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Clavaminols G–N, six new marine sphingoids from the Mediterranean ascidian *Clavelina phlegraea*

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ABSTRACT

An exhaustive examination of the chemical constituents of the Mediterranean ascidian *Clavelina phlegraea* led to the isolation of clavaminols G–N (**1**–**6**), a new series of amino alcohols, which expand the family of modified marine sphingoids. Structures of the novel compounds **1**–**6** have been elucidated by spectroscopic analysis and chemical derivatization; bioactivities of compounds **1**–**6** have also been investigated and comparison of their pharmacological properties with those of previously isolated clavaminols A–F allowed us to perform an assessment of simple structure–activity relationships.

1. Introduction

Simple long chain amino alcohols have drawn the attention of natural products scientists due to their interesting bioactivities, which range from antimicrobial activity to inhibition of cell proliferation through prevention of the formation of actin stress fibers in cultured cells.[1–11] Structurally, these compounds are related to the sphingosine derivatives (e.g., sphinganine, 4-sphingenine, phytosphingosine), long known as central structural elements of sphingolipids, which are important constituents of the lipid portion of cell membranes in living organisms. As part of our search for bioactive substances of marine origin, we have been investigating the chemistry of the Mediterranean ascidian *Clavelina phlegraea* Salfi, 1929. Recently, we have reported the isolation from this organism of six new 2-amino-3-alkanols, clavaminols A–F, with cytotoxic properties against A549 (lung carcinoma), T47D (breast carcinoma), and AGS (gastric carcinoma) cell lines, inducing cell death through activation of the apoptotic machinery.[12] These results prompted an in depth-examination of the ascidian and the isolation of a further six new amino alcohols, clavaminols G–N (**1**–**6**). The present report describes the isolation, the structure determination, and the bioactivities of compounds **1**–**6**; particularly, comparison of their pharmacological properties with those of clavaminols A–F allowed us to perform an assessment of simple structure–activity relationships.

2. Results and discussion

2.1. Isolation and structure elucidation of clavaminols G–N

Specimens of *C. 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 mixture of amino alcohols, as evidenced by NMR analysis. Subsequent purifications of this fraction by reversed phase HPLC yielded pure clavaminols G–N (Fig. 1).

Clavaminol G (**1**) was obtained as an optically inactive white amorphous solid. Its molecular formula $C_{13}H_{27}NO_2$ was obtained from HRFABMS measurements on the $[M+H]^+$ ion (*m*/*z* 230.2108, calculated value: 230.2120) and indicated one unsaturation degree. The ^{1}H NMR spectrum of **1** ($CDCl_3$) was interpreted also on the basis of ^{13}C NMR and HSQC information; besides the resonance of an acetyl group (δ_H 2.01, s, 3H; δ_C 23.5, 170.8), it contained an AB system attributed to the diastereotopic geminal protons of a nitrogen-substituted methylene group [δ_H: 3.50 (ddd, 1H, J=14.0, 6.5, 2.8 Hz, H-1$_a$) and 3.10 (1H, J=14.0, 7.7, 5.5 Hz, H-1$_b$); δ_C: 46.1 (C-1)]. An oxymethine proton signal (δ_H: 3.71, m, 1H, H-2; δ_C: 71.7, C-2) and a D_2O-exchangeable broad singlet at δ 5.88 (NH) were also present, as well as a methylene envelope (δ 1.25–1.45, 16H) and a methyl triplet at δ 0.89 (δ_C 14.4, C-11). Analysis of COSY connectivities indicated a single ^{1}H–^{1}H spin system, which started at the exchangeable signal at δ 5.88 coupled to both geminal protons at δ 3.10 and 3.50, in turn coupled to the hydrogen at δ 3.71 of the oxymethine group. This proton was further coupled to the hydrogens

