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Identification and Quantification, by NMR and LC-MS, of Sterols Isolated from the Marine Sponge *Aplysina aerophoba*

Pere Ferriol[1,2], Francisco J. Toledo Marante[1*], Ignacio Brouard Martin[3], José J. Santana Rodríguez[4], Rayco Guedes Alonso[4], Alba González Benkovics[1] and Roberto Mioso[4,5]

[1]*Department of Chemistry, University of Las Palmas de Gran Canaria, Gran Canaria, 35017, Spain*
[2]*Department of Biology, University of the Balearic Islands, Palma de Mallorca, 07122, Spain*
[3]*Institute of Natural Products and Agrobiology (IPNA), CSIC, Tenerife, 38206, Spain*
[4]*University Institute for Environmental Studies and Natural Resources (i-UNAT), University of Las Palmas de Gran Canaria, 35017, Spain*
[5]*Department of Biochemistry, Federal University of Pernambuco, Recife, PE, 50670-901, Brazil*

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Abstract: Aplysterol, several didehydroaplysterols, and their acetylated derivatives were isolated from samples of the marine sponge *Aplysina aerophoba*, which had been collected on the coast of the Canary Islands, and were identified by chromatography and ^{1}H- and ^{13}C-NMR spectroscopy. A quantitative analysis was done, based on the relative intensities of the signals corresponding to the olefinic quaternary carbons from the ^{13}C-NMR spectrum at 125 MHz. Finally, the results described were confirmed by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

Keywords: *Aplysina aerophoba*; marine sponge; sterols; quantitative analysis; NMR; UHLC-MS/MS.

1. Introduction

Sponges (Porifera) are multicellular organisms, and together with their associated microbial symbionts, they remain the most important marine source for new bioactive natural products of pharmaceutical and medical interest [1,2]. In particular, marine sponges belonging to the *Aplysina* genus (Aplysinidae, Verongida, Demospongiae) are characterized by a unique biochemistry profile, which includes: a lack of terpenes; the presence of brominated metabolites derived from tyrosine, which are considered to be molecular markers in the Verongida species; and the presence of a great variety of sterols-generally with the aplystane skeleton [3-5].

Steroids are a highly diverse group of metabolically active lipids found in marine sponges. The capacity to biosynthesize common and unusual sterols has been reported by various authors [6]. These compounds often form complex inseparable mixtures, and their identification (usually as neutral sterols) is typically done by GC-MS [7-9]. However, by virtue of their greater spread on the polarity

* Corresponding author: E-Mail: franciscojavier.toledo@ulpgc.es; Phone: + 34 928 454430 *Fax:* + 34 928 452922

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scale, some of these sterols can be isolated, characterized, and quantified in pure condition by liquid chromatography tandem mass spectrometry (LC–MS/MS) analysis [10,11].

Among the background on the chemistry of steroids isolated from *Aplysina* genus, two early publications written by De Luca *et al.* and Kelecom *et al.* stand out, in which they reported that aplysterol and 24(28)-didehydroaplysterol are the major sterols for *Aplysina aerophoba* [12,13]. In other studies, verongulasterol, 25-dehydroaplysterol, (24*R*)- and (24*S*)-24-isopropenylcholesterol, (24*R*)- and (24*S*)-24-methylcholesta-5,25-dien-3β-ol, and (24*R*)- and (24*S*)-24-methylcholesta-7,25-dien-3β-ol were isolated from the Caribbean sponge *V. cauliformis* [14]; 26-methylated sterols, aplysterol, 24(28)-didehydroaplysterol, and seven commonly occurring sterols were found in *A. fulva* (= *A. fistularis* var. *fulva*) [15]; aplysterol, verongulasterol, and 25-dehydroaplysterol were found in *A. archeri* [16]; and, finally, aplysterol and verongulasterol were reported as occurring in *A. lacunosa* and *A. fistularis* [17].

