Synthesis of Clionamine B, an Autophagy Stimulating Aminosteroid Isolated from the Sponge Cliona celata

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Autophagy-Modulating Aminosteroids Isolated from the Sponge *Cliona celata*

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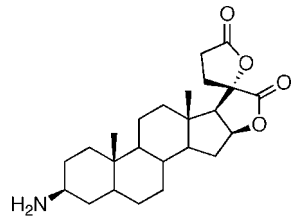
ABSTRACT



4 Clionamine D

Clionamines A−D (1−4), new aminosteroids that modulate autophagy, have been isolated from South African specimens of the sponge *Cliona celata*. Clionamine D (4) has an unprecedented spiro bislactone side chain.

Autophagy is a highly conserved catabolic process by which proteins and organelles in the cytoplasm are sequestered into double-membrane vesicles called autophagosomes and delivered to lysosomes for degradation and recycling of the low molecular weight products.[1] Autophagy is upregulated during periods of nutrient starvation, which facilitates cell survival.[1a] It is involved in mammalian developmental processes, innate and adaptive immunity, degradation of invading bacteria, and diseases such as neurodegeneration.[1b]

Although our understanding of autophagy is increasing rapidly, deeper insights into the regulation and molecular machinery of autophagy are required to clarify its role in diverse cellular processes. The chemical tools rapamycin, bafilomycin A1, and chloroquine, which permit rapid user-controlled activation or inhibition of autophagy, have proven useful reagents for studying this process.[1d] However, these reagents are limited because their targets are either only indirectly or nonexclusively involved in autophagy and their off-target effects can lead to ambiguous or misleading results. Studies in yeast have identified over 30 autophagy-related (Atg) genes.[1a] At present, there are no known direct inhibitors of any of the Atg proteins. Leading investigators in the field have proposed that new insights could be made if each definable step of autophagy could be independently blocked with chemical probes that operate without off-target effects.[1d]

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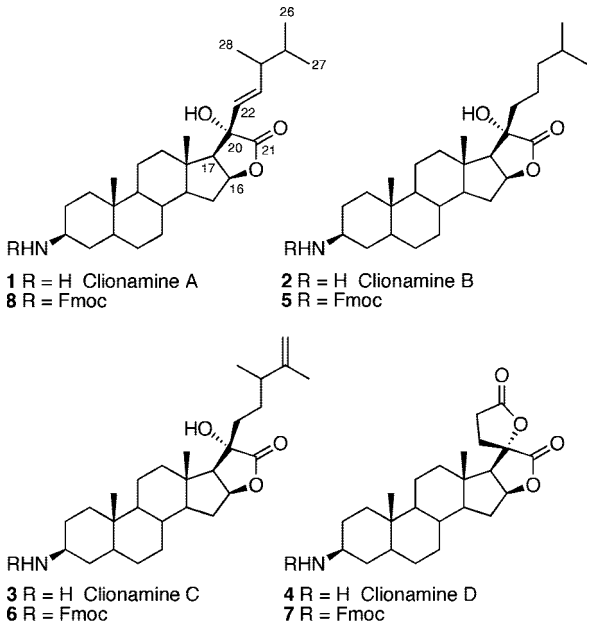
These compounds should facilitate the identification of new Atg genes, new biological roles for autophagy, and represent potential starting points in the development of therapeutics for neurodegenerative and other diseases.

In an effort to discover new chemical genetics tools for studying autophagy, we have screened a library of marine invertebrate extracts using a new "high content" assay designed to detect modulators of autophagosome accumulation.[2a] The crude MeOH extract of the sponge *Cliona celata* collected in South African marine waters showed promising activity in the screen. Assay-guided fractionation of the extract led to the identification of a family of novel aminosteroids **1**−**4**. One of the aminosteroid congeners, clionamine D (**4**), has an unprecedented spiro bislactone side chain. Details of the isolation and structure elucidation of clionamines A−D (**1**−**4**) are presented below.