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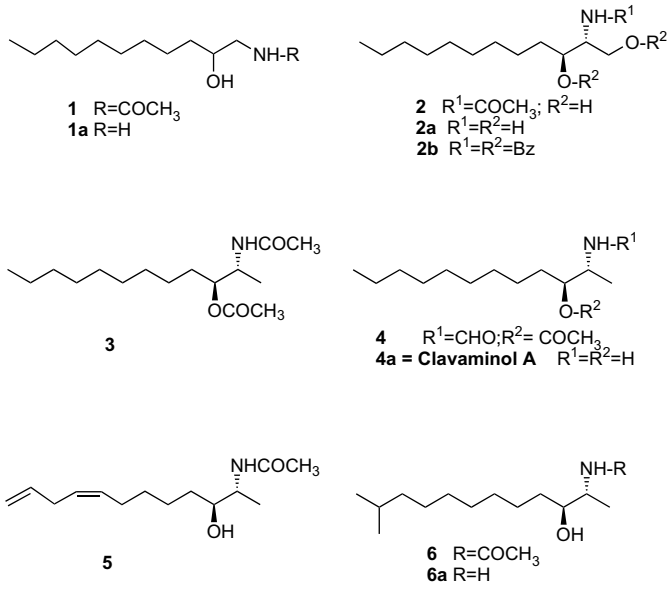

Figure 1. Structures of clavaminols G–N and derivatives.

of a methylene group at δ 1.45 (δ_C 35.4, C-3), which was substituted by a nine-carbon linear alkyl chain. A carbonyl resonance present in the ^{13}C NMR spectrum at δ 170.76 was correlated in the HMBC spectrum to the signals at δ 5.88 (NH) and 2.01 (*Me*CO), as well as to the methylene protons at δ 3.10 and 3.50, thus indicating the presence of an *N*-acetyl group linked at C-1. Therefore, the structure of **1** was established as *N*-(2-hydroxyundec-1-yl) acetamide. In spite of the presence of the stereogenic center at the C-2, the absence of optical rotation for **1** suggested that the compound has been isolated as a racemic mixture. This was confirmed by treatment of compound **1** with (*R*)-MTPA chloride, which gave a mixture of the two diasteroisomeric esters.

The high-resolution FAB mass spectrum of clavaminol H (**2**), which contained a quasi-molecular ion peak at *m*/*z*=260.2230 $[M+H]^+$, provided the formula $C_{14}H_{29}NO_3$ and indicated one degree of unsaturation. The ^{1}H NMR spectrum of **2** ($CDCl_3$) displayed some similarities with that of compound **1**; it also featured a large signal at δ 1.28–1.55 due to overlapping methylene signals as well as a methyl triplet at δ 0.88 (Me-12), a D_2O-exchangeable broad singlet resonating at low-field (δ 6.40, NH), and a methyl singlet at δ 2.05 (*Me*CO). In addition, the spectrum displayed two AB resonances at δ 4.02 (dd, *J*=11.3, 3.0 Hz, H-1$_a$) and 3.77 (submerged by other signals, H-1$_b$) and two multiplets integrating for one proton each at δ 3.85 (H-2) and 3.80(H-3), respectively. Analysis of the whole set of spectroscopic data (see Tables 1 and 2) clearly indicated that the differences between the two compounds were in the functionalized part of the molecule. Interpretation of COSY connectivities, aided by HSQC data, delineated a sphingosine type structure with two oxygenated functions: the mutually coupled methylene proton signals at δ 4.02 and 3.77, which were correlated in the HSQC spectrum to a carbon δ 62.7 (C-1), were both coupled to the signal at δ 3.85(δ_C: 53.9, C-2), assigned to a methine hydrogen bonded to an *N*-substituted carbon. The latter signal was coupled to the oxymethine proton at δ 3.80 (δ_C: 74.6, C-3), in turn coupled to a methylene signal at δ 1.56 (δ_C: 34.8, C-4) to which a saturated nine-carbon linear chain was attached. The methine proton at δ 3.85 was also coupled to the exchangeable signal at δ 6.40 (NH); the latter signal appeared to be correlated in the HMBC spectrum to the carbonyl resonance present in the ^{13}C NMR spectrum at δ 171.1, in turn correlated with the methyl singlet at δ 2.05. This clearly indicated the presence of an *N*-acetyl group linked at C-2. The absolute stereochemistry of clavaminol H was assigned by circular dichroism (CD) analysis on its perbenzoyl derivative **2b**.[11] To this purpose, compound **2** was subjected to acid hydrolysis, followed by exhaustive benzoylation to give the corresponding *N*,*O*,*O*′-tribenzoyl derivative **2b**. CD spectrum (MeOH) of **2b** (Fig. 2) was compared with those reported for (2*S*,3*R*)-*erythro* and (2*S*,3*S*)-*threo* stereoisomers of 2-aminohexane-1,3-diol[13] and it proved to be the mirror image of the CD spectrum of (2*S*,3*R*)-2-aminohexane-1,3-diol; thus, the absolute stereostructure of clavaminol H was defined as *N*-[(2*R*,3*S*)-1,3-dihydroxydodecan-2-yl]acetamide.

