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Clavaminols A–F, novel cytotoxic 2-amino-3-alkanols from the ascidian *Clavelina phlegraea*

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Abstract—The chemical investigation of the Mediterranean ascidian *Clavelina phlegraea* has led to the isolation of six new 2-amino-3-alkanol derivatives, clavaminols A–F (**1**–**6**). Their stereostructures were established by analysis of spectroscopic data and chemical conversion. Clavaminols A, B, C, and F were tested for their cytotoxic and pro-apoptotic properties and clavaminol A was shown to be the more potent cytotoxic compound of this series inducing cell death through activation of the apoptotic machinery.

1. Introduction

The marine environment is still an unexploited resource of toxic substances to kill proliferating tumour cells and several marine compounds with antitumoral activity have already been described. However, in most cases, only a general characterization has been performed, based on their capacity to inhibit cell proliferation or to promote cell death. During our search for new biologically active and significant metabolites from Mediterranean marine ascidians, we have examined specimens of the tunicate *Clavelina phlegraea* Salfi, 1929 collected in the Bay of Naples. This study led to the isolation of six new cytotoxic compounds, the 2-amino-3-alkanols clavaminols A and B (**1** and **2**) together with the acetamides **3**–**5** (clavaminols C–E) and the *O*-acetoxy derivative **6** (clavaminol F).

2-Amino-alkanols are commonly encountered in tunicates and some sponges. The structures of these molecules are generally related to the widely distributed amphiphilic sphingosine, the central structural element of sphingolipids; their carbon-chain length varies from C_{12} to C_{30} and polyunsaturated variants have been also reported. Ascidians from *Pseudodistoma* and *Clavelina* genera have been prolific in the production of 2-amino-3-hydroxyhydrocarbons. Examples are crucigasterins 277, 275, and 225,[1] and obscuraminols A–F,[2] unsaturated and polyunsaturated 2-amino-3-alkanols isolated as their diacetyl derivatives from the Mediterranean ascidians *Pseudodistoma crucigaster* and *Pseudodistoma obscurum*, respectively, and (2*S*,3*R*)-2-aminododecan-3-ol,[3] an antifungal agent isolated from the ascidian *Clavelina oblonga*. 2-Amino-3-alkanols have also been described as antimicrobial constituents of the Pacific sponge *Xestospongia* sp.[4,5] and of the tropical marine sponge *Haliclona* sp.[6] Finally, the isolation of the (2*S*,3*R*)-2-aminotetradecan-3-ol has been reported from the clam *Spisula polynima*; this compound, named spisulosine, is an inhibitor of cell proliferation that prevents the formation of actin stress fibres in cultured cells.[7]

In this paper, we describe the isolation and the structural elucidation of clavaminols A–F and the cytotoxic and pro-apoptotic characteristics of clavaminols A, B, C, and F. Our results indicate that all the compounds tested affect the viability of three different cell lines (A549, lung carcinoma; T47D, breast carcinoma and AGS, gastric carcinoma) and that clavaminol A is the more potent cytotoxic compound of this series inducing cell death through activation of the apoptotic machinery.

Keywords: Marine natural products; Amino alcohols; Cytotoxic compounds; Ascidians.
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2. Results and discussion

2.1. Isolation and structure elucidation of clavaminols A–F

Specimens of the tunicate *Clavelina phlegraea* collected in the bay of Naples were homogenized and extracted at room temperature with methanol and, subsequently, with chloroform. The combined extracts were then partitioned between water and ethyl acetate; the ethyl acetate soluble material was chromatographed on a normal phase flash column to give a complex amino alcohol mixture, as evidenced by NMR analysis. Subsequent purifications of this fraction by reversed phase HPLC yielded pure clavaminols A–F.

Clavaminol A (**1**) was obtained as an optically active yellow viscous oil, $[\alpha]_D^{25} = -4.25°$ (0.001, MeOH). ESI mass spectrum of **1** (positive in mode) gave a quasimolecular ion peak at *m/z* 202 $[M+H]^+$. HRFABMS measurements on the $[M+H]^+$ ion (*m/z* = 202.2182) indicated the molecular formula $C_{12}H_{28}NO$ for **1** (calculated value: 202.2171). The ^1H NMR spectrum (CD_3OD) of **1** contained two methine resonances at δ 3.27 and 3.70, consistent with protons next to nitrogen and oxygen atoms, respectively, as supported by the corresponding ^{13}C NMR signals at δ 52.36 and 71.38, respectively, in the HSQC spectrum. The remaining signals of the proton spectrum included a large signal (δ 1.3–1.5) due to a number of overlapping methylene signals as well as a methyl doublet at δ 1.21 (δ_C 11.87) and a methyl triplet at δ 0.90 (δ_C 14.04) (see Tables 1 and 2).