Specimens of *C. celata* were collected by hand using SCUBA at −20 m in Coffee Bay off the warm temperate Wild Coast of South Africa and frozen for storage.[3] Lyophilized sponge material was exhaustively extracted with MeOH to give a crude extract that was active in the autophagy assay. The residue from the MeOH extract was partitioned between H_2O and EtOAc, and the resulting EtOAc-soluble portion was further fractionated via Sephadex LH-20 chromatography eluting with MeOH. Reversed-phase HPLC separation (65% H_2O/35% acetonitrile/0.1% TFA) of the most bioactive LH-20 fraction gave pure clionamine A (**1**). Other bioactive LH-20 fractions contained mixtures of aminosteroids that could not be separated into pure components by either normal-phase or reversed-phase HPLC. These fractions were reacted with Fmoc-Cl, and the resulting Fmoc derivatives were purified by normal-phase HPLC (17% EtOAc/ 83% hexanes) to give pure samples of **5**−**7**. Pure clionamine A (**1**) was also reacted with Fmoc-Cl to give the derivative **8**, which was purified by normal-phase HPLC, for comparison with **5**−**7**.

Clionamine A (**1**) was isolated as an optically active white amorphous solid that gave a $[M + H]^+$ ion at *m*/*z* 444.3465 in the HRESIMS appropriate for a molecular formula of $C_{28}H_{45}NO_3$ (calcd for $C_{28}H_{46}NO_3$, 444.3478), requiring seven sites of unsaturation. The ^{13}C/DEPT data (CD_3OD) showed 27 clearly resolved resonances (5 × CH_3; 8 × CH_2; 11 × CH; 3 × C) accounting for 42 hydrogens attached to carbon. A very weak resonance in the 1D ^{13}C NMR spectrum at δ 76.6 (C-20) showed strong correlations in the HMBC spectrum, identifying it as the last outstanding carbon indicated by the HRESIMS measurement. A LRESIMS run in CD_3OD gave a $[M + D]^+$ ion at *m*/*z* 448, confirming three exchangeable protons, which in combination with the DEPT data accounted for all 45 hydrogen atoms required by the HRESIMS. Preliminary examination of the ^1H NMR spectrum obtained for clionamine A (**1**) revealed the presence of five upfield methyl resonances, two singlets [δ 0.81 (Me-18), 0.87 (Me-19)] and three doublets [δ 0.91 (Me-26), 0.91 (Me-27), 1.02 (Me28)], and a complex aliphatic proton resonance envelope between δ 1.1 and 2.3, typical of a steroid.

The C-3 carbinol methine ^1H resonance usually found in steroids was replaced in **1** by a more upfield methine at δ 3.08 that was correlated in the HSQC spectrum to a carbon at δ 51.7, suggestive of a C-3 amine, in agreement with the HRESIMS identification of a nitrogen atom in the molecular formula. Analysis of the COSY, HSQC, HMBC, HSQC-TOCSY, and ROESY data obtained for **1** (Supporting Information) confirmed the presence of a standard steroidal ABC ring system in **1** with a primary amine located at C-3 as shown in Figure 1. COSY and HMBC correlations showed

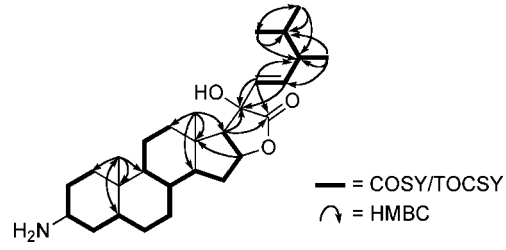


Figure 1. 2D NMR correlations observed for clionamine A (**1**).

that the three doublet methyl resonances could be assigned to an unsaturated seven-carbon fragment (C-22 to C-28), typical of C-24 methylated steroidal side chains. The resonances assigned to the alkene protons in the seven carbon fragment [δ 6.13 (H-23); 5.75 (H-22)] both showed HMBC correlations to the quaternary carbon resonance at δ 76.6 (C-20), and one of them (H-22) showed an additional HMBC correlation to a carbon resonance at δ 178.7 (C-21), assigned