Thus, the aim of this study was to reinvestigate the presence of the steroid metabolome (steroidome) in *A. aerophoba*, through the use of modern structural elucidation and chemical analysis such as NMR spectroscopy and ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

2. Materials and Methods

2.1. Chemicals

All of the chemical reagents used — for example, hexane, ethyl acetate (EtAcO), chloroform, methanol, glacial acetic acid, and sulfuric acid — were Panreac brand analytical grade or higher, and were purchased from Medina Cejudo (Las Palmas de Gran Canaria, Spain). The deuterated Sigma-Aldrich solvents for nuclear magnetic resonance spectroscopy were acquired from BioSigma (Santa Cruz de Tenerife, Spain). Pure water was produced by reverse osmosis in a Milli-Q water system (Millipore, Bedford, MA, USA). The Scharlau brand silica gel column chromatography was acquired from Melcan (Santa Cruz de Tenerife, Spain).

2.2. Collection and Identification of Biological Material

The material, which was identified as *A. aerophoba* Nardo, 1833 (Aplysinidae, Verongida, Demospongiae), was collected on the coast of Maspalomas, Gran Canaria, Spain, after having been torn from its habitat by a sea storm and subsequently deposited on the beach. A voucher specimen with the code AN SPON 01 was deposited in the sponge collection of the Organic Chemistry Laboratory, University of Las Palmas de Gran Canaria (ULPGC), Las Palmas de Gran Canaria, Spain. Samples of *A. aerophoba* were immediately soaked in acetone until extraction of the metabolites.

2.3. Apparatus and Analytical Methods

Thin-layer chromatography (TLC) analysis was performed using Macherey-Nagel (Germany) POLYGRAM® polyester sheets with SIL G/UV_{254} silica layers (5 × 10 cm or 10 × 20 cm). Normal phase column chromatography was performed on silica gel (Scharlau) with 0.06–0.2 mm particle size as the adsorbent, at the head of the chromatographic column; and 0.04–0.06 mm particle size for the stationary phase. Chromatography was performed at medium or low pressure using a Büchi chromatography system (Flawil, Switzerland) or Fluid Metering Inc. (USA) motors connected in series with an Ace Glass Inc. (USA) column. The motor was equipped with a controlling membrane to stabilize the pressure pulses.

^{1}H- and ^{13}C-NMR spectroscopy experiments were recorded at 300, 400, and 500 MHz on a Bruker apparatus. Tetramethylsilane (TMS) was used as an internal standard (δ 0.00) for deuterated chloroform (δ 77.00) or deuterated methanol (δ 49.00), for the calibration of the ^{13}C-NMR spectra. Electrospray ionization mass spectrometry was performed at either low or high resolution, with a

common electron impact mass spectrometer (EI-MS) or by fast atom bombardment (FAB). FAB-MS at 70 eV was performed in the positive mode, using a FISONS VG Micromass Autospec apparatus, with 3-nitrobenzyl alcohol (3-NBA) as the matrix.

Melting points were measured on a Gallenkamp melting point apparatus and are uncorrected.

2.4. Normal Phase TLC analysis

The dry residue of the crude extract of the *A. aerophoba* sponge or its successive chromatographic fractions (4 mg) was redissolved in 40 µL of acetone, and then 3 µL amounts from the resulting solutions were applied to the silica gel plates for TLC analysis. The chromatogram is typically eluted with a mixture of standard solvents for lipids — hexane and acetone at a 75:25 ratio, *v*/*v* [18].

The plates were air dried at 23 °C, then analyzed under UV light (λ = 254 nm), followed by the application of *oleum* spray — sulfuric acid (4%) + acetic acid (80%) + water (16%), and they were then heated at 120 °C for 20 min. Finally, the resulting spots were observed in daylight. The metabolites were identified on the plates by comparing their characteristic colorations and relative retention factors (Rf). The aplysterol mixture was revealed by the presence of a purple coloration (Rf = 0.43) when using *oleum*, as was the mixture of its acetates (Rf = 0.78).