The planar structure of **1** was easily assigned as the linear amino alcohol 2-aminododecan-3-ol by interpretation of 2D NMR data (COSY, HSQC, HMBC experiments). In fact, a single ^1H–^1H spin system from C-1 through C-12 was readily identified, starting from the methyl doublet at δ 1.21 (3H-1) coupled to the double quartet at δ 3.27 (H-2, J = 2.94, 6.9 Hz), which was in turn coupled to the multiplet at δ 3.70 (H-3). The methylene protons at δ 1.45 (2H-4, m) showed connectivity to this oxymethine proton and to an AB system at δ 1.53 and 1.36 assigned to C-5 methylene protons; both these

Table 1. ^1H NMR data for clavaminols A–F (**1**–**6**)

POS.	**1** δ_H (mult. J)[a]	**2** δ_H (mult. J)[a]	**3** δ_H (mult. J)[b]	**4** δ_H (mult. J)[b]	**5** δ_H (mult. J)[b]	**6** δ_H (mult. J)[b]
1	1.21 (d, 6.9)	1.21 (d, 6.9)	1.10 (d, 6.9)	1.09 (d, 6.9)	1.09 (d, 6.9)	1.40 (d, 6.6)
2	3.27 (dq, 2.94; 6.9)	3.25 (dq, 2.8; 6.9)	4.02 (m)	4.01 (m)	4.01 (m)	3.54 (m)
3	3.70 (m)	3.68 (m)	3.64 (m)	3.64 (m)	3.64 (m)	5.22 (m)
4	1.45 (m)	1.45 (m)	1.40 (m)	1.40 (m)	1.41 (m)	1.41 (m)
5	1.53 (m) 1.36 (m)	1.53 (m) 1.34 (m)	1.48 (m) 1.30 (m)	1.48 (m) 1.30 (m)	1.50 (m) 1.33 (m)	1.65 (m) 1.51 (m)
6	1.30–1.37 (m)	1.41 (m)	1.28–1.31(m)	1.28–1.31(m)	1.40 (m)	1.27–1.31 (m)
7	1.30–1.37 (m)	2.08 (m)	1.28–1.31(m)	1.28–1.31(m)	2.03 (m)	1.27–1.31 (m)
8	1.30–1.37 (m)	5.38 (m)	1.28–1.31(m)	1.28–1.31(m)	5.37 (m)	1.27–1.31 (m)
9	1.30–1.37 (m)	5.38 (m)	1.28–1.31(m)	1.27 (m)	5.37 (m)	1.27–1.31 (m)
10	1.29 (m)	2.03 (m)	1.26(m)	1.29 (m)	2.01 (m)	1.26 (m)
11	1.30 (m)	1.38(m)	1.29(m)	0.88 (t, 6.9)	1.36 (m)	1.29 (m)
12	0.90 (t, 7.0)	0.90 (t, 7.0)	0.88 (t, 7.0)	—	0.91 (t, 7.0)	0.88 (t, 7.0)
CO*Me*	—	—	1.99 (s)	1.99 (s)	1.99 (s)	2.20 (s)
N*H*	—	—	5.80 (br d, 7.1)	5.76 (br d, 7.0)	5.76 (br d, 7.0)	—

[a] Data recorded in CD_3OD.
[b] Data recorded in $CDCl_3$.

Table 2. ^{13}C NMR data for clavaminols A–F (**1**–**6**)

POS.	1[a]	2[a]	3[b]	4[b]	5[b]	6[b]
1	11.87	11.95	14.10	14.26	14.25	12.86
2	52.36	52.32	49.55	49.75	49.77	50.74
3	71.38	71.45	74.31	74.51	74.51	73.40
4	33.74	33.68	33.63	33.63	33.82	33.86
5	26.55	26.74	26.10	26.21	26.08	30.81
6	30.3	30.3	30.3	30.0	30.29	29.65
7	30.3	26.94	30.3	30.0	27.32	29.65
8	30.3	129.65	30.3	30.0	130.10	29.65
9	30.3	129.65	30.3	32.1	130.10	29.65
10	32.65	29.17	31.95	22.91	29.64	32.10
11	23.32	22.74	22.80	14.41	23.06	22.94
12	14.04	14.01	14.11	—	14.3	14.42
–CO*Me*	—	—	23.48	23.72	23.66	22.02
–CO–	—	—	170.26	170.17	170.26	171.82

