Published in final edited form as:

European J Org Chem. 2009 November ; 2009(31): 5327–5336. doi:10.1002/ejoc.200900775.

Aplysqualenols A and B: Squalene-Derived Polyethers with Antitumoral and Antiviral Activity from the Caribbean Sea Slug *Aplysia dactylomela*

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Abstract

The novel bromotriterpene polyethers aplysqualenol A (**1**) and aplysqualenol B (**2**) have been isolated from the Caribbean sea slug *Aplysia dactylomela* collected in Puerto Rico, and their structures and relative configurational assignments established from spectroscopic data aided by quantum mechanical calculations of NMR chemical shifts. Although both these compounds may be conceived as polyoxocyclic derivatives of the C30 squalene skeleton, remarkably **1** and **2** possess an unprecedented C15 to C24 flexible chain of 14.5° spatial disposition that contains a unique ether bridge between C16 and C19. Biological activity screening tests revealed that, although aplysqualenol A (**1**) does not have significant anti-infective properties, it possesses potent antitumoral and antiviral activities.

Keywords

Aplysia dactylomela; Sea slug; Polyether; Antitumor activity; Antiviral activity

Introduction

Previous chemical investigations of the Caribbean sea slug *Aplysia dactylomela* Rang (order Anaspiidea, family Aplysiidae) found worldwide in tropical to warm temperate waters, reported the isolation of two principal groups of compounds, terpenes (monoterpenes, sesquiterpenes, diterpenes and steroids) and nonterpenoid C-15 acetogenins.^[1] To date, over 60 terpenoid natural products have been isolated from *A. dactylomela*, and they exhibited various biological activities including anticancer, anti-HIV, algucidal, ichthyotoxic, nematocidal, antiparasitic, and antibacterial activities.^[2] As part of a recent exploratory survey of marine invertebrates for anticancer constituents, *A. dactylomela* was collected in Cabo Rojo, Puerto Rico (April, 2001), and extracts from this anaspidean mollusk gave evidence of significant cytotoxic activity in the brine shrimp lethality bioassay (BSLT).^[3] Thus, further examination of its constituents resulted in the isolation of two cytotoxic and structurally unique organic compounds. Herein, we report the isolation and structure

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Supporting information for this article is available on the WWW under <http://www.eurjoc.org/> or from the author.

Supporting Information: ¹H and ¹³C NMR spectra and representative 2D NMR data (¹H–¹H COSY, HMQC, HMBC, and NOESY) for compounds **1** and **2**. Complete ¹H and ¹³C NMR spectral data for aplysqualenol A acetate (**3**) (Table 4) and aplysqualenol B diacetate (**4**) (Table 5). Atom coordinates for the minimum energy structures of stereoisomer models **1a–1d**, as well as statistical information regarding computational results (such as the calculated ¹³C chemical shifts, the corrected values, and Δδ) are summarized in Tables 6–9.

determination of aplysqualenol A (**1**) and B (**2**), novel brominated triterpene polyethers with a tetracyclic skeleton, isolated from two specimens of this chemically prolific animal.^[4]

The MeOH–CHCl₃ (1:1) extract of freeze-dried *A. dactylomela* (54 g, dry wt) was partitioned between hexane and H₂O. The hexane soluble material, which exhibited potent cytotoxicity against *Artemia salina*,^[3] was subjected to fractionation using column chromatography (Silica gel) and polar bonded-phase HPLC to afford aplysqualenol A (**1**) and aplysqualenol B (**2**) as colorless oils (0.06% and 0.02% yield, respectively, based on crude extract weight). Aplysqualenol A (**1**) was evaluated in the National Cancer Institute (NCI) three-cell line, one-dose, primary anticancer assay displaying potent in vitro cytotoxicity against MCF-7 breast cancer, NCI-H460 nonsmall cell lung cancer, and SF-268 CNS cancer (the percent of growth of the treated cells when compared to the untreated cells was approximately 0, 0, and 4%, respectively).