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(3) The sponge specimens were collected as part of a collaboration between M. Davies-Coleman and D. J. Faulkner and transferred under contract to the Pfizer research laboratories in St. Louis. When Pfizer disbanded its natural products program, they generously donated their marine invertebrate collection to Andersen's group at UBC.

to an ester carbonyl. A methine resonance at δ 2.14 (H-17; HSQC to δ 65.8) was also correlated in the HMBC spectrum to both carbon resonances at δ 76.6 and 178.7, demonstrating that the side chain alkene (C-22), the ester carbonyl (C-21), and the methine carbon (C-17) were all substituents on the same oxygenated quaternary carbon (C-20). When the data for **1** were recorded in DMSO-d_6, there were HMBC correlations observed between an OH proton resonance at δ 6.07 (OH-20; no HSQC correlations) and carbon resonances at δ 74.7 and 176.1, assigned to C-20 and C-21, respectively, showing that there was also a hydroxyl substituent attached to the quaternary carbon (C-20).

COSY correlations were observed between the methine resonance at δ 2.14 (H-17) and a deshielded methine resonance at δ 5.07 (H-16; HSQC to δ 84.0), and from there in sequence to δ 1.48 (H-15α) and 2.25 (H-15β) and on to δ 1.17 (HSQC to δ 56.5), which had been assigned to H-14 as part of the COSY and HMBC confirmation of the ABC ring system. HMBC correlations observed between the Me-18 resonance (δ 0.81) and the carbon resonances assigned to C-14 (δ 56.5) and C-17 (65.8) confirmed that C-14 and C-17 were part of a standard five-membered steroid ring D. The downfield shift of the H-16 (δ 5.07) and C-16 (δ 84.0) resonances were consistent with the attachment of the C-21 ester alkoxy oxygen to C-16 to form a γ lactone, which provided the remaining required site of unsaturation.

ROESY correlations between the H-3 resonance (δ 3.08) and both the H-1α (δ 1.82) and H-5 (δ 1.23) resonances showed that the amino group at C-3 was equatorial. Additional ROESY correlations (Supporting Information) were consistent with the normal steroidal trans,anti,trans,anti,trans relative configuration in the ABCD ring system of **1**. Me-18 (δ 0.81) gave a ROESY correlation to H-22 (δ 5.75), requiring that C-20 was a β substituent on C-17 and C-22 was a β substituent on C-20. ROESY correlations observed between H-16 (δ 5.07) and H-17 (δ 2.14) in CD_3OD and between OH-20 (δ 6.07) and both H-16 (δ 4.98) and H-17 (δ 2.08) in DMSO-d_6 demonstrated that H-16, H-17, and OH-20 were cis as shown. H-22 (δ 5.75) and H-23 (δ 6.13) had a scalar coupling constant $J = 15.8$ Hz, and H-22 showed ROESY correlations to the H-24 (δ 2.09) and Me-28 (δ 1.02) resonances demonstrating that the $\Delta^{22,23}$ olefin had an *E* configuration. It was not possible to determine the relative configuration at C-24 by analysis of the NMR data obtained for **1**, so it remains unassigned.

Clionamine B (**2**) was isolated as its Fmoc derivative **5**, which was a white optically active amorphous solid. The HRESIMS obtained for **5** contained an $[M + Na]^+$ ion at *m*/*z* 676.3975 consistent with a molecular formula of $C_{42}H_{55}NO_5$ (calcd for $C_{42}H_{55}NO_5Na$, 676.3978). Subtracting the atoms accounted for by addition of the Fmoc fragment ($C_{15}H_{10}O_2$) gave a molecular formula of $C_{27}H_{45}NO_3$ for clionamine B (**2**), requiring six sites of unsaturation and differing from the formula of **1** simply by loss of C. Comparison of the 1D and 2D NMR data obtained for **5** in C_6D_6 (Supporting Information) with the data obtained for the Fmoc derivative **8** prepared from clionamine A (**1**) recorded in the same solvent revealed that **5** and **8** were nearly identical, and differed only in the side chain. COSY and HMBC correlations (Supporting Information) revealed that **5** had the saturated C-22 to C-27 side chain structure found in cholesterol. ROESY correlations observed between Me-18 (δ 0.70) and H-22 (δ 1.66) and between OH-20 (δ 1.98) and both H-16 (δ 4.91) and H-17 (δ 1.82) confirmed that **5** and **8** had the same relative configuration about the γ-lactone ring.

