“Polychlorinated Androstanes From the Burrowing Sponge *Cliona nigricans*”

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SUPPORTING INFORMATION

General methods. Low and high resolution EIMS spectra (70eV) were performed on a VG Prospec (Fisons) mass spectrometer. Optical rotations were measured at 589 nm on a Perkin-Elmer 192 polarimeter using a 10 cm microcell. IR (KBr) spectra were measured on a Bruker IFS-48 spectrophotometer. UV spectra were obtained in CH₃CN using a Beckman DU70 spectrophotometer. CD spectra (CH₃CN) were obtained on a JASCO 500A polarimeter. ¹H and ¹³C NMR spectra were determined on a Bruker AMX-500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts were referenced to the residual solvent signal (CDCl₃: δ_H 7.26, δ_C 77.0). Homonuclear ¹H connectivities were determined by the COSY experiment. Through-space ¹H connectivities were evidenced using a ROESY experiment with a mixing time of 500 ms. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bonded to ¹³C; the interpulse delays were adjusted for an average ¹J_{CH} of 140 Hz. Two and three bond ¹H-¹³C connectivities were determined by gradient 2D HMBC experiments optimized for a ^{2,3}J of 9 Hz. Medium pressure liquid chromatography was performed on a Büchi apparatus. HPLC were achieved on a Beckman apparatus equipped with a refractive index detector and LUNA (Phenomenex) SI60 (250 × 4 mm) columns.

Collection, extraction and isolation. A specimen of *Cliona nigricans* collected in summer of 2002 along the coasts of Gallinara (Italy) at a depth of 40 m and identified by G.B., was frozen immediately after collection and kept frozen until extraction. A voucher sample is deposited at the Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli “Federico II” (Ref. No. CN-01-02). The sponge (1700 g, dry weight after extraction) was homogenized and extracted with methanol (4 × 500 mL). Obtained extracts were pooled, evaporated in vacuo, and then partitioned between water and EtOAc to yield an organic extract weighing 12.8 g. This was subjected to MPLC purification over silica gel (230-400 mesh), eluting with a solvent gradient system of increasing polarity from *n*-hexane to MeOH. Fractions eluted with *n*-hexane-EtOAc 6:4 were combined and then further purified by HPLC (eluent *n*-hexane-EtOAc 6:4, flow 0.8 mL/min) yielding pure clionastatin A (**1**, 1.0 mg) and clionastatin B (**2**, 1.3 mg). A second specimen of *C. nigricans* (Ref. No. CN-01-03), collected in spring of 2003 in the Gulf of Genoa (Italy) at a depth of 35 m, identified by G.B., was treated exactly as above to furnish additional amounts of clionastatins A (**1**, 1.2 mg) and B (**2**, 1.9 mg).

Clionastatin A (1). amorphous solid, $[\alpha]_D^{25} + 66$ (*c* 0.02, CHCl₃); UV (CH₃CN): $\lambda_{\max}$ (ϵ) 214 (14500), 265 (12500), 303 (6000) nm; CD (CH₃CN): $\Delta\epsilon_{220} - 10$, $\Delta\epsilon_{244} + 41$; IR (KBr): $\nu_{\max}$ 1640, 1606, 1581, 880 cm⁻¹; EIMS (70 eV): *m/z* (relative intensity) 382/384/386 (1/1/0.3), 347/349/351 [*M*⁺ – Cl] (1.2/1/0.2), 333/335/337 [*M*⁺ – CH₂Cl] (1.2/1/0.2); HR-EIMS: *m/z* 382.0299 (calcd. for C₁₉H₁₇³⁵Cl₃O₂ 382.0294, 0.5 mDa error); ¹H and ¹³C NMR (CDCl₃): see Table 1.

Clionastatin B (2). amorphous solid; $[\alpha]_D^{25} + 54$ (*c* 0.02, CHCl₃); UV (CH₃CN): $\lambda_{\max}$ (ϵ) 215 (14500), 266 (12500), 303 (6000) nm; CD (CH₃CN): $\Delta\epsilon_{220} - 10$, $\Delta\epsilon_{244} + 41$; IR (KBr): $\nu_{\max}$ 1638, 1608, 1581 cm⁻¹; EIMS (70 eV): *m/z* (relative intensity) 416/418/420 (1.5/2/1), 381/383/385 [*M*⁺ – Cl] (1/1/0.3), 367/369/371 [*M*⁺ – CH₂Cl] (1/1/0.3); HR-EIMS: *m/z* 415.9940 (calcd. for C₁₉H₁₆³⁵Cl₄O₂ 415.9904, 3.6 mDa error). ¹H NMR (CDCl₃): δ 7.32 (s, H-17), 6.53 (s, H-6), 6.46 (d, *J* = 10.0 Hz, H-4), 6.08 (dd, *J* = 10.0, 2.0 Hz, H-3), 4.70 (dd, *J* = 9.0, 2.0 Hz, H-2), 4.24 (d, *J* = 11.3 Hz, H-19a), 4.20 (d, *J* = 9.0 Hz, H-1), 4.12 (s, H-14), 3.74 (d, *J* = 11.3 Hz, H-19b), 2.72 (ddd, *J* = 17.1, 3.3, 2.0 Hz, H-11a), 2.08 (ddd, *J* = 17.1, 15.2, 3.3 Hz, H-11b), 1.98 (ddd, *J* = 15.2, 3.3, 2.0 Hz, H-12a), 1.48 (ddd, *J* = 15.2, 15.2, 3.3 Hz, H-12b), 1.32 (s, H₃-18). ¹³C NMR (CDCl₃): δ 197.3 (s, C-15), 181.4 (s, C-7), 163.5 (d, C-17), 155.4 (s, C-9), 149.7 (s, C-5), 137.5 (s, C-8), 131.1 (d, C-3), 130.1 (d, C-6), 128.8 (d, C-4), 124.6 (s, C-16), 67.6 (d, C-1), 60.9 (d, C-2), 52.2 (s, C-10), 48.9 (d, C-14), 43.1 (s, C-13), 42.8 (t, C-19), 34.6 (t, C-12), 27.0 (q, C-18), 26.8 (t, C-11).

