclic Guanidine Moieties of the Crambescidins

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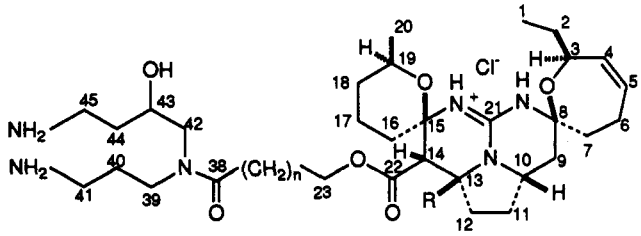
The absolute stereochemistry of the pentacyclic guanidine moieties of crambescidin 816 (**1**)[1] and of 13,14,15-isocrambescidin 800 (**4**), a new member of this family, was determined, based on chiral GC analysis of a derivative of 2-hydroxybutanoic acid, an ozonolysis product of the crambescidins. Significantly less antiviral activity and cytotoxicity were observed for **4**.

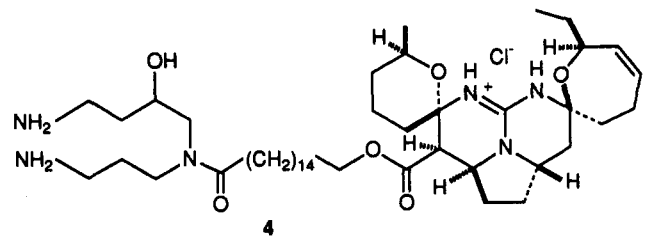
Introduction

Recently a new group of potent cytotoxic and antiviral compounds, the crambescidins (**1–3**), isolated from the red encrusting sponge *Crambe crambe*,[1] and ptilomycalin A, isolated from the sponges *Ptilocaulis spiculifer* and a *Hemimycale* sp.,[2,3] have been described. These compounds are of a unique structural type consisting of a pentacyclic guanidine moiety linked by a long-chain ω-hydroxy acid to a hydroxyspermidine or spermidine unit. While relative stereochemistry of the pentacyclic guanidine moiety has been established in all cases, based on NOE NMR data, the absolute stereochemistry has remained undetermined.[4]



1: n = 14, R = OH
2: n = 14, R = H
3: n = 16, R = OH

The present paper reports the structure of the new 13,-14,15-isocrambescidin 800 (**4**)[4] and the absolute stereochemistry of the pentacyclic guanidine moieties of crambescidins 816[1] and **4**.



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Results and Discussion

Isolation and Structure of 13,14,15-Isocrambescidin 800 (4). Compounds **1–4** were isolated following a procedure similar to that described previously (see Experimental Section).[1] The molecular formula $C_{45}H_{80}N_6O_6$ was assigned to 13,14,15-isocrambescidin 800 (**4**) from high-resolution fast atom bombardment mass spectrometry (HRFABMS) data [*m/z* 801.6242 (M + H, Δ −2.4 mmu)] and is identical to that of crambescidin 800 (**2**). Other mass spectral data [low-resolution FABMS, and FABMS/collision-induced dissociation (CID)/MS] for **4** were also identical to those for **2**. However, chromatographic properties and the NMR patterns for **2** and **4** were similar but clearly distinguishable, indicating they are isomers of each other.

The structures of the hydroxyspermidine and the long chain ω-hydroxy acid units of **4** were assigned to be the same as that of **2** based on FABMS and NMR data. The presence of a hydroxyspermidine was evident, since fragment ions for losses of aminohydroxybutyl (M + H − C_4H_9NO, 714.5552, Δ 1.9 mmu) and of acetylhydroxyspermidine (M + H − $C_9H_{20}N_3O_2$, 598.4582, Δ 0.2 mmu) were observed in FABMS and FABMS/CID/MS spectra of **4**. These losses were characteristic of the hydroxyspermidine unit in crambescidins, e.g. **1** and **2**.[1] The position of the hydroxyl group in the hydroxyspermidine unit was as-

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(2) Kashman, Y.; Hirsh, S.; McConnell, O. J.; Ohtani, I.; Kusumi, T.; Kakisawa, H. *J. Am. Chem. Soc.* **1989**, *111*, 8925–8926.

