ORGANIC LETTERS

2004
Vol. 6, No. 10
1633−1635

Polychlorinated Androstanes from the Burrowing Sponge *Cliona nigricans*

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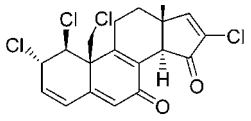
Received March 10, 2004

ABSTRACT



Clionastatin B

Two new steroidal derivatives, named clionastatins A and B, have been isolated from the burrowing sponge *Cliona nigricans*. These molecules are tri-and tetrachlorinated androstane derivatives, respectively, and they represent the first polyhalogenated steroids found in a natural organism, either marine or terrestrial, and the first examples of halogenated androstanes in nature. Both clionastatins proved to be potently cytotoxic.

Steroids are among the most represented secondary metabolites in living organisms; they are practically ubiquitous in all eukaryotic cells and many of them play essential and specific roles.[1] The incredible diversity found in their structures is the outcome of highly efficient biosynthetic modifications that result in the introduction of multiple oxygenated functionalities or, sometimes, in extensive degradation of the basic tetracyclic structure (e.g., secosteroids).[2] Among the thousands of naturally occurring steroidal derivatives, the halogenated ones are extremely rare,[3] although halogen-containing secondary metabolites are well-known and abundant in nature, particularly in marine organisms. The few natural halogenated steroids contain chlorine and include physalolactones from the Solanaceae plant *Physalis peruviana*,[4] blattellastanosides from the German cockroach *Blattella germanica*,[5] and kiheisterones from the sponge *Strongylacidon* sp.[6] Remarkably, all of these compounds possess a single chlorine atom, and almost invariably it is part of a chlorohydrin group, thus suggesting their origin from the corresponding epoxide. Interestingly, in many cases, the putative epoxide precursor co-occurs with the chlorinated derivative.[7]

In our ongoing search for biologically active compounds from marine organisms, we had the chance to analyze the burrowing sponge *Cliona nigricans*, for which there was no previous report of chemical investigation. Burrowing sponges are known to occur in tropical to temperate coastal waters

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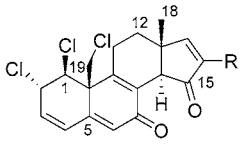
10.1021/ol049548r CCC: $27.50
Published on Web 04/08/2004

and to live within various kinds of calcareous substrates, excavating them by mechanical and chemical tools.[8] As a result of its spread and high etching activity, the genus *Cliona* has been recognized as an important negative factor in the balance of biodeposited carbonates. Previous chemical investigations on *Cliona* species yielded linear peptides,[9] pyrrole alkaloids,[10] and simple sterols.[11]



1 R = H
2 R = Cl

The organic extract obtained from a massive specimen of *C. nigricans*, collected along the coasts of Gallinara Island (Italy), showed activity in preliminary cytotoxicity assays. Bioassay guided fractionation of the organic material afforded two new steroidal derivatives named clionastatins A (**1**, 1.0 mg) and B (**2**, 1.3 mg), isolated as the main components responsible for the cytotoxic activity. Clionastatins A and B are tri-and tetrachlorinated androstane derivatives, respectively, and their structures appear exceptional in many respects. First of all, to date, no steroidal nucleus possessing the same oxidation pattern found in clionastatins [3,5,8(9),-16-tetraen-7,15-dione] has been described yet, among either natural or synthetic compounds. Furthermore, both the number and the arrangement of halogen atoms attached to the androstane nucleus of clionastatins are unique. Indeed, these molecules represent the first polyhalogenated steroids found in a natural organism, either marine or terrestrial and, moreover, can be regarded as the first examples of halogenated androstanes in nature.

EIMS of clionastatin A (**1**)[12] exhibited a molecular ion cluster at *m*/*z* 382/384/386 (in a ratio ca. 1/1/0.3) in accordance with the presence of three chlorine atoms. High-resolution measurement on the peak at lower mass established for **1** the molecular formula $C_{19}H_{17}Cl_3O_2$, requiring 10 sites of unsaturation. The relatively few resonances appearing in the ^{1}H NMR spectrum of **1** (Table 1) were spread over the whole spectrum without signal overlapping: four multiplets and a methyl singlet between δ 1.3 and 2.7, four doublets and a methine singlet in the midfield region (δ 3.2−4.8), and four doublets and a methine singlet in the sp^2 region, between δ 6.0 and 7.4. The ^{13}C NMR spectrum of **1** (Table 1) showed the following resonances: (i) two carbonyl groups (δ 181.6 and 205.9), accounting for both the oxygen atoms of the molecular formula; (ii) eight additional sp^2 carbons (between δ 128.0 and 171.0), building up four double bonds; and (iii) nine carbon atoms in the sp^3 region of the spectrum. Thus, to complete its unsaturation requirement, **1** must possess four rings. HMQC experiment established all the C−H connectivities and revealed that the 17 hydrogen atoms of clionastatin A (**1**) are actually distributed in one methyl, three methylene, and eight methine groups. Additionally, five sp^2 (two of which are the carbonyls) and two sp^3 carbon atoms are unprotonated.