Assignments were aided by HMBC and HSQC experiments.
[a] Data recorded in CD_3OD.
[b] Data recorded in $CDCl_3$.

protons were coupled to the cluster at δ 1.30–1.37 which was sequentially coupled to the methyl triplet at δ 0.90. Thus, spectral properties of compound **1** appeared to be very similar to those reported for a 2-amino-3-alkanol previously isolated from *C. oblonga*;[3] however, the optical rotation of **1** showed opposite sign with similar value as compared with that reported for the metabolite isolated from *C. oblonga*, suggesting clavaminol A could be its enantiomer. This was confirmed by application of the circular dichroism method recently proposed by Kossuga et al.,[3] applicable to microscale analysis, which provides reliable assignments of both relative and absolute configurations of 2-amino-3-alkanols. This method exploits large differences in the CD spectra of diastereomeric *N*,*O*-dibenzoyl-2-amino-3-alkanols. Exciton coupling between the benzoyl groups in the *erythro* and *threo* acyclic dibenzoyl vicinal amino alcohols gives rise to bisignate CD spectra opposite in sign and differing in band magnitude and fine structure.[8–10]

The treatment of **1** with benzoyl chloride in pyridine, followed by HPLC purification, gave the corresponding *N*,*O*-dibenzoyl derivative **1a**. Comparison of CD spectrum of **1a** with those reported by Kossuga et al.[3] for the four stereoisomers of 2-amino-3-hexanol revealed an excellent match for the (2*R*,3*S*) *erythro* diastereomer. Actually, CD spectrum of compound **1a** was the mirror image of that of (2*S*,3*R*)-2-aminododecan-3-ol isolated from *C. oblonga* and, therefore, the absolute stereostructure of clavaminol A was defined as (2*R*,3*S*)-2-aminododecan-3-ol.

The molecular formula $C_{12}H_{26}NO$, obtained by HRFABMS for clavaminol B (**2**), appeared related to that of **1**, differing only in having one unsaturation. ^1H and ^{13}C NMR spectra supported this information with signals for two sp^2 methines [δ_C: 129.65 (2C); δ_H: 5.38 (m, 2H)] and two allylic methilenes [δ_C : 26.94 and 29.17; δ_H: 2.03 (m, 2H) and 2.08 (m, 2H)]. Chemical shifts and coupling patterns of the remaining signals for **2**, assigned by aid of COSY, HSQC, and HMBC experiments were very similar to those of **1**. Thus, compound **2** was deduced to be a monounsaturated derivative of clavaminol A. The location of the disubstituted double bond at Δ^8 was established upon observation of COSY and HMBC correlations. The signal of the two allylic methylene protons at δ 2.03 (2H-10) was coupled to the signal at δ 1.38 assigned to 2H-11 on the basis of its coupling to the terminal methyl signal at δ 0.90. Furthermore, in the HMBC spectrum, a cross peak was observed between the methyl carbon resonating at δ 14.01 (C-12) and the allylic protons at δ 2.03 (2H-10). The *Z* configuration of this double bond was deduced by the upfield resonances of the allylic carbon signals (δ 26.94, C-7; δ 29.17, C-10).[11–13] The very limited amounts of clavaminol B in our hands and the need of material for biological assays prevented us to apply the CD method to establish the absolute configuration at C-2 and C-3. However, clavaminol B showed a negative sign of optical rotation as that of clavaminol A with a similar value. This suggested the same (2*R*,3*S*) stereochemistry, on account of the close structural relationship between the compounds.