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(4) It has come to our attention that Berlinck *et al.* have assigned the stereochemistry of **1** at C-43 as *S* and have also isolated 13,14,15-epicrambescidin 800 independently and have assigned the same structure, **4**. [Berlinck, R. G. S.; Braekman, J. C.; Bruno, I.; Daloze, D.; Ferri, S.; Riccio, R.; Spampinato, S.; Speroni, E. 7th Interational Symposium on Marine Natural Products; Societa Chimica Italiana, Capri, Italy, July 5–10, 1992; Abstract P-56.]

0022-3263/93/1958-4805$04.00/0

Table I. ^1H NMR and ^{13}C NMR Signals for 4 (CD_3OD)

position	^1H, δ (ppm), mult, J (Hz)	^{13}C, δ (ppm), mult
1	0.90, t, 7	10.7, q
2a	1.45, m	30.2, t
2b	1.51, m	
3	4.45, br s[a]	71.9, d
4	5.50, br d[a]	134.0, d
5	5.68, br t[a]	131.2, d
6a	2.12, m	24.9, t
6b	2.35, m	
7a	1.94, m	39.0, t
7b	2.38, m	
8		86.6, s
9a	1.45, t, 13	38.0, t
9b	2.56, dd, 13, 2.3	
10	3.74, m	54.6, d
11a	1.68, m	30.8, t
11b	2.24, m	
12a	1.65, m	29.6, t
12b	2.29, m	
13	4.00, m	54.2, d
14	3.80, d, 3.4	42.5, d
15		84.4, s
16a	1.52, m	33.6, t
16b	1.80, m	
17	1.75, m	20.9, t
18a	1.15, m	32.9, t
18b	1.60, m	
19	4.02, m	70.0, d
20	1.13, d, 6.5	22.3, q
21		150.1, s
22		167.7, s
23a	3.96, m	66.1, t
23b	4.10, m	
24	1.63, m	28.4, t
36	1.57, m	26.5, t
37	2.35, m	34.1, t
38		177.3, s
39a	3.40, m	42.8, t
39b	3.65, m	
40a	1.90, m	26.5, t
40b	1.90, m	
41	2.85, m	38.2, t
42a	3.32, m	54.7, t
42b	3.45, m	
43	3.99, m	68.4, d
44a	1.65, m	32.8, t
44b	1.75, m	
45	3.12, m	38.4, t

[a] Broad signal; coupling constant not measured.

Table II. ^1H NMR Data of 4[a]

	δ (ppm)		
	CD_3OD[b]	C_5D_5N[c]	$CDCl_3$[d]
H-11a	1.68	1.50	1.70
H-11b	2.24	2.00	2.24
H-12a	1.65	1.70	1.72
H-12b	2.29	2.15	2.24
H-13	4.00	4.20	4.00
H-14	3.80, d, J = 3.4 Hz	3.88, d, J = 3.4 Hz	3.50, d, J = 3.4 Hz

[a] All signals except H-14 are observed as multiplets. [b] Assignments based on DQFCOSY (see supplementary material S-6a, S-7, and S-8a, respectively). [c] Assignments based on 45-COSY and HMBC (S-6c and S-8b). [d] Assignments based on 45-COSY (S-6b).