Mass and NMR spectroscopic data of clavaminol I (**3**), as well as sign and value of its optical rotation (see Experimental section), exactly matched those of the diacetyl derivative we previously obtained by treatment of clavaminol A with acetic anhydride and pyridine.[12] Thus, the structure of **3** was identified as (2*R*,3*S*)-2-acetamido-3-acetoxydodecane.

HRFABMS for clavaminol L (**4**) gave a quasi-molecular ion at *m*/*z*=272.2231 $[M+H]^+$, indicating the molecular formula $C_{15}H_{29}NO_3$ with two unsaturation degrees. The ^{1}H NMR spectrum

Table 1
^{1}H NMR ($CDCl_3$) data for clavaminols G–N (**1**–**6**)[a]

Pos.	**1** δ (mult., *J*)	**2** δ (mult., *J*)	**3** δ (mult., *J*)	**4** δ (mult., *J*)	**5** δ (mult., *J*)	**6** δ (mult., *J*)
1	3.10 (ddd, 14.0, 7.7, 5.5); 3.50 (ddd, 14.0, 6.5, 2.8)	4.02 (dd, 11.3, 3.0); 3.77[b]	1.10 (d, 7.0)	1.14 (d, 6.8)	1.09 (d, 6.9)	1.08 (d, 6.9)
2	3.71 (m)	3.85 (m)[b]	4.16 (m)	4.27 (m)	4.03 (m)	4.02 (m)
3	1.45 (m)	3.80 (m)[b]	4.83 (m)	4.84 (m)	3.65 (m)	3.65 (m)
4	1.43;[b] 1.31[b]	1.56 (m)	1.55 (m); 1.50 (m)	1.43 (m)	1.41 (m)	1.41 (m)
5	1.27–1.30	1.52 (m) 1.35[b]	1.27[b]	1.58 (m); 1.49 (m)	1.51(m); 1.37(m)	1.49 (m); 1.32 (m)
6	1.27–1.30	1.28–1.30	1.25–1.28	1.26–1.29	1.40 (m)	1.26–1.28
7	1.27–1.30	1.28–1.30	1.25–1.28	1.26–1.29	2.06 (m)	1.26–1.28
8	1.27–1.30	1.28–1.30	1.25–1.28	1.26–1.29	5.43 (m)	1.26–1.28
9	1.26[b]	1.28–1.30	1.25–1.28	1.26–1.29	5.42 (m)	1.27[b]
10	1.29[b]	1.26[b]	1.26[b]	1.26[b]	2.79 (t,6.6)	1.18 (m)
11	0.89 (t, 6.9)	1.29[b]	1.30[b]	1.28[b]	5.82 (m)	1.53 (m)
12	—	0.88 (t, 7.0)	0.88 (t, 7.0)	0.88 (t, 7.0)	5.04 (br d, 17.0); 4.98 (br d, 10.2)	0.88 (d, 6.6)
13	—	—	—	—	—	0.88 (d,6.6)
–NH–	5.88 (m)	6.40	5.78 (br d, 7.2)	5.88 (br d, 6.5)	5.74 (m)	5.77 (br d, 7.0)
–NHCOCH$_3$	2.01 (s)	2.05	1.95 (s)	—	1.99 (s)	1.97 (s)
–OCOCH$_3$	—	—	2.10 (s)	2.10 (s)	—	—
–NHCHO	—	—	—	8.11 (br s)	—	—

[a] Submerged by other signals.
[b] Assignments were aided by HSQC experiments.