Results and Discussion

The molecular formula of aplysqualenol A (**1**), [α]_D²⁰ +48.1° (c1.6, CHCl₃), was determined to be C₃₁H₅₃BrO₇ by HRFABMS and ¹³C NMR. Treatment of **1** with acetic anhydride and pyridine at 55 °C for 5h gave the corresponding monoacetate **3**, C₃₃H₅₅BrO₈, ν_{max} 1740 cm⁻¹ and δ_{H} 2.05 (3H, s), whose IR spectrum still showed an absorption due to hydroxyl group at 3549 cm⁻¹, thus proving the presence of secondary and tertiary hydroxyl groups in **1**. The presence of two hydroxyl groups was further indicated by two D₂O exchangeable resonances (δ 2.97 and 3.47) in the ¹H NMR spectrum. The ¹H and ¹³C NMR spectral data revealed that aplysqualenol A (**1**) possessed 7 tertiary methyls, 5 oxygenated quaternary carbons, 6 oxygenated methine carbons, and a brominated methine carbon (δ 58.9). Since aplysqualenol A displayed signals for only one unsaturated bond [δ_{C} 144.4 (C) and 113.8 (CH₂); δ_{H} 4.87 (1H, br s) and 4.93 (1H, br s)], **1** was assumed to comprise four oxacyclic units. The detailed analysis of the ¹H–¹H COSY, HMQC, and HMBC spectra of **1** showed the presence of the same partial structural units in the molecule (rings A–B–C) as those in thyriferol (**5**) and venustatriol (**6**), isolated from red algae of the genus *Laurencia*,^[5,6] and aplysiol A (**7**) and B (**8**), isolated recently from South China Sea specimens of *A. dactylomela*.^[7] Furthermore, the ¹H–¹H COSY and HMBC data, summarized in Table 1, allowed the foregoing partial structures (C1 through C15 and C25 through C28) and the remaining ring system D (C16 through C24 and C29 through C31) to be connected, establishing the connectivities of all the carbon atoms in **1** as shown.

This unprecedented architecture required further confirmation, which was provided by unambiguous determination of the locations of the two hydroxyl groups in **1**. The positions of these pivotal functionalities were initially determined by HMBC correlations (C15/15-OH and C18/18-OH), and later confirmed by a deuterium shift experiment, which revealed that among all oxygenated carbons, only the two signals for C15 and C18 shifted significantly to lower field when measured in CD_3OH as compared to CD_3OD .^[8] Having definitively located the two hydroxyl groups at C15 and C18, we inferred that the other 9 oxycarbons in aplysqualenol A all participate in ether linkages. Thus, the locus of oxolane ring D arising from an ether bridge between C16 and C19, was indicated by the HMBC correlations (C15/H-16, C15/H-17 $\alpha\beta$, C16/H-18, C16/H₃-28, C19/H-17 β , C19/H-20 $\alpha\beta$, and C-19/H₃-29). The one additional ethereal group (-OCH₃) present in **1** was shown to be located at C22 based on the HMBC correlations (C22/-OCH₃, C22/H-24 $\alpha\beta$, C22/H₃-30, and C22/H-21 $\alpha\beta$), leaving the bromine atom to reside at C3.