The Fmoc derivative **6** of clionamine C (**3**) was isolated as a colorless glass that gave a $[M + Na]^+$ ion at *m*/*z* 688.3961 in the HRESIMS consistent with a molecular formula of $C_{43}H_{55}NO_5$ (calcd for $C_{43}H_{55}NO_5Na$, 688.3978), identical with the molecular formula of the Fmoc derivative **8** of clionamine A (**1**). Analysis of the COSY, HMBC, and ROESY data (Supporting Information) obtained for **6** in C_6D_6 showed that clionamine C (**3**) differed from clionamine A only by having a $\Delta^{25,26}$ olefin in place of the $\Delta^{22,23}$ olefin present in **1**.

Clionamine D (**4**) was isolated as its Fmoc derivative **7**, which gave a $[M + Na]^+$ ion at *m*/*z* 646.3130 in the HRESIMS consistent with a molecular formula of $C_{39}H_{45}NO_6$ (calcd for $C_{39}H_{45}NO_6Na$, 646.3145). Subtracting the atoms present in the Fmoc residue in **7** gave a molecular formula of $C_{24}H_{35}NO_4$ for the underivatized natural product clionamine D (**4**), requiring eight sites of unsaturation. The ^{13}C NMR spectrum showed thirty three well-resolved resonances, nine assigned to the symmetrical Fmoc residue and twenty four to the steroidal moiety. Analysis of the DEPT data showed that there were 44 hydrogen atoms attached to carbon ($16 \times CH$, $11 \times CH_2$, $2 \times CH_3$) and, therefore, only one exchangeable hydrogen, which had to be associated with the Fmoc amide functionality.

A comparison of the 1D and 2D NMR data obtained for **7** and **8** in C_6D_6 (Supporting Information) showed that clionamine D (**4**) and clionamine A (**1**) had identical steroidal ABCD ring systems and differed only in their side chains. The molecular formula of clionamine D (**4**) contained only 24 carbons, 3 less than the standard 27 carbon steroidal skeleton, indicating that its side chain was degraded. In addition, the 1D ^1H NMR spectrum of the Fmoc derivative **7** contained only two methyl resonances, and they could be assigned to Me-18 (δ 0.43, s) and Me-19 (δ 0.46, s), providing further support for a degraded side chain in **4**.

A series of four mutually coupled resonances in the COSY spectrum of **7** [(δ 2.13 (H-22); 1.60 (H-22′); 2.58 (H-23); 1.91 (H-23′)], which were correlated in pairs to two carbon resonances at δ 25.0 (C-22) and 28.3 (C-23) in the HSQC spectrum, could be assigned to an isolated spin system comprised of two vicinal methylene carbons (Figure 2). All four of the C-22 and C-23 methylene proton resonances showed HMBC correlations to resonances at δ 86.0 (C-20) and 173.9 (C-24), assigned to an oxygenated quaternary carbon and an ester carbonyl, respectively, that had to be the substituents on either end of the $-CH_2CH_2-$ fragment. The geminal proton resonances at δ 2.13 (H-22) and 1.60 (H-22′) showed additional HMBC correlations to a second deshielded resonance at δ 174.5 (C-21), also assigned to an ester carbonyl. A proton resonance at δ 1.69 (d, $J = 6.4$

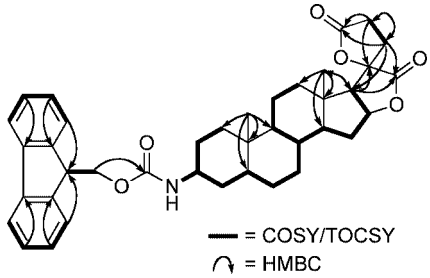

Figure 2. 2D NMR correlations observed for Fmoc clionamine D (**7**).