Table 2
^{13}C NMR data ($CDCl_3$) for clavaminols G–N (**1**–**6**). Assignments were aided by HMBC and HSQC experiments

Pos.	1	2	3	4	5	6
1	46.1	62.7	14.8	14.8	14.0	14.4
2	71.7	53.9	47.7	46.2	49.4	49.7
3	35.4	74.6	77.2	76.4	74.3	74.5
4	25.7	34.8	31.5	32.6	33.5	33.8
5	29.7	26.2	25.8	31.0	25.5	26.2
6	29.7	29.7	29.6	29.3	29.4	29.9
7	29.7	29.7	29.6	29.3	27.1	29.9
8	29.7	29.7	29.6	29.3	130.5	29.9
9	32.1	29.7	29.6	29.2	126.8	27.6
10	23.0	32.2	32.0	31.9	31.2	39.2
11	14.4	22.9	22.9	22.7	137.0	28.1
12	—	14.4	14.1	14.1	114.5	22.9
13	—	—	—	—	—	22.9
–NHCO*C*H$_3$	23.5	23.7	23.7	—	23.4	23.7
–NHCOCH$_3$	170.8	171.1	168.9	—	169.8	170.2
–OCO*C*H$_3$	—	—	21.4	20.9	—	—
–OCOCH$_3$	—	—	171.3	171.5	—	—
–NH*C*HO	—	—	—	160.5	—	—

of **4** strongly resembled that of **3** (see Table 1), displaying, in addition to the signals due to an aliphatic saturated alkyl chain, two methine signals at δ 4.27 (m, H-2) and 4.84 (m, H-3), a D_2O-exchangeable signal at δ 5.88 (NH) and a methyl signal resonating as a doublet at δ 1.14 (Me-1). The substantial difference between the spectra of **3** and **4** was the presence in **4** of only one acetyl methyl singlet at δ 2.10 as well as the presence of a downfield shifted broad singlet at δ 8.11, which was correlated, in the HSQC spectrum, to a carbonyl resonance present in the ^{13}C NMR spectrum at δ 160.5. COSY connectivities revealed that the signal at δ 8.11 was coupled to the exchangeable signal at δ 5.88, in turn coupled to the proton resonating at δ 4.27 (H-2); in addition, the HMBC correlation map evidenced long range couplings between the signal at δ 8.11 and the carbon resonance at δ 46.2 (C-2) and between the proton signal at δ 4.27 (H-2) and the carbonyl resonance at δ 160.5. The presence of an *N*-formyl group at C-2 was thus deduced. Furthermore, the HMBC correlation between the proton resonance at δ 4.84 (H-3) and the carbonyl resonance δ 171.5 indicated the presence of the acetoxy group at C-3. Thus, the planar structure of compound **4** was determined as 2-formylamido-3-acetoxydodecane. In order to establish the stereochemistry of **4**, the compound was hydrolyzed and yielded compound **4a**, which was shown to be identical to clavaminol A, as indicated by comparison of their spectral properties and optical rotation.[12]

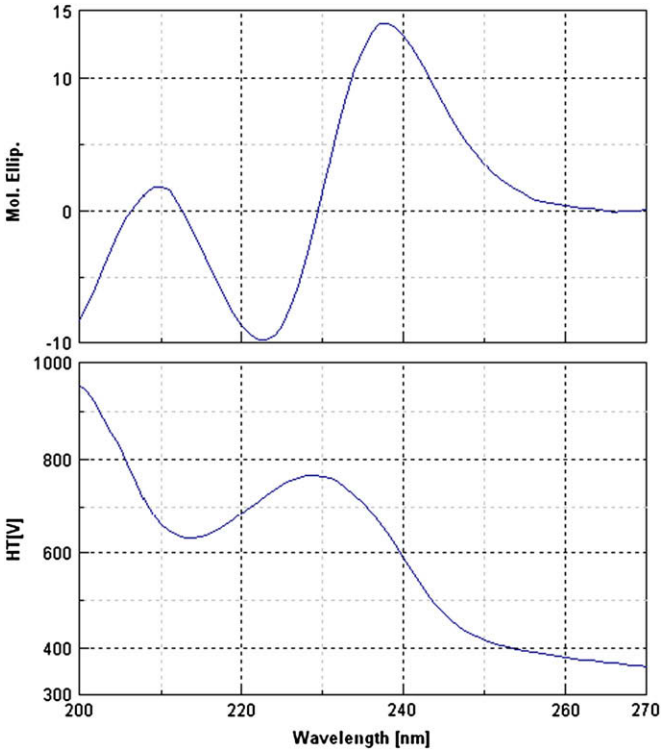

Figure 2. CD spectrum of *N,O,O′*-tribenzoyl clavaminol H (**2b**) in MeOH (25 °C).

