

Ptilomycalin A, Crambescidin 800 and Related New Highly Cytotoxic Guanidine Alkaloids from the Starfishes *Fromia monilis* and *Celerina heffernani*.

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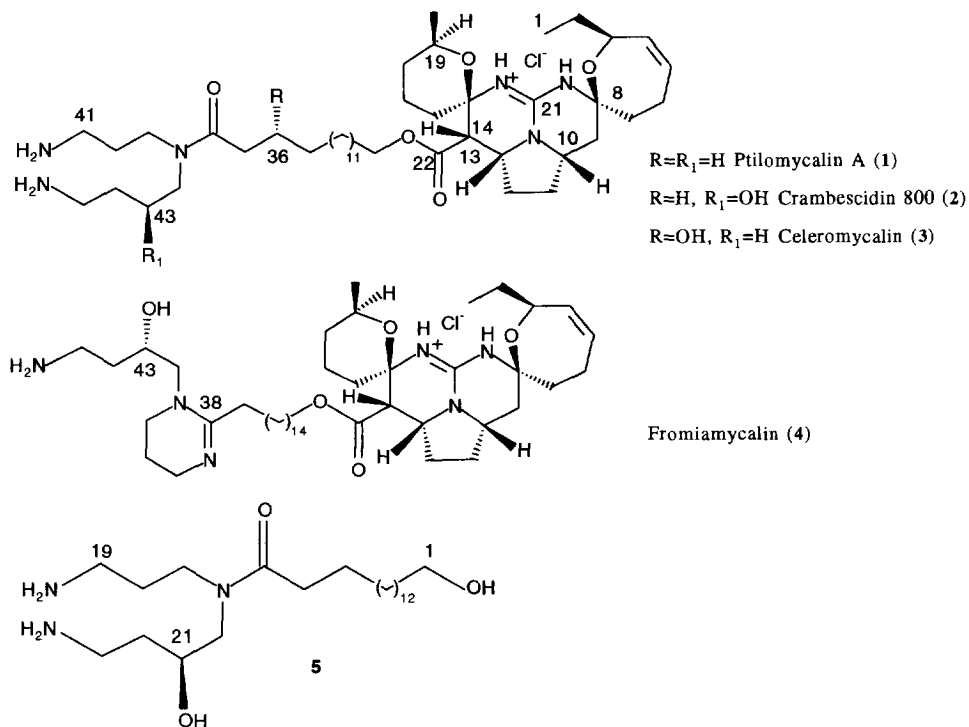
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Abstract: Two novel pentacyclic guanidine alkaloids, celeromycalin **3** and fromiamycalin **4**, have been isolated from the New Caledonian starfishes *Celerina heffernani* and *Fromia monilis*, respectively. Also found in *Celerina heffernani* are the known ptilomycalin A (**1**) and crambescidin 800 (**2**), which latter has been also isolated from *Fromia monilis*. The new compounds exhibited an high cytotoxic activity like the previous crambescidins. These complex pentacyclic guanidines are typical sponges metabolites and their occurrence in starfishes is noteworthy. *Fromia monilis* also contained the less active component **5**, made up from an hydroxyspermidine residue linked to a long chain ω -hydroxyacid.

Ptilomycalin A (**1**) from the sponges *Ptilocaulis spiculifer* and *Hemimycale sp.*^{1,2} and the related crambescidins (*e. g.* crambescidin 800, **2**) from the sponge *Crambe crambe*^{3,4} are an unique family of complex pentacyclic guanidines linked by a linear ω -hydroxy fatty acid to a spermidine or hydroxyspermidine unit. These compounds, which differ from one another in the chain length of the long-chain hydroxy-acid and in the presence or absence of a hydroxyl group at C-13 in the guanidine-containing portion and/or in the spermidine moiety, were found to be very antiviral, cytotoxic and antifungal agents. In connection with our systematic investigation of the chemical constituents of echinoderms⁶ we have examined the polar extracts of the starfishes *Fromia monilis* and *Celerina heffernani*, collected off New Caledonia, which both showed marked activity in antiviral assays vs. *Herpes simplex virus* HSV-1. In a previous paper we described the steroidal