Cytotoxic Activity. WEHI 164 cells (murine fibrosarcoma cell line), RAW 264-7 cells (murine macrophage), and THP-1 cells (human monocytes) were maintained in adhesion on Petri dishes with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (heat-inactivated only for WEHI 164 cells) (FBS), 25 mM HEPES, penicillin (100 U/ml) and streptomycin (100 µg/ml). Then they were plated on 96-well plates and allowed to adhere at 37 °C in 5% CO₂/95% air for 2 h. Thereafter, the medium was replaced with 50 µL of fresh medium and then 75 µL aliquot of 1:2 v/v serial dilutions of clionastatins A-B (**1-2**) were added and the cells incubated for 72 h. The cells viability was assessed through an MTT conversion assay (Mossmann, T. *J. Immunol. Methods* **1983**, *139*, 55-63). After 72 h, 25 µL of MTT (5 mg/mL) was added and the cells were incubated for additional 3 h. Following this time the cells were lysed and the dark blue crystals dissolved with 100 µL of a solution containing 50% (v:v) DMF and 20% (v:v) SDS with an adjusted pH of 4.5. The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with compounds **1** and **2** was calculated as: % dead cells = 100 - (OD treated/OD control) × 100. The results of cytotoxic activity, expressed as IC₅₀ (µg/µL) are: WEHI-164 (**1**: 0.8; **2**: 1.1); RAW 264-7 (**1**: 1.1; **2**: 1.4); THP-1 (**1**: 1.5; **2**: 2.0).

Table . ^1H (500 MHz), ^{13}C (125 MHz), and 2D NMR data for Clionastatin A (**1**) recorded in CDCl_3

Pos.	δC , mult.	δH , mult., J in Hz	COSY ($\text{H}\rightarrow\text{H}$)	g-HMBC ($\text{H}\rightarrow\text{C}$)	ROESY ($\text{H}\rightarrow\text{H}$)
1	67.5, CH	4.20, d, 9.0	2	2, 3, 4, 19	
2	61.3, CH	4.71, dd, 9.0, 2.0	1, 3	1, 3, 4, 10	19a
3	132.8, CH	6.06, dd, 10.0, 2.0	2, 4	1, 2, 4, 5	4
4	128.6, CH	6.46, d, 10.0	3	2, 3, 6, 10	3
5	149.3, C				
6	130.1, CH	6.53, s		4, 5, 7, 8, 10	
7	181.6, C				
8	138.5, C				
9	154.9, C				
10	52.3, C				
11a	27.3, CH_2	2.69, ddd, 17.1, 3.3, 2.0	11b, 12a, 12b	8, 9, 10, 12	19b, 18
11b		2.08, ddd, 17.1, 15.2, 3.3	11a, 12a, 12b	8, 9, 10, 12	12b
12a	35.0, CH_2	1.92, ddd, 15.2, 3.3, 2.0	11a, 11b, 12b	11, 13, 14, 18	
12b		1.48, ddd, 15.2, 15.2, 3.3	11a, 11b, 12a	11	11b, 14
13	45.5, C				
14	50.1, CH	3.99, s		7, 8, 9, 13, 16, 18	12b
15	205.9, C				
16	134.0, CH	6.31, d, 6.5	17	13, 14, 15	17
17	170.5, CH	7.40, d, 6.5	16	13, 15, 18	16
18	27.0, CH_3	1.32, s		12, 14, 17	11a
19a	43.6, CH_2	3.76, d, 11.3	19b	1, 5, 9, 10	2
19b		4.24, d, 11.3	19a	1, 5, 9, 10	11a

EIMS spectrum of clonastatin B

^1H NMR spectrum (CDCl_3) of clionastatin A

Low-field region of ^1H NMR spectrum (CDCl_3) of clionastatin A

Gradient acquired 2D HMBC spectrum of clonastatin A