Structures of the minor compounds clavaminols M (**5**) and N (**6**) were easily defined also by comparison of their spectroscopic data with those of the previously isolated clavaminols C and E.[12] The molecular formula $C_{14}H_{25}NO_2$ of **5**, obtained by HRFABMS measurements, indicated three unsaturation degrees. Its proton spectrum closely matched that of clavaminol E[12] apart from the presence of characteristic ABX resonances [three one-proton signals at δ 4.98 (br d, J=10.2 Hz, H-12$_a$), 5.04 (br d, J=17.0 Hz, H-12$_b$), and 5.82 (m, H-11)] indicative of a terminal vinyl group, whose presence was confirmed by HSQC correlations of the above protons with the carbons at δ 114.5 (C-12) and 137.0 (C-11), respectively. Analysis of COSY and HMBC correlations allowed assignment of the whole spin system C1/C-12 and indicated the location of the two double bonds at Δ^8 and Δ^{11} positions, respectively. Stereochemistry of the C8 double bond was deduced by the chemical shift of allylic carbons (δ 27.1, C-7; δ 31.2, C-10) indicative of a *Z* configuration.[14–16]

Clavaminol N (**6**) has a molecular formula of $C_{15}H_{31}NO_2$, as established by HRFABMS. Proton and carbon NMR spectra were almost identical to those of clavaminol C,[12] differing only for the resonance relative to the end group of the alkyl chain, which was shown to be an isopropyl moiety [δ_H: 0.88 (d, J=6.6 Hz, 6H, Me-12/13), 1.53 (m, 1H, H-11); δ_C: 22.9 (C-12/C-13), 28.1 (C-11)].

The stereochemical features of compounds **5** and **6** were deduced on the basis of comparison of their optical rotation values with those of clavaminols E and C, respectively, since the slight structural differences between the two compounds shouldn't significantly affect their optical properties. This comparison suggested the same (2*R*,3*S*) stereochemistry for both compounds **5** and **6**, and thus their structures were assigned as *N*-[(2*R*,3*S*,*Z*)-3-hydroxydodeca-8,11-dien-2-yl] acetamide (**5**) and *N*-[(2*R*,3*S*)-3-hydroxy-11-methyldodecan-2-yl] acetamide.

The whole set of amino alcohols isolated from *C. phlegraea*, clavaminols A–N, expands the family of modified sphingoids from marine organisms; by analogy with the biosynthesis of sphingosine, which requires palmitoyl CoA and L-serine,[17] we may postulate that appropriate fatty acids and (*R*)-alanine are implied as biogenetic precursors for clavaminols A–F and I–L, while biosynthesis of clavaminols G and H appears to involve a C_{10} fatty acid coupled with glycine and (*R*)-serine, respectively.

2.2. Biological activities of clavaminols G–N

We studied the effects of clavaminols G–N on the viability of two different cell lines, A549 (lung carcinoma) and AGS (gastric carcinoma), using the sensitive calcein-AM assay; the hydrolysis products of clavaminols H, G, and N (compounds **1a**, **2a**, and **6a**) were also tested. As showed in Figure 3, none of the new clavaminols was significantly cytotoxic; in contrast, compound **6a** showed a cytotoxic effect comparable to that of clavaminol A in both cell lines. Interestingly, compound **2a**, which is related to clavaminol A by the presence of an extra hydroxyl group, showed significant cytotoxic activity in AGS cells, even if lower than that of clavaminol A; however, it was inactive against lung cancer cell line A549. The pharmacological properties of clavaminols G–N, compared to those of clavaminols A–F (**1**–**6**), allowed us to perform an assessment of simple structure–activity relationships of the novel structures. Previously, we showed that clavaminol A was cytotoxic against a number of tumoral cell lines, with an IC_{50} close to 5 μg/mL for AGS