Clavaminol C (**3**) has the molecular formula $C_{14}H_{30}NO_2$, as indicated by HRFABMS. Comparison of its NMR spectra ($CDCl_3$) with those of clavaminol A recorded in same solvent (see Section 3), readily allowed identifying the planar structure of this compound as the *N*-acetyl-clavaminol A. In fact, besides the resonance of an acetyl group [δ_H 1.99 (s, 3H); δ_C: 23.48 (CO*Me*), 170.26 (*CO*Me)], a significant downfield shift was observed for the signal due to the N-linked methine proton (δ 4.02, H-2). The ^1H NMR spectrum also revealed an exchangeable signal at δ 5.80 (NH) coupled with the proton resonating at δ 4.02. A long range correlation between this amidic proton and the carbonyl resonance at δ 170.26 observed in the HMBC spectrum of **3** definitively confirmed the presence of the *N*-acetyl group. The proposed structure for clavaminol C was in full accord with the complete assignments of its ^1H and ^{13}C resonances performed by 2D NMR analysis (see Tables 1 and 2). In order to determine the absolute stereochemistry of clavaminol C, an aliquot of both acetamide **3** and clavaminol A was acetylated by treatment with acetic anhydride and pyridine at room temperature. After HPLC purification, each reaction mixture afforded a diacetyl derivative. They were shown to be identical (compound **1a**) by comparison of their spectral properties and optical rotations. This result allowed us to assign also to clavaminol C the (2*R*,3*S*) absolute configuration.

Clavaminol D (**4**) has a molecular formula of $C_{13}H_{27}NO_2$, as established by HRFABMS; its proton and carbon NMR spectra, as well as optical rotation, were almost identical to those of **3**, thus suggesting it could possess the same structure of clavaminol C with one methylene less. This structure, including the stereochemical features of the functionalized part of the molecule, was confirmed by comparison of its NMR data and optical rotation value with those of **3**.

For clavaminol E (**5**) the molecular formula of $C_{14}H_{28}NO_2$ was also established by HRFABMS. Its NMR spectra displayed a close similarity with those of clavaminol C; main differences were confined to the presence of proton and carbon signals due to a disubstituted double bond (δ_H: 5.37, m, 2H; δ_C: 130.1, 2C). As previously described for clavaminol B, the Δ^8 position of the double bond was established through 2D NMR analysis and the *Z* stereochemistry was indicated by the ^{13}C NMR resonances at δ 27.32 and 29.64 of C7 and C10. The (2*R*,3*S*) absolute configuration was proposed for **5** by comparing its optical rotation value and sign to those of **3**. Clavaminol E (**5**) was thus identified as the *N*-acetamide of clavaminol B.

HRFABMS indicated for the last metabolite clavaminol F (**6**) the same molecular formula $C_{14}H_{30}NO_2$ of clavaminol C; thus, the two compounds must be isomers. The analysis of the spectroscopic data clearly indicated the differences between the two compounds were in the functionalized part of the molecule. In fact, the ^1H NMR of **6** exhibited a downfield multiplet at δ 5.22 (H-3), which, in the COSY map, showed connectivity with a methylene signal at δ 1.41 and with a signal at

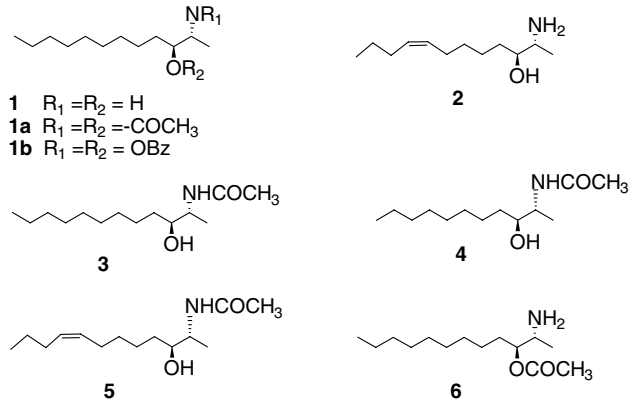

Figure 1. Structures of clavaminols A–F.

δ 3.54 (H-2), in turn coupled to the methyl signal resonating as a doublet at δ 1.40. All these ^{1}H NMR signals were correlated by HSQC to the relevant ^{13}C NMR resonances at δ 73.40 (C-3), 33.86 (C-4), 50.74 (C-2), and 12.86 (C-1), respectively. The presence of an acetyl group was indicated by the methyl proton signal at δ 2.20 (s) and by the ^{13}C NMR resonance at δ 171.82. These data were consistent with the presence of a 2-amino-3-acetoxy functionality in **6** instead of the 2-acetamido-3-hydroxy function present in **3**. This structure was confirmed by the correlations present in the HMBC spectrum, as well as by acetylation of clavaminol F, which yielded a diacetyl derivative with the same spectroscopic properties of compound **1a**. Measurement of optical rotation of the acetylation product obtained from clavaminol F and comparison with that of **1a** allowed us also to assign to clavaminol F the (2*R*,3*S*) stereochemistry Figure 1.