2.5. UHPLC-MS Analysis

Specific reagents: A standard solution (1000 $mg \cdot L^{-1}$ in methanol) of the aplysterol mixture was prepared and stored in amber vials at -20 ºC. This standard solution was diluted in methanol on a daily basis so that it could be introduced by direct infusion into the mass spectrometry equipment. All the solvents used as mobile phases — water (LC-MS grade), methanol (LC-MS grade), and formic acid — were sourced from Panreac Química (Barcelona, Spain).

Instrumentation: The mass determination of the compounds being studied (aplysterol and didehydroaplysterols) was done using a Waters ACQUITY UPLC© system (Madrid, Spain) coupled to: a model 2777 autosampler with a 25 µL syringe and a tray for 2 mL vials, and an ACQUITY tandem quadrupole (triple quadrupole) mass spectrometer with an electrospray ionization (ESI+) interface. All of the Waters components were controlled using the MassLynx Mass Spectrometry software. The electrospray ionization parameters were fixed as follows: capillary voltage of 3 kV, cone voltage of 40 V, source temperature of 120 ºC, and desolvation temperature of 400 ºC. Nitrogen was used as the desolvation gas, at a flow rate of 800 $L \cdot h^{-1}$, while argon was employed as the collision gas, at a flow rate of 50 $L \cdot h^{-1}$. MS/MS detection parameters were optimized by direct infusion of a 5 $mg \cdot L^{-1}$ standard solution of each analyte into the detector, at a flow rate of 10 $\mu L \cdot min^{-1}$.

Chromatographic conditions: The chromatographic separations were performed with the use of a 50 × 2.1 mm Waters ACQUITY BEH C18 (Madrid, Spain) UHPLC analytical column with a particle size of 1.7 µm, which operated at a temperature of 30 °C. Elution chromatography was performed in gradient mode at a flow rate of 0.3 $mL \cdot min^{-1}$, using water (phase A) and methanol (phase B), which were both acidified with formic acid (0.1%) to encourage ionization of the molecules in the mass spectrometer. The injected sample volume was 10 µL. The gradient used is shown in Table S3 (see supplementary material).

3. Results and Discussion

3.1. Structure Elucidation

Maceration of pieces of the marine sponge *A. aerophoba* in acetone, followed by filtration and rotaevaporation, furnished the crude extract. Through column chromatography, eluting with hexane/ethyl acetate, successive fractions with increasing polarity were obtained, and the analogous fractions were combined on the basis of their TLC band. Finally, it was obtained a fraction which

showed a green colour in the Liebermann-Burchard test [19] — this assay indicates the presence of sterols.

The fractions containing the above compounds were eluted with hexane and ethyl acetate (80:20 ratio), and they were repeatedly rechromatographed with the same eluent until purification of the mixture, in which the steroids aplysterol (**1**, 62.65%), 24(28)-didehydroaplysterol (**3**, 22.89%), 23(24)-didehydroaplysterol (**5**, 7.23%), and 24(25)-didehydroaplysterol (**7**, 7.23%) were identified (Figure 1). This expands upon the works described in the bibliography [12,13], due to compounds **5** and **7** being new additions to the literature. On the other hand, these results are very interesting since the mixture of aplysterol (**1**) and 24,28-didehydroaplysterol (**3**) was recently tested in vitro and has shown inhibitory activity on human DNA topoisomerase II-α [20]. Using the same chromatography, a mixture of acetylated derivatives (**2**, **4**, **6**, and **8**) was also isolated — they were isolated here for the first time as natural products.