Table 1. NMR Data for Clionastatin A (**1**) Recorded in $CDCl_3$ at 500 MHz for ^{1}H and 125 MHz for ^{13}C

carbon	δ_H, mult, *J* in Hz	δ_C, mult
1	4.20, d, 9.0	67.5, CH
2	4.71, dd, 9.0, 2.0	61.3, CH
3	6.06, dd, 10.0, 2.0	132.8, CH
4	6.46, d, 10.0	128.6, CH
5		149.3, C
6	6.53, s	130.1, CH
7		181.6, C
8		138.5, C
9		154.9, C
10		52.3, C
11a	2.69, ddd, 17.1, 3.3, 2.0	27.3, CH_2
11b	2.08, ddd, 17.1, 15.2, 3.3	
12a	1.92, ddd, 15.2, 3.3, 2.0	35.0, CH_2
12b	1.48, ddd, 15.2, 15.2, 3.3	
13		45.5, C
14	3.99, s	50.1, CH
15		205.9, C
16	6.31, d, 6.5	134.0, CH
17	7.40, d, 6.5	170.5, CH
18	1.32, s	27.0, CH_3
19a	3.76, d, 11.3	43.6, CH_2
19b	4.24, d, 11.3	

Inspection of the ^{1}H−^{1}H COSY spectrum indicated that the molecule contains few spin systems, all being restricted to few resonances, and therefore providing scarce information about the carbon framework of **1**. However, key correlations appearing in the gradient-selected HMBC spectrum allowed us to extend some of these spin systems, leading to five quite informative partial structures (Figure 1). Fragment **A** is a simple spin system made up of two methylenes, both linking diastereotopic protons, whereas fragment **B** contains a *cis*-double bond (J = 6.5 Hz) conjugated with a ketone carbonyl also flanked by an uncoupled methine group (^3J HMBC correlations: δ_H 7.40/δ_C 205.9; δ_H 6.31/δ_C 50.1. ^2J HMBC

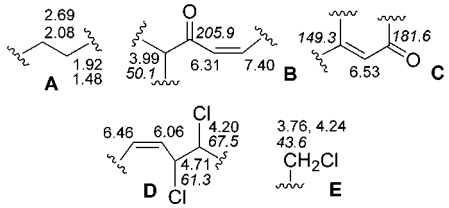


Figure 1. Partial structures deduced by COSY and g-HMBC.

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(12) Clionastatin A (**1**): amorphous solid; $[\alpha]^{25}_D$ +66 (*c* 0.02, $CHCl_3$); UV (CH_3CN) λ_{max} (ϵ) 214 (14500), 265 (12500), 303 (6000) nm; CD (CH_3CN) $\Delta\epsilon_{220}$ −10, $\Delta\epsilon_{244}$ +41; IR (KBr) ν_{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$^+$ − CH_2Cl] (1.2/1/0.2); HR-EIMS *m*/*z* 382.0299, calcd for $C_{19}H_{17}{}^{35}Cl_3O_2$ 382.0294.

correlations: δ_H 6.31/δ_C 205.9; δ_H 3.99/δ_C 205.9). A second conjugated ketone carbonyl was unveiled in fragment **C**, but in this case the double bond is trisubstituted and the single sp^2 proton ($\delta_H = 6.53$) showed ^2J HMBC correlations with the two singlet carbon signals at δ_C 149.3 and 181.6. Fragment **D** contains the third double bond (also *cis*, $J =$ 10.0 Hz) and two vicinal methine groups whose proton and carbon chemical shifts (δ_H 4.20, δ_C 67.5; δ_H 4.71, δ_C 61.3) are both indicative of linkage with chlorine atoms. The third chlorine atom was confidently linked to the isolated methylene group constituting fragment **E**. The existence of fragment **E** was also strongly indicated by the presence of an intense peak at *m*/*z* 333/335/337 [$M^+ - CH_2Cl$] (in a ratio 1.2/1/0.2) in the EI mass spectrum of **1**.