2.2. Biological activities of clavaminols A–F

Next, we studied the effects of compounds **1**, **2**, **3**, and **6** on AGS cell viability using the sensitive calcein-AM assay and we found that clavaminol A (**1**) was a moderately cytotoxic compound with an IC_{50} close to 5 μg/mL. Interestingly, compound **2** was less active indicating that additional unsaturation was detrimental for the cytotoxic activity. Compounds **3** and **6** were completely inactive at the concentrations tested demonstrating a definite structure activity relationship for the amino and hydroxyl groups (Fig. 2a). Notably, spisulosine, a close analogue of clavaminol A endowed with cytotoxic activity, possesses, in addition to a larger alkyl chain, the (2*S*,3*R*) configuration, opposite to that of clavaminol A, and this could explain its higher potency compared to clavaminol A.[7] Whether spisulosine and clavaminol A induce cytotoxicity by similar mechanisms is an interesting open question that remains to be investigated. The cytotoxic activity of clavaminol A was not restricted to the gastric tumoral cell line AGS and similar anti-tumoral activity was found in the two other cell lines tested, A549 (lung cancer) and T47D (breast cancer) suggesting that clavaminol A targets a common pathway in tumoral cells (Fig. 2b).

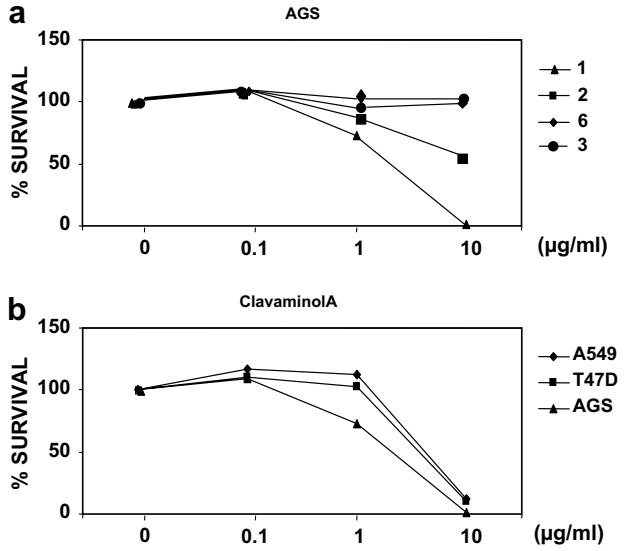

Figure 2. (a) Cytotoxic effects of clavaminols in the AGS cell line. The cells were treated with increasing concentrations of the compounds for 24 h and the cellular viability measured by the calcein-AM assay. (b) Clavaminol A induces cytotoxicity in different tumoral cell lines.

The calcein-AM assay is a powerful method for the screening of cytotoxic compound but does not discriminate between the two major biological pathways that induce cell death such are necrosis and apoptosis and therefore the effect of clavaminol A on cellular apoptosis was investigated in the leukaemia cell line Jurkat. The cells were incubated with this compound (10 μg/mL) for 18 h and the hypodiploidy (i.e., loss of fragmented DNA) was detected by flow cytometry as a marker for apoptosis. In Figure 3a it is shown that clavaminol A was able to induce a strong increase (44.6%) in the percentage of hypodiploid cells. DNA fragmentation is the hallmark for the apoptotic pathway[14,15] and therefore we studied whether or not clavaminol A could induce DNA fragmentation and measured it by the TUNEL assay. Moreover, the involvement of cell cycle control in apoptosis induced by this compound in Jurkat cells was also studied by double staining with PI and FITC–dUTP, as described in Section 3. This method allowed us to determine in which phase of the cell cycle DNA fragmentation occurs.[16] Thus, in Figure 3b we show that most of the DNA fragmentation was detected in G1/S phase in clavaminol A-treated Jurkat cells.

3. Experimental

3.1. General experimental procedures

ESI mass spectra were obtained on an API 2000 mass spectrometer. HRFABMS (glycerol matrix) were performed on a VG Prospec (FISONS) mass spectrometer. Optical rotations were measured with a Perkin-Elmer 192 polarimeter. CD spectra were recorded on an J-710 spectropolarimeter (Jasco, Tokyo, Japan) equipped with a J-710 for Windows software (Jasco). NMR experiments were performed on a Varian Unity INOVA 500 spectrometer; chemical shifts are referred