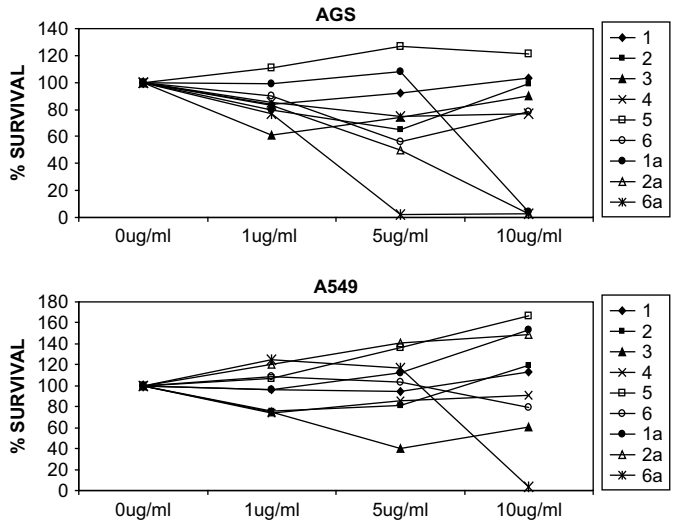

Figure 3. Cytotoxic effects of clavaminols G–N (compounds **1**–**6**) and of compounds **1a**, **2a**, and **6a** in gastric carcinoma cells (AGS) and in lung carcinoma cells (A549).

cells; however, it was less active than the related antitumoral compound spisulosine possessing the opposite configuration at carbons 2 and 3.[8,12] Remarkably, clavaminol B was less potent than clavaminol A, indicating that additional unsaturation was detrimental for the cytotoxic activity: clavaminols C and F were indeed completely inactive at the concentrations tested, demonstrating a definite structure–activity relationship for the amino and hydroxyl groups.[12] In the new series of clavaminols (G–N) we emphasize that free OH and NH_2 groups are critical for the cytotoxic activity, which on the other hand is not affected by branching of the alkyl chain. Above all, we proved that additional hydroxylation admittedly decreases the activity but may confer cell selectivity to clavaminols. Compound **2a** was indeed cytotoxic only in AGS cells, while clavaminols A and B were cytotoxic in both cell lines.[12]

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 a 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 to the residual solvent signal (CD_3OD: δ_H=3.31, δ_C=49.0; $CDCl_3$: δ_H=7.26, δ_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 chromatography (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 was 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). The 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** (3 mg), **5** (2 mg), and **6** (2 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** (10 mg).

3.2.1. Clavaminol G (*1*)

White powder; ESI-MS (positive ion mode): m/z=230 $[M+H]^+$; HRFABMS (positive ion mode): m/z=230.2108 $[M+H]^+$; the molecular formula $C_{13}H_{28}NO_2$ requires 230.2120.

3.2.2. Clavaminol H (*2*)

White powder; $[\alpha]_D^{25}$ +3.19 (0.0013, MeOH); ESI-MS (positive ion mode): m/z=260 $[M+H]^+$; HRFABMS (positive ion mode): m/z=260.2230 $[M+H]^+$; the molecular formula $C_{14}H_{30}NO_3$ requires 260.2226.

3.2.3. Clavaminol I (*3*)

White powder; $[\alpha]_D^{25}$ +10.45 (0.0005, MeOH); ESI-MS (positive ion mode): m/z=286 $[M+H]^+$; HRFABMS (positive ion mode): m/z=286.2376 $[M+H]^+$; the molecular formula $C_{16}H_{32}NO_3$ requires 286.2382.

3.2.4. Clavaminol L (*4*)

White powder; $[\alpha]_D^{25}$ +12.17 (0.0009, MeOH); ESI-MS (positive ion mode): m/z=272 $[M+H]^+$; HRFABMS (positive ion mode): m/z=272.2231 $[M+H]^+$; the molecular formula $C_{15}H_{30}NO_3$ requires 272.2226.

3.2.5. Clavaminol M (*5*)

White powder; $[\alpha]_D^{25}$ +9.05 (0.0005, MeOH); ESI-MS (positive ion mode): m/z=240 $[M+H]^+$; HRFABMS (positive ion mode): m/z=240.1975 $[M+H]^+$; the molecular formula $C_{14}H_{26}NO_2$ requires 240.1964.