glycoside constituents from *Fromia monilis* ⁷, which were modestly cytotoxic, and in this paper we report the discovery of crambescidin 800 (**2**) in both *Fromia monilis* and *Celerina heffernani*, which latter also contained ptilomycalin A (**1**), and of two new related guanidine alkaloids, named celeromycalin **3**, from *Celerina heffernani*, and fromiamycalin **4**, from *Fromia monilis*, as the highly cytotoxic agents. *Fromia monilis* also contained the modestly active compound **5**, made up from the hydroxyspermidine residue linked to long chain ω -hydroxyacid, the so called "anchor part" of this unique family of compounds, the pentacyclic guanidine moiety having been indicated the "vessel part" ².



These compounds were submitted to anti-HIV assays on cells CEM 4 infected by HIV-1 by the ANTI-AIDS UNIT of Rhône Poulenc Rorer and compounds **1-4** were found to be highly cytotoxic to the target cells with CC-50 of 0.11 $\mu\text{g/ml}$ compounds (**1**, **2** and **4**) and 0.32 $\mu\text{g/ml}$ (compound **3**) without cytoprotective effects at a dose of < 0.1 $\mu\text{g/ml}$. Interestingly compound **5** exhibited a weaker cytotoxicity with CC-50 of 2.7 $\mu\text{g/ml}$. These results related a structure activity relationship among these compounds, showing that the activity is mostly due to the pentacyclic guanidine portions of the molecules.

RESULTS AND DISCUSSION

Compounds **1-5** were isolated from the methanol soluble portion of the acetone extracts of the fresh organisms by DCCC separations, the solvents used being CHCl_3 -MeOH- H_2O 7:13:8 (ascending) and *n*-BuOH- Me_2CO - H_2O 3:1:5 (descending), followed by reverse phase HPLC (see Experimental). The known compounds **1** and **2** were identified by FABMS and NMR spectra and comparison with the literature data ^{2,3}.

Celeromycalin **3**, $[\alpha]_D^{25} = -4.5^\circ$, isolated from *C. heffermani*, exhibited in the FAB mass spectrum a pseudomolecular ion at m/z 801 $[\text{M} + \text{H}]^+$, already observed in the spectrum of crambescidin 800 (**2**). Comparison of the ^1H and ^{13}C NMR data of crambescidin 800 (**2**) and ptilomycalin A (**1**) with those for **3** (Table 1) argues the presence of the same ring system, and probably, the same relative stereochemistry, which was then confirmed by NOE difference spectra (CD_3OD). NOE's between H-10 and H-13, between CH_3 -1 and both H-10, H-13, and between H-13 and H-14, established the relative stereochemistry at C-8, C-10, C-13 and C-14. Similarly, NOE between the CH_3 -1 and H-19 assigned the stereochemistry at C-15 and C-19, in agreement with the stereochemistry reported for crambescidins and ptilomycalin A (**1**), and eliminated that reported for isocrambescidin 800, which requires inversion of the configuration of C-13, C-14 and C-15 with respect to **1** and **2** ⁴. Further analysis of the ^1H and ^{13}C NMR data for **3** established the presence of the spermidine residue, like in ptilomycalin A (**1**). The position of the hydroxyl group in the polymethylene chain of **3** was assigned by HMBC NMR experiment (Fig. 1), which indicated that the proton at δ 2.62 ppm (H-37) correlated with both the carbonyl carbon at δ 175.3 ppm (C-38) and the hydroxymethine carbon at δ 70.1 ppm (C-36). The carbon signal at 175.3 ppm, in turn, correlated with the protons on C-39 and C-42 of the spermidine residue, thus confirming unambiguously the assignment of signal at δ 175.3 ppm to the amide carbonyl-38, and accordingly located the hydroxyl group at C-36. The configuration 36R was assigned based on the results of the application of the Mosher's method ^{8, 9, 10}. In the ^1H NMR spectrum of the 36-(S)- α -methoxy- α -(trifluoromethyl)phenyl acetate (MTPA ester), the C-37 methylene proton signal were observed upfield shifted relative to the corresponding ones in the ^1H NMR spectrum of the 36-(R)-MTPA ester [$\Delta\delta$ ($\delta_S - \delta_R$); for H-37 $_\alpha$ = -0.03; and H-37 $_\beta$ = -0.06], in agreement with the 36R configuration. The proton signals at C-35 overlap with those of the polymethylene chain and were not observed as separate peaks. The absolute configuration of the pentacyclic guanidine moiety of one member of the crambescidin family was recently determined ⁴. Thus, assuming the same absolute configuration, formula **3** can be regarded as the absolute stereostructure of the novel celeromycalin.