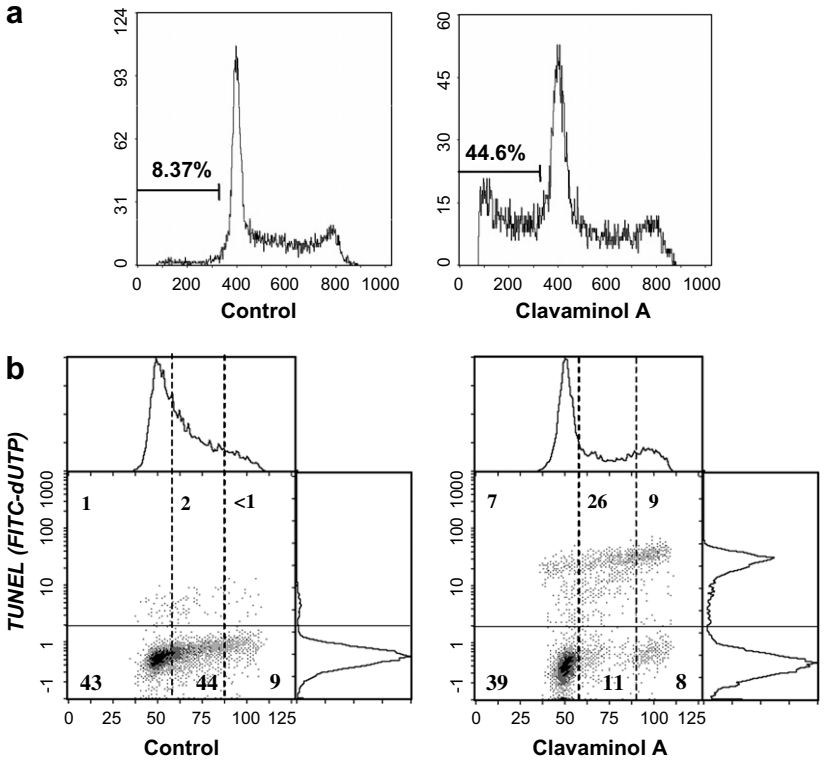

Figure 3. (a) Clavaminol A induces apoptosis. Jurkat cells were treated with clavaminol A and the percentage of subdiploid cells was detected using flow cytometry. (b) G_1/S phase dependence for apoptosis induced by clavaminol A. Jurkat cells were incubated with clavaminol A (10 μg/mL) for 18 h and the cell cycle and the DNA strand breaks analyzed by the TUNEL method using flow cytometry.

to the residual solvent signal (CD_3OD: $\delta_H = 3.31$, $\delta_C = 49.0$; $CDCl_3$: $\delta_H = 7.26$; $\delta_C = 77.0$). Medium-pressure liquid chromatographies (MPLC) were carried out on a Buchi 861 apparatus with SiO_2 (230–400 mesh) packed columns. High-performance liquid chromatographic (HPLC) separations were achieved on a Knauer 501 apparatus equipped with an RI detector.

3.2. Extraction and isolation

Specimens of *C. phlegraea* were collected in spring 2005 in the bay of Naples and kept frozen until used. The freshly thawed tunicate (42.25 g dry weight after extraction) was homogenized and treated at room temperature with methanol (3× 1 L) and, subsequently, with chloroform (3× 1 L). The combined extracts were concentrated *in vacuo* and the resulting aqueous residue partitioned between water and ethyl acetate. The ethyl acetate soluble material (2 g), obtained after evaporation of the solvent, was chromatographed on a silica gel flash column using a gradient elution (hexane → AcOEt → MeOH).

Fraction eluted with AcOEt/MeOH 6:4 was chromatographed by HPLC on an RP-18 column (Gemini, 5 μm, 250 × 4.60 mm), using MeOH/ H_2O 63:37 as the eluent (flow 1 mL/min). This separation afforded compounds **3** (15 mg), **4** (2 mg), **5** (3 mg), and **6** (6 mg) in a pure form. A mixture of compounds **1** and **2** was also obtained, and was separated by a further HPLC on RP-18 column (Gemini, 5 μm, 250 × 4.60 mm), with MeOH/ H_2O (55:45) as the eluent, to give pure compounds **1** (25 mg) and **2** (1.0 mg).

Clavaminol A (**1**). Yellow viscous oil; $[\alpha]_D^{25}$: −4.25° (0.0094, MeOH); ESI-MS (positive ion mode): $m/z = 202$ $[M+H]^+$; HRFABMS (positive ion mode): $m/z = 202.2182$ $[M+H]^+$; the molecular formula $C_{12}H_{28}NO$ requires 202.2171; selected ^1H NMR data in $CDCl_3$: δ_H 3.97 (m, 1H), 3.42 (m, 1H), 0.88 (t, J = 6.0 Hz), 1.30 (d, J = 6.0 Hz).