Table III. Some ROESY Correlations of 4[a]

proton	correlated to
Me-1	H-2a, H-2b, H-3, H-4, H-5, H-10
H-3	H-1, H-4, H-5, H-6a, H-6b, H-7a, H-7b, NH-1
H-4	Me-1, H-2, H-2, H-5, H-6a, H-6b, H-7a
H-5	Me-1, H-3, H-4, H-6a, H-6b, H-7a
H-6a	H-3, H-4, H-5, H-7a
H-6b	H-3, H-4, H-5, H-7a
H-7a	H-3, H-4, H-5, H-6a, H-6b
H-7b	H-3
H-9a	H-9b, H-11a
H-9b	H-9a, H-10, H-11b
H-10	H-9b, H-11b
H-11a	H-12b, H-11b, H-13
H-11b	H-10, H-11a, H-9b
H-12a	H-11b, H-12b
H-12b	H-12a, H-11a
H-13	H-14, H-12, H-19, H-11a
H-14	H-19, H-13, H-12b
H-19	H-14, H-13
H-23a	H-24
H-23b	H-24
H-39a	H-40a, H-40b, H-43
H-93b	H-40a, H-40b
H-40a	H-39a, H-39b, H-41
H-41	H-40a, H-40b
H-42a	H-42b, H-43
H-42b	H-44a
H-43	H-42a, H-39a, H-39b
H-44a	H-42b, H-45
H-44b	H-45
H-45	H-44a, H-44b

[a] 500 MHz in $CDCl_3$ and C_5D_5N. See supplementary material (S-9a and S-9b).

signed by 2D NMR data. ^1H and ^{13}C NMR chemical shifts in the hydroxyspermidine unit in 4 were nearly identical to those in crambescidins 816 (1), 844 (3), and 800 (2), suggesting that the stereochemistry of the secondary alcohol in all crambescidins is the same.[5]

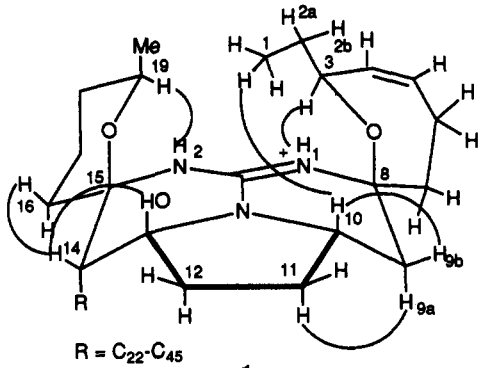
As in the other crambescidins a polymethylene chain extending from C-37 to C-23 was indicated by NMR and, especially, by MS data, which provided a nearly unbroken series of fragment ions from cleavage at successive methylene groups, from *m/z* 598 to 404. The polycyclic guanidine region of isocrambescidin 800 was observed in the fragments at *m/z* 404.2551 ($C_{22}H_{34}N_3O_4$, Δ 0.2 mmu) and 358.2498 ($C_{21}H_{32}N_3O_2$, Δ 0.4 mmu).

Further evidence for the pentacyclic moiety of isocrambescidin 800 (4) was provided by NMR spectra. The ^{13}C and ^1H chemical shifts for 4, assigned by COSY, HMQC, and HMBC NMR experiments, were very similar to those for 2; however, H-14 (3.80 ppm, d, J = 3.4 Hz) was deshielded by 0.6 ppm and the coupling constant $J_{13,14}$ changed from 5.6 Hz in 2 to 3.4 Hz in 4. In the ^{13}C NMR spectrum, a significant upfield shift of C-14 (from 49.4 ppm in 2 to 42.5 ppm in 4) was also observed, suggesting some change near C-14. It should be noted that $J_{9b,10}$ also changed from 4.5 Hz in 2 to 2.3 Hz in 4. All the above data supported the idea that 4 is stereoisomeric to 2 in the pentacyclic guanidine region.

Relative stereochemistry for the pentacyclic guanidine moiety of 4 was assigned based on NOE data. NOE difference and ROESY experiments were recorded both in $CDCl_3$ and C_5D_5N since the signals for H-11 and H-12 were superimposed in solvents other than C_5D_5N. Assignments for H-11 to H-14 (Table II) were based on COSY and HMBC (supplementary material); NOE's between *H*-10 and C*H*$_3$-1 and between N*H*-1 (9.85 ppm in $CDCl_3$) and *H*-3 indicated that the C-1 to C-10 portion of the molecule has the same relative stereochemistry at C-3, C-8, and C-10 as in 1 and 2 (Scheme I).[1] However, NOE's observed between *H*-19 and C*H*$_3$-1, *H*-19 and N*H*-2 in 1 and 2, or *H*-10 and *H*-13 in 2 were absent in the case of 4, indicating that the stereochemistry of the left portion of the pentacyclic guanidine moiety is different from the former.