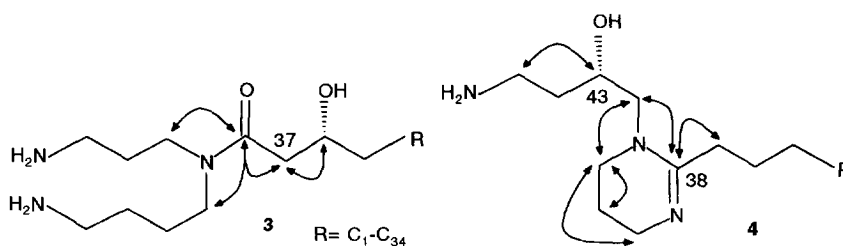


Fig. 1 \curvearrowright Some HMBC correlations of Celeromycalin (**3**) and Fromiamycalin (**4**)

Table 1. ^1H ^{13}C NMR data for Celeromycalin **3** and Fromiamycalin **4** (CD_3OD).

Position	3		4	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
1	0.89 t (7.3)	10.7	0.89 t (7.3)	10.8
2	1.48 m, 1.58 m	30.7	1.48 m, 1.58 m	30.6
3	4.45 brd (10.3)	72.3	4.45 brd (10.3)	72.3
4	5.55 brd (10.5)	134.3	5.55 brd (10.5)	134.3
5	5.75 brt (9.5)	131.3	5.75 brt (9.5)	131.3
6	2.22 m, 2.45 m	24.4	2.22 m, 2.45 m	24.4
7	2.04 dd (13.4, 5.7), 2.41 m	38.1	2.04 dd (13.4, 5.7), 2.41 m	38.1
8	-	85.1	-	85.1
9	2.68 dd, 1.45 t	38.4	2.68 dd, 1.45 t	37.8
10	4.08 m	55.5	4.08 m	55.5
11	2.32 m, 1.60	31.5	2.32 m, 1.60	31.5
12	2.38, 1.85 m	27.6	2.38, 1.85 m	27.6
13	4.38 m	54.0	4.38 m	54.0
14	3.12 d (5.0)	50.9	3.12 d (5.0)	50.7
15	-	82.1	-	82.1
16	1.75 m, 1.55 m	32.7	1.75 m, 1.55 m	32.6
17	1.87 m, 1.95 m	19.4	1.87 m, 1.95 m	19.5
18	1.32 m, 1.72 m	33.0	1.32 m, 1.72 m	33.0
19	3.89 m	68.4	3.89 m	68.4
20	1.13 d (6.3)	21.7	1.13 d (6.3)	21.8
21	-	150.1	-	150.1
22	-	170.0	-	170.2
23	4.17 t (6.6)	66.5	4.17 t (6.6)	66.5
24	1.63 m	30.2	1.63 m	29.6
25	1.32 m	30.1-30.6 ^a	1.32 m	27.0
26-34	1.32 m	30.1-30.6 ^a	1.32 m	30.1-30.6 ^a
35	1.43 m, 1.55 m	38.5	1.32 m	30.1-30.6 ^a
36	4.04 m	70.1	2.1 m	27.7
37	2.62 dd (14.5, 9.3), 2.42 dd	41.3	2.80 m, 2.52 m	32.8
38	-	175.3	-	166.2
39	3.51 m, 3.62 m	43.3	3.54 m, 3.61 m	47.8
40	2.1 m	29.6	2.1 m	19.8
41	2.94 m	37.9	3.42 m, 3.64 m	39.9
42	3.40 dd (14.0, 3.5), 3.54 dd (14.0, 10.0)	48.6	3.42 dd (14.0, 3.5), 3.64 dd (14.0, 10.0)	58.5
43	1.98 m, 2.05 m	26.8	4.08 m	67.1
44	1.68 m, 1.74m	26.9	1.88 m, 1.78 m	32.6
45	3.00 m	40.5	3.16 m	38.4

^a: several signals between 30.12 and 30.62. The coupling constants are given in Hz and are enclosed in parentheses. The assignments were aided by 2D-COSY, 2D-HOHAHA, HMQC, HMBC experiments, and comparison with the spectra described for the known **1** and **2**.