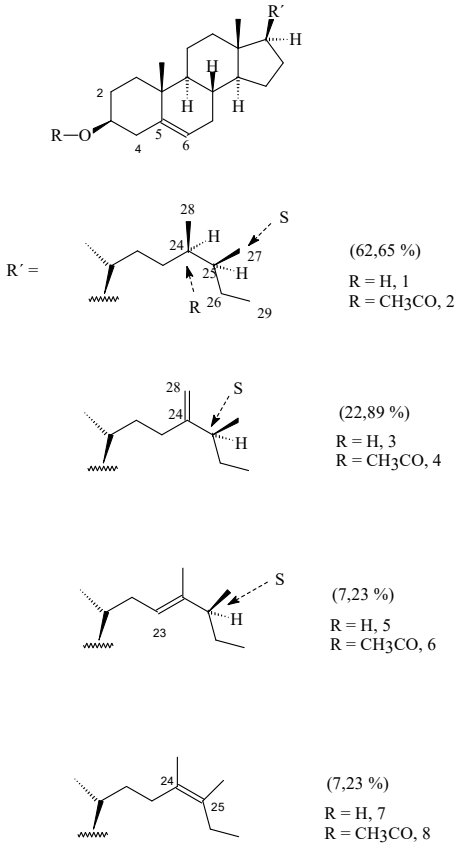

Figure 1. Sterols identified in *Aplysina aerophoba*

Both the mixture of aplysterols and the mixture of their acetates have the appearance of a white solid, and according to TLC they are homogeneous. Their melting points, which were determined after crystallization from methanol, were as follows: 135–136 ºC for the mixture of aplysterols, and 119–120 ºC for the mixture of their acetates.

The high-resolution mass spectrum of the aplysterol mixture displayed: a signal for the molecular ion peak at *m/z* 414.383263, which corresponds to the molecular formula $C_{29}H_{50}O$; and a signal for the base peak at *m/z* 396.372177 ($C_{29}H_{48}$), which was due to the loss of a water molecule and indicates the presence of a hydroxyl group. The molecular formula indicates that the aplysterol mixture has two carbons more than cholesterol (cholesterol is a 27-carbon compound). A detailed

study of the mass spectrum indicates that two additional carbons are in the side chain, which would, therefore, be formed by ten carbon atoms. Thus, for example, peaks at *m/z* 273.2133 (loss of ten-carbon side chain) and 255.2041 (loss of ten-carbon side chain and water moiety) lead to propose to the aplysterol mixture a tetracyclic nucleus identical to the nucleus of cholesterol. The presence of secondary products with unsaturation in the side chain is evidenced by the observation of the molecular ion at *m/z* 412.364784 ($C_{29}H_{48}O$) and water loss at *m/z* 394.353569 ($C_{29}H_{46}$). A significant peak at *m/z* 314.2478, which is characteristic of unsaturation at C-24 [12], is assigned to the ion resulting from the McLafferty rearrangement (cleavage of the C-22/C-23 bond with hydrogen transfer from C-20) — see Figure 2.

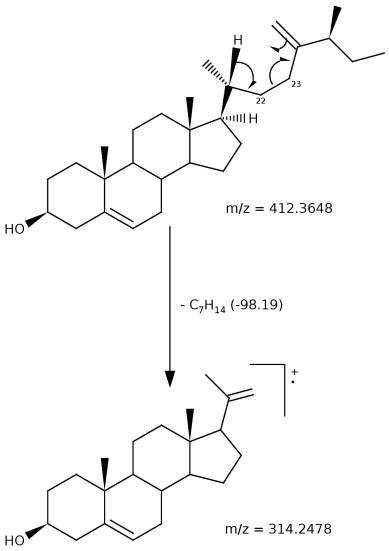

Figure 2. McLafferty rearrangement of the 24(28)–didehydroaplysterol

According to the IR spectrum, the aplysterol mixture has an alcohol group, because an absorption band is observed at 3608 cm^{-1} (stretching frequency of a free hydroxyl group). The absorption bands at 3027 and 3006 cm^{-1} are due to the stretching vibration of the =CH group, which suggests the presence of double bonds, in which the wide band with several peaks in the region between 2961 and 2870 cm^{-1} corresponds to the stretching vibrations of C-H bonds of the methyl, methylene, and methine groups. The two sharp peaks appearing at 1464 and 1379 cm^{-1} belong to the so-called "fingerprint", within which an intense absorption band is observed at 1209 cm^{-1}, which corresponds to the stretching vibration of the C-O bond of the alcohol group.