Clavaminol B (**2**). Yellow viscous oil; $[\alpha]_D^{25}$: −5° (0.001, MeOH); ESI-MS (positive ion mode): $m/z = 200$ $[M+H]^+$; HRFABMS (positive ion mode): m/z = 200.2027 $[M+H]^+$; the molecular formula $C_{12}H_{26}NO$ requires 200.2014.

Clavaminol C (**3**). White powder; $[\alpha]_D^{25}$: +11.4° (0.0022, MeOH); ESI-MS (positive ion mode): $m/z = 244$ $[M+H]^+$; HRFABMS (positive ion mode): m/z = 244.2281 $[M+H]^+$; the molecular formula $C_{14}H_{30}NO_2$ requires 244.22765.

Selected ^1H NMR data in CD_3OD: δ_H 3.83 (m, 1H), 3.48 (m, 1H), 1.94 (s, 3H), 1.09 (d, 3H, J = 6.9 Hz), 0.90 (t, 3H, J = 6.9 Hz).

Clavaminol D (**4**). White powder; $[\alpha]_D^{25}$: +8° (0.0013, MeOH); ESI-MS (positive ion mode): $m/z = 230$ $[M+H]^+$; HRFABMS (positive ion mode): m/z = 230.2128 $[M+H]^+$; the molecular formula $C_{13}H_{27}NO_2$ requires 230.2120.

Clavaminol E (**5**). White powder; $[\alpha]_D^{25}$: +3° (0.0012, MeOH); ESI-MS (positive ion mode): $m/z = 242$

$[M+H]^+$; HRFABMS (positive ion mode): m/z = 242.2125 $[M+H]^+$; the molecular formula $C_{14}H_{28}NO_2$ requires 242.2120.

Clavaminol F (**6**). White powder; $[\alpha]_D^{25}$: −4° (0.004, MeOH); ESI-MS (positive ion mode): m/z = 244 $[M+H]^+$; HRFABMS (positive ion mode): m/z = 244.2279 $[M+H]^+$; the molecular formula $C_{14}H_{30}NO_2$ requires 244.22765.

3.3. Preparation of dibenzoyl derivatives

Benzoyl chloride (15 μL, 0.13 mmol) was added to a solution of **1** (3.3 mg, 0.016 mmol) in pyridine (0.5 mL). The reaction mixture was heated at 50 °C for 12 h at which time the pyridine was removed under vacuum and the residue purified by SiO_2 HPLC (LUNA, 3 μm, 150 × 4.60 mm, 25:75 EtOAc/*n*-hexane, 0.4 mL/min) to afford compound **1b** (2.3 mg, 0.0056 mmol).

Erythro-(2R,3S)-N,O-dibenzoyl-2-amino-3-dodecanol (**1b**). ^{1}H NMR (500 MHz, $CDCl_3$): δ_H 8.09 (d, J = 7.9 Hz 2H), 7.77 (d, J = 7.9 Hz 2H), 7.61 (m,1H), 7.42–7.48 (m,5H), 6.96 (br d, 1H, NH), 5.22 (m, 1H), 4.47 (m, 1H), 1.84 (m, 1H), 1.71 (m, 1H), 1.45 (m, 2H), 1.25 (m, 12H) 1.30 (d, J = 7 Hz, 3H), 0.87 (t, J = 7.1 Hz, 3H). ^{13}C NMR (500 MHz, $CDCl_3$): δ_C 167, 166.8, 133.2, 132, 130, 128.5, 127.0, 77.6, 49.0, 31.2, 33.8, 29.5, 14.8, 14.1. ESI-MS (positive ion mode): m/z = 410 $[M+H]^+$; HRFABMS (positive ion mode): m/z = 410.2702 $[M+H]^+$; the molecular formula $C_{26}H_{36}NO_3$ requires 410.2695.

3.4. Preparation of diacetyl derivatives

A solution of **1** (0.9 mg, 0.0045 mmol) was stirred in a solution of pyridine (0.3 mL) and acetic anhydride (0.3 mL) for 5 h at room temperature. The pyridine was removed under vacuum and the residue purified by reverse HPLC (LUNA, 3 μm C18, 150 × 4.60 mm, 8:2 MeOH/H_2O, 0.6 mL/min) to give the diacetate derivative **1a** (0.8 mg, 0.003 mmol). Compound **3** (1.4 mg, 0.0058 mmol) was acetylated using the same procedure reported for **1** affording the diacetate derivative **1a** (1.2 mg, 0.0042 mmol).