The FABMS spectrum of fromiamycalin **4**, $[\alpha]_D = -12^\circ$, isolated from *F. monilis*, exhibited a pseudomolecular ion at m/z 783 $[M + H]^+$, eighteen mass units shifted relative to **2** and **3** (m/z 801). The presence of a 3-hydroxyspermidine residue was at first indicated by a fragment peak in the FAB mass spectrum at m/z 696 $[M + H - 87]$, corresponding to the loss of a C_4H_9NO (aminohydroxybutyl residue) from $[M + H]^{3,4}$. The 1H and ^{13}C NMR chemical shifts in the C-42/C-45 residue in **4** were nearly identical to those in crambescidin 800 (**2**), and related crambescidins 3,4 , suggesting that the stereochemistry of the secondary alcohol in all compounds is the same 5 . The presence of the pentacyclic guanidine moiety was derived from NMR measurements (Table 1) and as in the other crambescidins a polymethylene chain from C-37 to C-23 was also indicated by NMR. In the ^{13}C NMR spectrum of **4** the carbonyl signal observed in the spectra of all crambescidins at *ca.* 175.0 ppm was found upfield shifted to 166.2 ppm. Thus, the structure of fromiamycalin was, consequently, concluded to be **4**. The HMBC spectrum (Fig. 1) gave further support to this conclusion by showing that the H_2 -42 proton signals exhibited cross peaks to C-39, to the hydroxymethine carbon-43 at 67.1 ppm, and to the amidine carbon-38 at 166.2 ppm, which latter is also correlated with H_2 -37 (δ 2.5 and 2.8 ppm).

Compound **5**, $[\alpha]_D = +3.5^\circ$, isolated from *F. monilis*, exhibited in the FABMS a pseudomolecular ion at m/z 416 $[M+H]^+$, accompanied by a fragment at m/z 329 $[M + H - 87]^+$, corresponding to the loss of an aminohydroxybutyl residue, which is characteristic of the hydroxyspermidine in the previous crambescidins 3,4 . The structure of the hydroxyspermidine and the long chain ω -hydroxyacid units were then confirmed by NMR data (Table 2). 1H and ^{13}C chemical shifts in the hydroxyspermidine unit in **5** were nearly identical to those in crambescidins 3,4,5 and fromiamycalin **4** suggesting that the stereochemistry of the secondary alcohol is the same.

Table 2. 1H and ^{13}C NMR data of Compound **5** (CD_3OD)

Position	δ_C	δ_H
1	63.0	3.57 t (7)
2	33.6	1.55 m
3-13	30.5-30.8 ^a	1.32 m
14	26.5	1.32 m
15	34.1	2.52 m
16	177.5	-
17	43.7	3.70 m
18	27.0	1.98 m
19	38.0	2.91 m
20	54.7	3.50 dd (13, 5), 3.41 (10)
21	68.6	3.98 m
22	32.9	1.72 m, 1.85 m
23	38.6	3.14 m

^a : several signals between 30.5 and 30.8. The coupling constants are given in Hz and are enclosed in parentheses.

EXPERIMENTAL

General Methods.

NMR measurements were performed on a Bruker AMX-500 spectrometer equipped with a Bruker X-32 computer, using the UXNMR software package. Some of the HMBC experiments, using field gradients, were performed on a Bruker DRX-500 spectrometer at Bruker, Karlsruhe (Germany), and on a Varian Unity 500 at Varian, Darmstadt (Germany).

Two-dimensional homonuclear proton chemical shift correlation (COSY) experiments were measured by employing the conventional sequence. The COSY spectra were obtained using a data set ($t_1 \times t_2$) of 1024 X 512 points for a spectral data width of 2994.05 Hz (relaxation delay 1 sec.). The data matrix was processed using an unshifted sine bell window function, following transformation to give a magnitude spectrum with symmetrization (digital resolution in both F2 and F1 dimensions was 2.994 Hz/pt).