The ^{1}H-NMR spectrum shifts a signal at δ 5.35 (1H, overlapping double doublets), which is characteristic of a double bond between the C-5 and C-6 carbons of the four sterols with the same carbon skeleton (the aplysterol). A signal, at δ 5.16, as a triplet with J = 5.45 Hz reveals the presence of a minor regioisomer 23(24)-didehydroaplysterol (**5**). A broad singlet at δ = 4.70 and a multiplet at δ 4.68 — both at a 1:1 ratio — reveal the methylene of the olefinic carbon C-28 of the 24(28)-didehydroaplysterol (**3**). The hydrogen signal geminal to the alcohol group (δ 3.51, 1H, m) suggests a 3β-hydroxyl group typical of sterols (Figure 3). Additionally, singlets of the hydrogens were observed on the C-18 and C-19 angular methyls (δ 0.68 and 1.00, respectively), which correspond to those found in the literature for aplysterol.

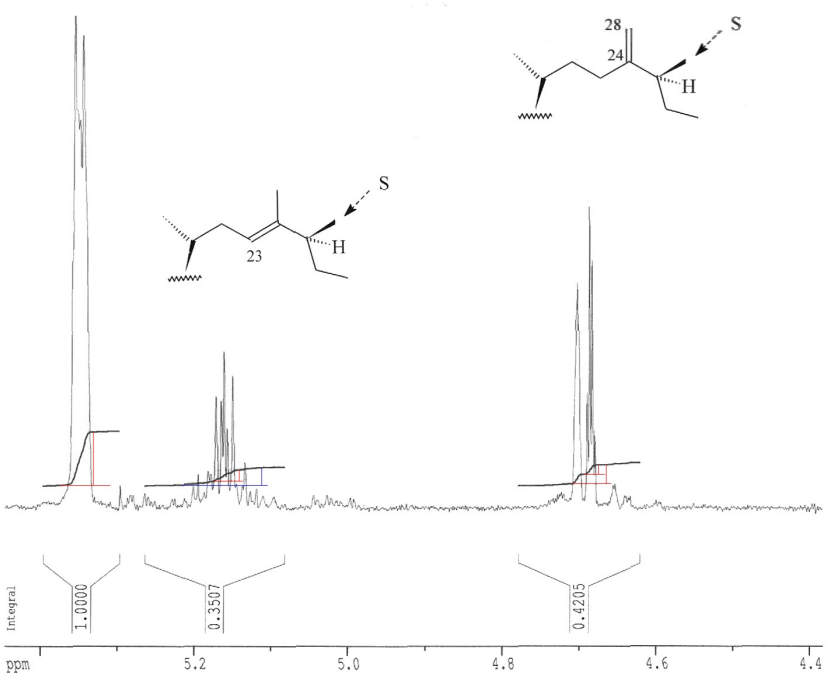

Figure 3. ^{1}H-NMR data (500 MHz, $CDCl_3$) of the olefinic protons for a mixture of aplysterol (**1**), 24(28)-didehydroaplysterol (**3**), 23(24)-didehydroaplysterol (**5**), and 24(25)-didehydroaplysterol (**7**)

The ^{1}H-^{1}H connectivities deduced by two-dimensional correlation experiments (COSY, TOCSY, and NOESY) are also consistent with the assigned structures. Thus, the signal at δ 2.24 (2H, m) is correlated with the olefinic proton at δ 5.35 (1H, m) and, therefore, it corresponds to the second ring of the carbon skeleton; that is, to the allylic hydrogens in C-7. Correlations were also observed between the signals at δ 2.28 (m), δ 1.84 (m) and δ 1.51 (m) with the signal at δ 3.51, of the hydrogen geminal to the hydroxyl group at C-3, whereby these signals must belong to hydrogens of the first ring, or more specifically, the hydrogens in C-2 and C-4.

The ^{13}C-NMR spectrum shows the presence of: peaks assignable to the six methyl groups of the proposed main structure, the aplysterol; as well as eleven methylenes, nine methines, and three quaternary carbons observed in the DEPT spectrum. These data are summarized in Table S1. The signals of the C-5 and C-6 carbons are characteristics because they are part of the double bond contained in the second cyclohexane ring (δ 140.7 and 121.7, respectively). They are also two characteristic signals assigned to the C-24 and C-28 (δ 39.8 and 16.5, respectively).