The oxacyclic systems of **1** were also deduced from the specific fragment ions in the EI mass spectrum (Figure 1). The mass spectrum showed fragment ions at m/z 503/505 [$\text{M} - \text{C}_7\text{H}_{13}\text{O}$]⁺, 403/405 [$\text{M} - \text{C}_{12}\text{H}_{21}\text{O}_3$]⁺, 359/361 [$\text{M} - \text{C}_{14}\text{H}_{25}\text{O}_4$]⁺, and 205/207 [$\text{M} - \text{C}_{23}\text{H}_{39}\text{O}_6$]⁺ due to cleavage at C19–C20, C15–C16, C14–C15, and C6–C7 bonds, respectively. Hence, the remaining two ether linkages consisted of a 2,7-dioxabicyclo[4.4.0]decane ring as evidenced from the intense (100%) fragment ion at m/z 153 [$\text{C}_9\text{H}_{13}\text{O}_2$]⁺. The presence of an oxolane ether bridge encompassing positions C16 and C19 was corroborated from the rearranged fragment ion at m/z 214 [$\text{M} - \text{C}_{19}\text{H}_{32}\text{BrO}_4 + \text{H}$]⁺ resulting from cleavage of the C15–C16 bond. Furthermore, the fragment ion at m/z 257 [$\text{M} - \text{C}_{17}\text{H}_{28}\text{BrO}_3$]⁺ arising from cleavage at C14–C15, confirmed the presence in **1** of the partial structural unit from C15 through C24 (including C28 through C31). Consequently, aplysqualenol A (**1**) was found to be a new member of squalene-derived bromotriterpene with novel structural features not found in known congeners of this class of natural products.^[4,7]

The assignment of the relative stereochemistry of **1**, except at C22, was straightforward. The equatorial orientation of the bromine atom at C3 on the A ring was evident from the J -values with vicinal axial-axial coupling constant of H-3 (12.3 Hz) in the ¹H NMR spectrum of **1**. From this point forward, the relative stereochemistries were determined by the NOESY spectrum as summarized in Table 1. NOEs about the ABC-rings were detected between H-3/

H₃-25, H₃-1/H₃-26, H-5 α /H-7, H₃-26/H-8 β , H-7/H-11, H-14/H-12 β , and, most importantly, H-14/H₃-27, respectively, establishing a *cis*-relationship among these sets of protons. No NOE, however, was detected between H-7/H₃-26, H-11/H₃-27, or H-11/H-14, thus suggesting that these pairs of protons were *trans* oriented. In addition, NOEs about the C/D rings were detected between H-13 α /H₃-28, H-16/H₃-28, H-16/H-17 α , H-17 α /H-18, and H-14/H₃-29, suggesting a close spatial proximity among these protons. Moreover, no NOE between H-14/H₃-28, H-14/H-16, H-16/H₃-29, and H-18/H₃-29 suggested the stereochemistries of these protons to be *trans* (Figure 2). The observation that in compound **1** the proton signal for H-14 was resolved as a doublet of doublets with two different coupling constant values (one much larger than the other indicating a chair/chair B–C rings system) confirmed the axial orientation of this methine and, therefore, that the relative stereochemistry at carbon C-14 would be *S*^{*} for aplysqualenol A (**1**). As far as we know, this is the first example of a squalene-polyether derivative displaying a C15 to C24 flexible side chain of equatorial spatial disposition.^[9] In order to corroborate the relative configuration shown for aplysqualenol A molecular mechanics/dynamics calculations were performed to establish the dominant conformations of **1**.^[10] The results of the conformational analysis inferred that the secondary alcohol at C18 was fixed with the tertiary hydroxyl oxygen at C15 in a H-bond. In turn, 15-OH is hydrogen bonded to the oxygen atom of oxane ring C. Indeed, the presence of intramolecular hydrogen bonding in aplysqualenol A was supported experimentally by the strong couplings of H-18 and 18-OH observed in the ¹H–¹H COSY spectrum, the fact that acetylation of **1** did not take place at 25 °C, and the strong NOEs detected between H₃-28/15-OH, H-17 β /18-OH, and H₃-29/18-OH. Furthermore, the coupling constants of H-22 with the diastereotopic protons H₂-21 (*J*_{22–21 α} = 6.5 Hz and *J*_{22–21 β} = 6.8 Hz) indicated that free rotation about the C21–C22 bond renders them equivalent, thus strengthening the contention that the secondary alcohol is fixed in a H-bond with 15-OH and not the methoxy-ether at C22. Insofar as the relative configuration for C22 is concerned, it was ultimately assigned as *R*^{*} on the basis of a combined approach, which included NOESY NMR data acquired with aplysqualenol B (**2**), an integrated QM (Quantum Mechanical)–NMR approach based on DFT (density functional theory)^[11] calculations of ¹³C NMR chemical shifts and the analysis of the experimental NMR data of aplysqualenol A (**1**), as well as biogenetical considerations (*vide infra*). Figure 2 shows a plausible conformation of rings A–D based on the coupling constants and NOE correlations observed which indicated the relative stereochemistry of aplysqualenol A (**1**) to be 3*R*^{*}, 6*S*^{*}, 7*R*^{*}, 10*S*^{*}, 11*R*^{*}, 14*S*^{*}, 15*R*^{*}, 16*R*^{*}, 18*R*^{*}, 19*S*^{*} and 22*R*^{*}.^[12] Sadly, all of our attempts to determine absolute stereochemistry by mixing **1** with a solid matrix-bound auxiliary reagent (MTPA) directly in the NMR tube were unsuccessful.^[13,14]