(5) These spectra showed signals for a minor conformer, as observed previously in crambescidins and in ptilomycalin. (See refs 2 and 3).

Scheme I



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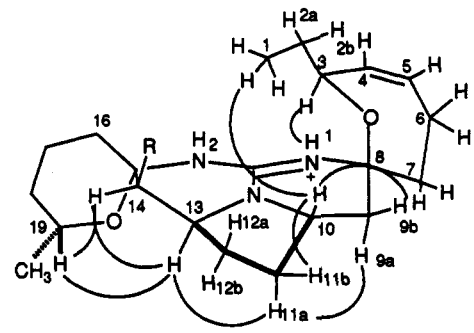

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The position of H-9a (α-axial) was assigned unambiguously by its coupling constant with H-10 (13 Hz). NOE's between H-9a and H-11a ($CDCl_3$) and H-11a and H-13 (C_5D_5N) by ROESY indicated all the above protons are on the same face, and therefore H-13 must be α. The coupling constant between H-14 and H-13 (J = 3.4 Hz) indicated that these protons are in a *cis* relationship.[6] This requires inversion of configuration at C-14 with respect to the reported crambescidins and ptilomycalin A. NOE's observed in the ROESY spectrum of 4 ($CDCl_3$ and C_5D_5N) between H-19 and H-13 and between H-19 and H-14 only accommodate the inverted configuration at C-15 in which H-19, H-14, and H-13 can make a small triangle as depicted in Scheme I. No other possible stereoisomer meets all the above observations.[7]

Absolute Stereochemistry of Crambescidins. The absolute configurations of the pentacyclic guanidine moieties of crambescidin 816 (1) and isocrambescidin 800 (4) were determined, based on the absolute stereochemistry of methyl 2-hydroxybutanoate, an ozonolysis product derived from the C-1 to C-4 portion of the crambescidins. Treatment of 1 or 4 with O_3, followed by H_2O_2, acid hydrolysis, and methylation gave a mixture containing methyl 2-hydroxybutanoate. Chiral GC/MS analysis of this product using Chirasil-Val III[8] and authentic methyl (2*S*)- and (2*R*)-2-hydroxybutanoates demonstrated that the methyl 2-hydroxybutanoate derived from 1 and 4 coeluted with the synthetic 2*S* sample. A GC/MS spectrum of the ozonolysis-derived ester was identical to that of the authentic compound.

Therefore, the absolute stereochemistry of crambescidins was determined to be 3*S*, 8*S*, 10*S*, 13*S*, 14*R*, 15*S*, 19*R*, which is the mirror image of the structure arbitrarily shown previously for crambescidins and ptilomycalin A.[1-3]

Biological Activity. Crambescidins 816 (1), 800, and 844 inhibited the growth of L1210 cells (98% at 0.1 μg/mL, IC_{50} 0.04 μg/mL), and 1 exhibited antiviral activity against *Herpes simplex*, Type I virus [HSV-1 (+++) at 1.25 μg/well]. Crambescidin 816 has also been claimed to be a strong Ca^{2+} channel blocker,[4] and ptilomycalin A has recently been shown to complex to several *N*-acetylamino acid anions, with affinity dependent on their ability to fit in the pentacyclic guanidine cavity.[3]

Crambescidin 800 (2) and ptilomycalin A both lack the hydroxyl group at C-13 but the latter also lacks the hydroxyl group at C-43 and is reported to be somewhat less cytotoxic to L1210 cells (IC_{50} 0.4 μg/mL).[2,3] Crambescidin 844 bears two extra methylene groups in the long chain when compared with crambescidin 816.