*3.2. Study of the Natural Mixture of Aplysterol (**2**), 24(28)-didehydroaplysterol (**4**), 23(24)-didehydroaplysterol (**6**), and 24(25)-didehydroaplysterol (**8**) acetates*

Spectroscopic studies conducted for the second mixture confirm that the isolated steroid is aplysterol acetate (**2**), unpurified by the three didehydroderivatives (**4**, **6**, and **8**). First, it exhibits an intense absorption band in IR spectrum at 1724.24 cm^{-1} corresponding to the stretching frequency of the C=O group of the esters that, by the strong peak at 1255.57 cm^{-1} corresponding to the stretching frequency of the C-O bond, indicates the presence of an acetoxy group at C-3.

The ^{1}H-NMR spectrum also shows the typical olefinic proton signal at δ 5.37 in the C6, as well as the hydrogen signal geminal to the acetoxy group at δ 4.62 (1H, m), and the signal of the methyl in the acetate group at δ 2.03 (3H, s). The rest of the spectrum-shifted signals are consistent with the steroidal skeleton of the aplysterol acetate, its majority sterol. A triplet centered at δ 5.16

reveals the skeleton of 23(24)-didehydroaplysterol, while two signals at δ 4.70 and δ 4.68 reveal the methylene of the 24(28)-didehydroaplysterol. The integral curves of these signals are consistent with the proportion assigned to the mixture of the non-acetylated aplysterols.

In the ^{13}C-NMR spectrum, the signal of the carbonyl carbon of the acetate was observed at δ 170.5, while the signals of the olefinic carbons C-5 and C-6 of the aplysterol skeleton (δ 139.6 and 122.6, respectively), as well as other expected signals, were observed in accordance with the literature [12]. Minority signals in the olefinic region of the spectrum were due to acetates of the 24(28)-didehydroaplysterol (δ 156.5 and 106.0), 23(24)-didehydroaplysterol (δ 136.0 and 132.5), and 24(25)-didehydroaplysterol (δ 135.8 and 129.1).

In the mass spectrum, the molecular ion peak at *m/z* 456 (M^+, $C_{31}H_{52}O_2$) was not observed, which was due to the easy loss of ketene (CH_2=C=O) producing peaks at *m/z* 414.3866 ($C_{29}H_{50}O$) (base peak) and 412.3690 ($C_{29}H_{48}O$), as well as the loss of a molecule of acetic acid — this latter phenomenon justifies the abundant peak at *m/z* 396.3708 (M^+-CH_3COOH; $C_{29}H_{48}$).

The COSY, TOCSY, and NOESY spectra are consistent with the given structures, showing, among other things, the correlation between: the olefinic proton in C-6 (δ 5.37) and the allylic methylene in C-7 (δ 2.31), or the olefinic methylene in C-28 (δ 4.69) and the allylic methylene in C-23 (δ 2.00).

In order to confirm the hypothesis that the compound isolated from the sponge was the acetylated derivative from the aplysterol mixture described above, a sample of the aplysterol mixture was acetylated with pyridine and acetic anhydride (1 h at 80 ºC), extracted in the usual manner, and then purified by chromatography on a silica gel column with solvents of increasing polarity, from hexane to hexane-EtAcO (1:1 ratio). The comparison, via TLC and spectroscopy, of the natural and synthetic mixtures, revealed that they contained indeed the same mixture of compounds (**2**, **4**, **6**, and **8**) (Figure 1).