The network of g-HMBC correlations depicted in Figure 2 was instrumental to establish the correct assembly of the

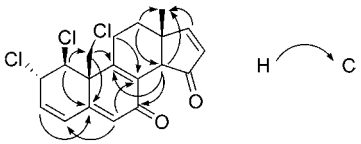

Figure 2. Key g-HMBC correlations observed for clionastatin A.

above structural subunits, thus building up the tetracyclic carbon framework of clionastatin A (**1**), which turned out to be a highly unsaturated androstane nucleus bearing three chlorine atoms, all located on ring A. This skeleton comprises an α,α′-dienone (the ^{13}C NMR resonance at δ_C 181.6 of the ketone group at C-7 is well explained by the presence of a cross-conjugated enone) and an α,β-unsaturated ketone carbonyl placed in a five-membered ring (perfectly consistent with the relatively high ^{13}C NMR value for C-15 (δ_C 205.9)[13]). The large proton−proton coupling constant between H-1 and H-2 ($J_{H-1/H-2} = 9.0$ Hz) indicated that the involved protons must be in *trans*-diaxial relationship. On the other hand, correlations in a ROESY (Figure 3) spectrum

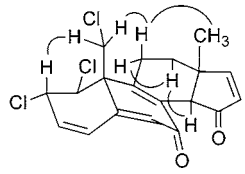

Figure 3. Key ROESY correlations detected for clionastatin A.

from H-2 (δ 4.71) to H-19a (δ 3.76), from both H-19b (δ 4.24) and H_3-18 (δ 1.32) to H-11a (δ 2.69), and from both H-11b (δ 2.08) and H-14 (δ 3.99) to H-12b (δ 1.48) defined the relative stereochemistry for clionastatin A (**1**). The absolute configuration reported for **1** is that invariably found for androstane derivatives.

Analysis of the MS data of clionastatin B (**2**) revealed that in its structure a fourth chlorine atom replaces one of the hydrogens of clionastatin A (**1**). Indeed, EIMS of clionastatin B (**2**) exhibited molecular ions at *m*/*z* 416/418/420 in a ratio ca. 1.5/2/1, a pattern of isotopic peaks consistent with the presence of four chlorine atoms. HR measurement on the peak at lower mass indicated the molecular formula $C_{19}H_{16}Cl_4O_2$ for **2**. Both ^{1}H and ^{13}C NMR spectra of clionastatin B (**2**) showed a very strong resemblance to parallel data assigned for **1**, except for ^{1}H/^{13}C resonances of ring D atoms. Routine inspection of these spectra, assisted by 2D NMR experiments, indicated that substitution of hydrogen with chlorine must occur at position 16. Indeed, ^{1}H NMR spectrum of **2** is lacking in the signal attributed to H-16, and H-17 (position secured by HMBC coupling with C-12, C-13, and C-14) resonates as a singlet at δ_H 7.32. Additionally, the chemical shift of C-17 is shifted upfield by 7 ppm compared with the same carbon in **1** (δ_C 163.5 in **2**, δ_C 170.5 in **1**). This is consistent with the proposed structure; indeed, substitution of a proton with chlorine in an alkene is known to cause an upfield shift of about 6 ppm of the adjacent alkene carbon.[6,13] Clionastatin B (**2**) showed the same pattern of coupling constants and of ROESY correlations above-reported for clionastatin A (**1**); thus, the two molecules must share the same stereochemistry.

The exceptionality of the structures of the two clionastatins raised the doubt that they could be artifacts. To check this possibility, a second specimen of *C. nigricans* was then collected in a different location of the Gulf of Genoa (Italy). Extraction and partitioning, carried out following the same procedure used before, afforded **1** and **2** in almost the same yield, thus pointing to clionastatins as genuine secondary metabolites. However, to understand the origin of these unique molecules, the symbiotic population of *Cliona nigricans* should be taken into account. Indeed, this organism is characterized by a strong and stable association with zooxanthellae,[14] and therefore it cannot be excluded that **1** and **2** are actually produced by the microalga or by a cooperative biosynthesis.

Clionastatins A (**1**) and B (**2**) were isolated as the main components responsible for the cytotoxic activity of the organic extract and, therefore, were evaluated for cytotoxicity against three different tumor cell lines: WEHI 164 (murine fibrosarcoma), RAW 264-7 (murine macrophage), and THP-1 (human monocytes). Both of them proved to be potently active against all the tested cell lines, with IC_{50} values ranging from 0.8 to 2.0 μg/mL (Supporting Information).

Acknowledgment. Financial support was provided by M.I.U.R., PRIN 2003.

Supporting Information Available: Experimental details and spectral data for **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL049548R

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