(2R,3S)-2-acetamido-3-acetoxydodecane (**1a**). $[\alpha]_D^{25}$: +18.8° (0.0008, MeOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ_H 5.78 (br d, J = 7.4 Hz, 1 H, NH), 4.83 (ddd, 1H, H-3), 4.16 (m, 1H, H-2), 2.09 (s, 3H, *Me*COO–), 1.95 (s, 3H, *Me*-CONH–), 1.56 (m, 1H, H-4a), 1.50 (m, 1H, H-4b), 1.29 (m, 2H, H-5), 1.25–1.29 (m, 8H, H6-H9), 1.26 (m, 2H, H-10), 1.30 (m, 2H, H-11), 1.10 (d, J = 7.06 Hz, 3H, H-1), 0.88 (t, J = 6.8 Hz, 3H, H-12); ^{13}C NMR (500 MHz, $CDCl_3$) δ_C 171.34 (s, *Me*COO–), 168.9 (s, *Me*CONH–), 77.2 (d, C-3), 47.7 (d, C-2), 32.05 (t, C-10), 31.51 (t, C-4), 29.4–29.6 (t, C-6, C-7, C-8, C-9), 25.8 (t, C-5), 23.74 (q, *Me*CONH–), 22.93 (t, C-11), 21.4 (q, *Me*COO–), 14.8 (q, C-1), 14.1 (q, C-12). ESI-MS (positive ion mode): m/z = 286 $[M+H]^+$. HRFABMS (positive ion mode): m/z = 286.2391; the molecular formula $C_{16}H_{32}NO_3$ requires 286.2382.

3.5. Cell cultures

The adherent tumoral cell lines AGS (gastric carcinoma), T47D (breast carcinoma) and A549 (lung carcinoma) were maintained in DMEM (Invitrogen, Barcelona, Spain) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL). The human leukaemia T cell line Jurkat was grown in suspension and was maintained in complete RPMI 1640. The cell cultures were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

3.6. Calcein uptake assay

Calcein-AM is a fluorogenic, highly lipid-soluble dye that rapidly penetrates the plasma membrane. Inside the cell, endogenous esterases cleave the ester bonds, producing the hydrophilic and fluorescent dye calcein, which cannot leave the cell via the plasma membrane. AGS, A549 and T47D cells were cultured in complete medium in 96-well plates (10^4 cells) and incubated with increasing concentrations of the compounds for another 24 h. After treatment, calcein-AM was added (final concentration 1 μM) and the cells were incubated for 60 min. The uptake was then stopped by transferring the plates on ice and washing the cells twice with HBSS pre-cooled to 4 °C. The fluorescence of the calcein generated within the cells was analyzed in a Tecan Pro fluorometer with 485-nm excitation and 535-nm emission filters.

3.7. Detection of cell cycle and DNA strand breaks

The percentage of apoptotic cells was measured using the TUNEL method. Control or treated Jurkat cells (1×10^6) were fixed in 4% paraformaldehyde in PBS for 24 h at 4 °C, washed twice in PBS and permeabilized in 0.1% sodium citrate containing 0.1% Triton X-100 for 2 min. Fixed cells were washed three times in PBS and resuspended in a final volume of 50 μl TUNEL buffer (0.3 nmol FITC-dUTP, 3 nmol dATP, 50 nmol $CoCl_2$, 5 U TdT, 200 mM potassium cacodylate, 250 mg/mL bovine serum albumen (BSA) and 25 mM Tris–HCl, pH 6.6). The cells were incubated for 1 h at 37 °C and then washed twice in PBS and analyzed by flow cytometry. To determine both DNA strand breaks and the phase of the cell cycle cells were in, TUNEL-stained cells were counterstained with PI (20 mg/mL) and treated with RNase (50 U/mL) in PBS for 1 h at RT, prior to cytofluorimetric analysis. In this method, fixation in formaldehyde prevents extraction of low molecular weight DNA from apoptotic cells and thus the cell cycle distribution estimates both apoptotic and nonapoptotic cells. After incubation, cells were immediately analyzed by flow cytometry using an EPIC XL analyzer (Beckman-Coulter, Hialeah, MI).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.02.015.

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