3.3. Quantification of Aplysterols by ^{13}C-NMR

The ^{13}C-NMR spectrum shifted eight signals corresponding to the olefinic carbons, in a δ range of 105–160 ppm (Figure S1). Of these, four signals were chosen due to their olefinic quaternary carbon status furnishing approximately the same response in the ^{13}C-NMR experiment (a C-5 carbon which was present in each of the four sterols, and three C-24 carbons which were present in each of the three didehydrosterols). The relative intensities of the signals corresponding to the said quaternary carbons (δ 140.74, 156.75, 136.04, and 135.77) — see Table 1 — led to a composition of 62.65% of aplysterol (**1**), 22.89% of 24(28)-didehydroaplysterol (**3**), 7.23% of 23(24)-didehydroaplysterol (**5**), and 7.23% of 24(25)-didehydroaplysterol (**7**). (Figure 1). Following, quantitative calculation equations are given:

$$\%\ (\mathbf{1}) = [(h_{1+3+5+7} - h_3 - h_5 - h_7) / (h_{1+3+5+7})] \times 100$$

$$\%\ (\mathbf{i}) = [h_i / (h_{1+3+5+7})] \times 100 \quad (i = \mathbf{3}, \mathbf{5}, \mathbf{7};\ h_i \text{ data are in Table 1})$$

Table 1. Chemical shift and signal intensity of the olefinic quaternary carbons observed in the ^{13}C-NMR (125 MHz, $CDCl_3$) used for determining the percentages in the aplysterol mixture (Figure 1).

Carbon (Compound)	**δ (ppm)**	**h_i (mm)**	**(Quaternary Carbon) (Signal intensity, %)**
C-5 (**1**+**3**+**5**+**7**)	140.74	41.5	100.00
C-24 (**3**)	156.75	9.5	22.89
C-24 (**5**)	136.04	3.0	7.23
C-24 (**7**)	135.77	3.0	7.23

*3.4. Chemical Analysis of the Aplysterol Mixture (**1** + **3** + **5** + **7**) by UHPLC-MS.*

Determination by direct infusion: After directly infusing the mixture of aplysterols at a concentration of 1 $mg \cdot L^{-1}$, the masses of the parent ions shown in Table S2 were obtained. The majority mass corresponds to aplysterol, as established by the previous spectroscopic study. The remaining peaks correspond to didehydroaplysterol in its different regioisomers. The fragmentation of the selected parent or precursor ions was subsequently performed in order to obtain the respective daughter or product ions for each compound. Table S2 shows the possible molecular structures of the ions obtained.

Chromatographic determination: Once the masses of the compounds being studied were determined, their mixture was injected into the chromatograph and passed through the column to produce the chromatographic separation. However, of all the compounds that compose the sample-mixture, it was able to chromatographically determine only the main component, the aplysterol (**1**). This compound was identified by both, SIR mode (monitoring parent ion) and MRM mode (monitoring ions fragmentation). This main compound was detected at a retention time of 5.4 min as shown in Figure S2.

4. Conclusions

Maceration of pieces of the marine sponge *A. aerophoba* with acetone, followed by filtration and rotaevaporation, furnished a crude extract. By column chromatography eluting with hexane and ethyl acetate, successive fractions with progressive polarity were obtained, which were monitored by TLC in order to obtain a fraction whose residue furnished a green-blue colored product in the Liebermann-Burchard test, which indicates a positive response for sterols. Successive fractions containing the above compounds were combined and repeatedly rechromatographed (hexane and ethyl acetate at a 80:20 ratio) in order to achieve maximum purification of the mixture, in which the steroids aplysterol (**1**, 62.65%), 24(28)-didehydroaplysterol (**3**, 22.89%), 23(24)-didehydroaplysterol (**5**, 7.23%), and 24(25)-didehydroaplysterol (**7**, 7.23%) were identified. Compounds **5** and **7** are new to the literature. The corresponding mixture of acetylated derivatives (**2**, **4**, **6**, and **8**) was also isolated at approximately the same percentage composition. These last four compounds were isolated here for the first time as natural products.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

ORCID
Pere Ferriol: 0000-0002-6847-8460
Francisco J. Toledo Marante: 0000-0002-7842-591X
Ignacio Brouard Martin: 0000-0002-9409-1534
José J. Santana Rodríguez: 0000-0002-5635-7215
Rayco Guedes Alonso: 0000-0002-9189-9589
Alba González Benkovics: 0000-0001-9365-8263
Roberto Mioso: 0000-0003-1369-2037

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