Compound 4, which we have determined to have the same absolute configuration at C-3 and, therefore, opposite configurations at C-13, C-14, and C-15 to those in crambescidin 816, is substantially less cytotoxic to L1210 cells (10% inhibition at 10 μg/mL) than other crambescidins and has no observed antiviral activity against HSV-1. (At 12.5 and 25 μg/well, the highest concentrations tested, 4 induced a cytotoxic zone vs CV-1 cells of 16 mm/well).

These observations revealed that the cage-like structure of the pentacyclic guanidine moiety in the crambescidins plays a major role in their strong biological activity.

Experimental Section

General. NMR chemical shifts (δ) are reported in ppm from TMS calibrated to the solvent peak. High- and low-resolution fast atom bombardment (FAB) mass spectra were measured on a ZAB-SE spectrometer and FABMS/CID/MS spectra on a 70 SE-4F instrument using dithiothreitol–dithioerythritol as matrix.[9] A C-18 column (25 × 0.8 cm, 5-μm particle size) and CH_3OH/0.1 M NaCl (8:2) solvent were used for HPLC separation. TLC on C-18 was developed with CH_3OH/0.1 M NaCl (8:2) solvent. Gas chromatography (GC) was carried out with a Chirasil Val III column (0.25 mm × 25 m) flow rate 1 mL/min; split ratio 20:1; temperature program 50 °C (6 min) → 5 °C/min → 180 °C (10 min). GC/MS was performed on a 5890 A gas chromatograph equipped with a 5970 MSD quadrupole mass detector, with other conditions the same as above. For each sample, 1 μL was injected.

Extraction and Isolation. The specimen was collected by SCUBA (−9 m) in October 1988 at Isla de Formentor (Cueva), Palma de Mallorca, Spain, and was identified by Dr. M. Uriz-Lespé (Centre d' Estudis Avançats de Blanes, Blanes, Spain). The frozen sample was extracted with CH_3OH–toluene (3:1). The extract was evaporated to give an oil (13 g), which was partitioned between $CHCl_3$ and 1 M NaCl (1:1, 100 mL × 3). The bioactive organic layer (6.6 g) was partitioned between the lower phase (4.2 g) and upper phase (2.4 g) of hexane–EtOAc–CH_3OH–H_2O (4:7:4:3). Half of the active lower phase was purified by flash chromatography (C-18, CH_3OH/0.1 M NaCl (15:4)), affording five fractions. Separation of the active fraction 4 (R_f 0.4, ninhydrin-positive spot) by HPLC afforded 1 (t_R 30.4 min, 30 mg). Similar separation of fraction 3 (R_f 0.45, ninhydrin-positive spot) afforded 4 (t_R 30.1 min, 11.2 mg).

(6) Similar systems showed J_{trans} = 11 Hz and J_{cis} = 4 Hz [see Snider, B. B.; Shi, Z. *J. Org. Chem.* **1992**, *57*, 2526–2528].

(7) It should be noted that the change in stereochemistry involves an increase in the dihedral angles H-9b/C-9/C-10/H-10 and H-13/C-13/C-14/H-14 of approximately 15° in a Dreiding model, which explains the change in coupling constants J_{14-13} and J_{9-10} observed in 4.

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13,14,15-Isocrambescidin 800 (4): colorless oil, $[\alpha]^{25}_D$ −48° (*c* 0.53, CH_3OH); LRFABMS see supplementary material, S-1; FABMS/CID/MS see supplementary material, S-2; ^{1}H-NMR (500 MHz, CD_3OD) see Table I and supplementary material, S-3; ^{13}C-NMR (125 MHz, CD_3OD) see Table I and supplementary material, S-4; ^{13}C-NMR (125 MHz, C_5D_5N) see supplementary material, S-5; DQFCOSY (500 MHz, CD_3OD) see supplementary material, S-6; HMQC (CD_3OD) see supplementary material, S-7; HMBC (CD_3OD, C_5D_5N) see supplementary material, S-8a and S-8b; ROESY (500 MHz, C_5D_5N, $CDCl_3$) see supplementary material, S-9a and S-9b. Anal. Calcd for $C_{45}H_{81}N_6O_6$: M_r 801.6220 (M + H). Found: M_r 801.6242 (HRFABMS).