The 2D-HOHAHA experiment was performed in the phase-sensitive mode (TPPI) using an MLEV-17 sequence for mixing ¹¹. The spectral width (t_2) was 2.994 Hz; 512 experiments of 160 scans each (relaxation delay 1.5 sec., mixing time 100 ms) acquired in 1K data points. For processing, an unshifted sine bell window function was applied in both dimensions before transformation. Resulting digital resolution in F2 was 2.9 Hz/pt. The {¹H, ¹³C} shift correlation experiments, at 300 K, utilized a 5-mm probe with reverse geometry and the sample was not spun.

¹H-detected heteronuclear multiple quantum coherence (HMQC) experiments were performed according to Bax and Subramanian ¹², using an initial BIRD pulse to suppress ¹H resonances not coupled to ¹³C and a GARP sequence for ¹³C decoupling during data acquisition. The spectral width in ¹H dimension was 2994.05 Hz; 256 experiments of 240 scans each (relaxation delay 1.5 s, delay after BIRD pulse 0.4 s, fixed delay t_1 3.3 ms) were acquired in 1K points. A sine square function was applied in t_2 dimension and a trapezoidal window in t_1 dimension (TM₁ 0.03 Hz, TM₂ 0.6 Hz) before Fourier transformation (digital resolution in F2 dimension 2.994 Hz/pt).

¹H detected heteronuclear multiple bond correlation (HMBC) spectroscopy was performed according to Bax and co-workers ^{13,14}.

Optical rotation were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm. Fast atom bombardment mass spectra (FABMS) were recorded in a glycerol-thioglycerol matrix (with 10 μ l of a solution of NaCl 0.1 N) in the positive ion mode on a VG ZAB instrument (Argon atoms of energy of 2-6 kV).

Animal Collection and Preliminary Experiments.

Fromia monilis (family Ophidiasteridae) Perrier and *Celerina heffermani* (family Ophidiasteridae) Livingstone were collected in the South of New Caledonia at depth of 5-40 m, in January. Taxonomic identification was performed by Jean Louis Menou of the ORSTOM Centre de Nouméa where reference specimens are on file (reference EA 13 and EA 17, respectively). Preliminary assays for antiviral activity were done on *Herpes simplex*, grown on Viro cells (monkey kidney epithelium cells) and showed marked activity [minimum viral inhibition concentration 3 μ g/ml (100 %)] of both the acetone extracts of both starfishes.

Extraction and Isolation.

The fresh organisms (*C. heffernani* and *F. monilis*), were extracted with acetone to give, after evaporation under reduced pressure, 4.57 g and 5.54 g residues, respectively, which were then partitioned between *n*-hexane and MeOH. The MeOH layer of *C. heffernani* (1.8 g) was chromatographed by DCCC using CHCl₃-MeOH-H₂O (7:13:8) in the ascending mode (the lower phase was stationary phase). Fractions were collected and combined into three main ones: 8-16 contained a mixture of guanidine alkaloids (160 mg), whereas fractions 17-30 (15 mg) and 31-70 (35 mg) were a complex mixture of sulphated steroidal glycosides. Fractionation of 8-16 (160 mg) was continued by DCCC using BuOH-Me₂CO-H₂O (3:2:5) in the descending mode (the upper phase was used as stationary phase) to give three main fractions. All fractions were separated by HPLC on a Waters C-18 μ -Bondapak column (30 cm x 3.9 mm i.d.; flow rate 1.5 ml min⁻¹) with MeOH-H₂O 43:57 as eluent to give ptilomycalin A (**1**, 6.0 mg), crambescidin 800 (**2**, 45 mg) and celeromycalin (**3**, 13 mg).

The MeOH layer of *F. monilis* (2.06 g) was chromatographed by DCCC in the same conditions as above and each fraction was purified straight by HPLC (C₁₈- μ Bondapak column; MeOH:H₂O 43:57) to give crambescidin 800 (**2**, 3.9 mg), celeromycalin (**3**, 3.8 mg), fromiamycalin (**4**, 3.5 mg) and compound (**5**, 3.2 mg).

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