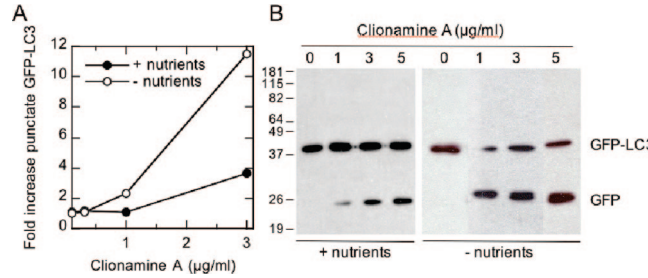

Figure 3. Induction of autophagosome accumulation by different concentrations of clionamine A (**1**) during 4 h incubation.

Hz), assigned to H-17, showed HMBC correlations to the Me-18 (δ 14.2) and C-12 (δ 39.1) carbon resonances and also to the resonances at δ 86.0 and 173.9, revealing that the later resonances could be assigned to C-20 and C-21, respectively. Although no HMBC correlation was observed between the H-16 resonance at δ 4.58 and the C-21 carbonyl resonance (δ 174.5), the downfield shifts of H-16 and C-16 (δ 83.4) were consistent with the attachment of the C-21 ester alkoxy oxygen to C-16 to form the E ring γ-lactone linkage found in the other clionamines. Connection of the final two unsatisfied valences, the alkoxy oxygen of the C-24 ester and the quaternary oxygenated carbon C-20, to give a second γ-lactone generated the final required site of unsaturation and established the complete constitution of **7**.

ROESY correlations observed between Me18 (δ 0.43) and both H-22 (δ 2.13) and H-22′ (δ 1.60) established that C-20 was β on C-17 and that C-22 was β on C-21 as shown. An additional ROESY correlation between H-16 (δ 4.58) and H-17 (δ 1.69) confirmed that they were cis as in clionamines A (**1**) to C (**3**).

The Fmoc derivatives **5**−**7** were treated with piperidine in DMF at rt which effectively liberated the underivatized natural products clionamines B to D (**2**− **4**) for biological testing and spectroscopic characterization. The NMR spectra obtained for **2**−**4** are available in the Supporting Information.

Clionamines A to D induced autophagosome accumulation as measured by the formation of cytoplasmic punctate Green Fluorescent Protein (GFP)-LC3, an autophagy marker, using automated microscopy (Figure 3A). This effect was increased in medium lacking amino acids and serum. Immunoblotting using an anti-GFP antibody[2b] showed that clionamine A (**1**) caused a decrease in the level of GFP-LC3 and an increase in GFP, indicating that the LC3 moiety of the fusion protein was degraded (Figure 3B), and that clionamine A stimulates autophagy, particularly in starvation conditions.

Clionamines A−D (**1**−**4**) combine several structural features that are rare or previously unprecedented in natural products. Only a very small number of 3-aminosteroids are known from nature. Marine natural product examples include the plakinamines[4] and lokysterolamines[5] isolated from sponges in the genera *Plakina* and *Corticum*. The E-ring γ-lactone found in all the clionamines is a well-known substructure generated in degradation products of steroidal saponins[6] but appears to be unknown as a feature of a marine natural product. Undoubtedly the most unique structural feature of the clionamines is the spiro bislactone side chain found in clionamine D (**4**), which has no precedent in natural or synthetic steroids. The novel structures of the clionamines combined with their ability to induce autophagy should make them useful chemical biology tools.

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Supporting Information Available: Experimental details, tables of NMR asignments, and 1D and 2D NMR spectra for **1** to **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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