3.2.6. Clavaminol N (*6*)

White powder; $[\alpha]_D^{25}$ +6.60 (0.0014, MeOH); ESI-MS (positive ion mode): m/z=258 $[M+H]^+$; HRFABMS (positive ion mode): m/z=258.2448 $[M+H]^+$; the molecular formula $C_{15}H_{31}NO_2$ requires 258.2433.

3.3. Hydrolysis of clavaminols G, H, L, and N

A solution of 1 mg of each compound **1**, **2**, **4**, and **6** was stirred with 1 M HCl in methanol 91% at 80 °C overnight. The reaction mixtures were evaporated under nitrogen yielding compounds **1a**, **2a**, **4a**, and **6a**, respectively.

3.3.1. Compound *1a*

ESI-MS: m/z 188 $[M+H]^+$; ^1H NMR (CD_3OD): δ 3.70 (m), 3.01 (dd, J=12.8, 2.5 Hz), 2.74 (dd, 12.8, 9.5 Hz), 1.48 (m), 1.30–1.37 (br), 0.90 (t, J=7 Hz).

3.3.2. Compound *2a*

ESI-MS: m/z 218 $[M+H]^+$; ^1H NMR (CD_3OD): δ 3.83 (dd, J=11.3, 3.8 Hz), 3.77 (m), 3.70 (dd, J=11.3, 8.7 Hz), 3.19 (m), 1.49 (m), 1.30–1.37 (br), 0.91 (t, J=7 Hz).

*3.3.3. Compound **4a***

$[\alpha]_D^{25}$ −4.25 (0.0009, MeOH); ESI-MS: *m*/*z* 202 $[M+H]^+$; ^1H NMR (CD_3OD): δ 3.70 (m), 3.26 (dq, *J*=2.95, 6.9 Hz), 1.53 (m), 1.44 (m), 1.30–1.37 (br), 1.22 (d, *J*=6.8 Hz), 0.90 (t, *J*=7 Hz).

*3.3.4. Compound **6a***

ESI-MS: *m*/*z* 216 $[M+H]^+$; ^1H NMR (CD_3OD): δ 3.70 (m), 3.26 (dq,), 1.53 (m), 1.45 (m), 1.33 (m), 1.22 (d, *J*=6.8 Hz), 0.88 (d, *J*=6.8 Hz).

3.4. Synthesis of tribenzoyl derivative 2b

Benzoyl chloride (15 μL, 0.13 mmol) was added to a solution of **2a** (0.8 mg, 0.0031 mmol) in pyridine (0.2 mL). The reaction was heated at 40 °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/hexane, 0.4 mL/min) to afford compound **2b** (0.5 mg, 0.0009 mmol).

3.4.1. Erythro-(2R,3S)-N,N,O-Tribenzoyl-2-amino-1,3-dodecandiol (2b)

ESI-MS (positive ion mode): *m*/*z*=530 $[M+H]^+$; HRFABMS (positive ion mode): *m*/*z*=530.6788 $[M+H]^+$; the molecular formula $C_{33}H_{40}NO5$ requires 530.6762. ^1H NMR ($CDCl_3$): δ 8.03 (d, *J*=7.9 Hz, 2H), 7.96 (d, *J*=7.9 Hz, 2H), 7.78 (d, *J*=7.9 Hz, 2H), 7.36–7.56 (m, 9H), 7.08 (d, 1H, NH), 5.38 (m, 1H), 4.88 (m, 1H), 4.63 (m, 2H), 1.88 (m, 1H), 1.96 (m, 1H), 1.55 (m, 2H), 1.45 (m, 2H), 1.23 (m, 12H), 1.30 (d, *J*=7 Hz, 3H), 0.86 (t, *J*=7.5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 167, 166.8, 166, 132, 130, 128.5, 127.0, 77.6, 49.0, 31.2, 33.8, 29.5, 13.8, 14.1.

3.5. Cell cultures

The adherent tumoral cell lines AGS (gastric carcinoma) and A549 (lung carcinoma) were maintained in DMEM medium (Cambrex Co, Barcelona, Spain) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (50 U/mL) and streptomycin (50 μg/mL). 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 and A549 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.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2009.03.056.

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