Methyl (2*S*)-2-Hydroxybutanoate. A. From Crambescidin 816 (1). Excess O_3 was bubbled through a solution of 1 (1.4 mg, 1.7 × 10^{-3} mmol) in CH_3OH (1 mL) at −78 °C. The solution was allowed to warm to room temperature and excess O_3 was removed by bubbling N_2 through the solution. Hydrogen peroxide (30% 50 μL, 0.4 mmol) was added and the reaction mixture was immediately concentrated (N_2). To the mixture was added trifluoroacetic acid–H_2O (1:1, 20 μL), and the solution was left at rt for 20 min. The mixture was concentrated (N_2) and dissolved in AcCl/CH_3OH (9:1; 100 μL) and stirred at 110 °C in a sealed vial for 30 min. The product was concentrated (N_2) and redissolved in CH_2Cl_2 (10 μL) for GC; t_R 6.45 min; GC/MS, *m/z* (%) 118 (2), 59 (96), 31 (100).

B. From 13,14,15-Isocrambescidin 800 (4). Methyl (2*S*)-2-hydroxybutanoate was obtained from 4 (1.5 mg) by following the procedure described above: GC t_R 6.46 min; GC/MS, *m/z* (%) 118 (1), 59 (90), 31 (100).

C. From (2*S*)-2-Aminobutanoic Acid.[10] To a solution of (2*S*)-2-aminobutanoic acid (0.25 g, 2.4 mmol) in H_2SO_4 (1 N, 5 mL), $NaNO_2$ (0.278 g, 4.0 mmol in 1 mL of H_2O) was added slowly at 0 °C. The solution was allowed to stand at rt for 12 h, NaCl (1 g) was added and the aqueous solution was extracted with ether. Concentration of the ether extract produced a pale yellow oil (128 mg, 51%), $[\alpha]^{25}_D$ −4.8° (*c* 0.96, H_2O). A portion of the oil (5 mg) was dissolved in AcCl/CH_3OH (9:1, 1 mL), stirred at 110 °C for 30 min, evaporated (N_2), and dissolved in CH_2Cl_2 (1 mg/mL) for GC: t_R 6.45 min; GC/MS, *m/z* (%) 118 (2), 59 (60), 31 (100). Anal. Calcd for $C_4H_8O_3$: C, 46.13; H, 7.75. Found: C, 46.01; H, 7.79.

Methyl (2*R*)-2-Hydroxybutanoate from (2*R*)-2-Aminobutanoic Acid. (2*R*)-2-Aminobutanoic acid (0.25 g, 2.4 mmol) was treated as described for the (2*S*) analog, giving a colorless oil (85 mg, 34%) $[\alpha]^{25}_D$ + 4.1° (*c* 1.6, H_2O); lit.[11] $[\alpha]^{20}_D$ + 2° (*c*, 1, H_2O). A portion of the oil (5 mg) was derivatized as was the 2*S* isomer and then dissolved in CH_2Cl_2 (1 mg/mL) for GC: t_R = 6.85 min; GC/MS, *m/z* (%): 118 (1), 59 (40), 31 (100). Anal. Found: C, 45.92; H, 7.80.

Acknowledgment. This work was supported in part under a research agreement with PharmaMar, S. A. We are grateful to Drs. P. J. Scheuer and W. Y. Yoshida for HMBC and ROESY spectra performed on an Omega 500-MHz NMR spectrometer and to Dr. M. Uriz-Lespé for sponge taxonomy.

Supplementary Material Available: Copies of NMR and mass spectra of 13,14,15-isocrambescidin 800 and copies of chiral GC traces (16 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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