Compound **2**, aplysqualenol B, was also isolated as a colorless oil, [α]_D²⁰ +27.1° (*c* 1.4, CHCl₃). Mass spectral analysis of this metabolite showed a molecular ion consistent with the molecular formula C₃₁H₅₃BrO₈ (observed [M + H]⁺ 633.3003; calculated 633.3002). Assignment of the structure for this metabolite was aided by the considerable spectroscopic analogy to aplysqualenol A (**1**). The only significant variations in the ¹H NMR spectrum between compound **2** and **1** were the disappearance of the tertiary methyl signal ascribable in **1** to H₃-30 (δ _H 1.63) and the appearance of a pair of AB doublets in **2** at δ _H 4.21 (1H, *J* = 13.5 Hz) and 4.09 (1H, *J* = 14.3 Hz), as well as the downfield shifts in the protons H-22 (δ _H 3.62, dd, *J* = 6.5, 6.8 Hz) and H₂-24 (δ _H 5.06, 1H, br s, H-24 α and 5.24, 1H, br s, H-24 β). These data suggested that the modifications in this compound were mainly located in the C₆ side chain appended to ring D. The connectivities observed in ¹H–¹H COSY, HMQC, and HMBC experiments (Table 2) made it possible to assign the fragment C20→C24 (including C30) as follows: δ _C: 34.2 (CH₂, C20), 28.6 (CH₂, C21), 85.0 (CH, C22), 146.9 (C, C23), 114.0 (CH₂, C24) and 62.9 (CH₂, C30). These facts can only be explained by the presence in **2** of a primary hydroxyl group at C30. The isolated hydroxymethylene group was

connected to the ring D side chain through C23 by HMBC correlations (C30/H-22, C30/H-24 $\alpha\beta$, C23/H-30 $\alpha\beta$ and C24/H-30 $\alpha\beta$). In order to confirm the presence of this system, compound **2** gave the 18,30-diacetate **4** by overnight treatment with Ac₂O/Py at 25 °C, C₃₅H₅₇BrO₁₀, $\nu_{\max}$ 3541, 1740 cm⁻¹ and δ_{H} 2.05 (3H, s) and 2.10 (3H, s), thus confirming its structure as an aplysqualenol A derivative. As in compound **1**, strong NOE correlations established the *cis* orientation between the methyl group H₃-27 (δ_{H} 1.15) and the oxymethine proton H-14 (δ_{H} 3.54), indicating that the relative configuration at C14 in compound **2** was *S**. The relative stereochemistry of the remaining carbons C3, C6, C7, C10, C11, C15, C16, C18, and C19 in compound **2** was established as identical with that observed for aplysqualenol A (**1**) on the basis of the observed NOE data in the NOESY experiments in CDCl₃ (Table 2). The relative stereochemistry of C22 in compound **2** was established as follows. The fact that in aplysqualenol B (**2**) the H₂-30 protons were diastereotopic (they appeared as an isolated AB system indicating rotational constraints along the C23–C30 bond), inferred that the primary alcohol was fixed with the methoxy-ether oxygen in a H-bond. This contention was supported by molecular mechanics calculations, which predicted identical coupling constant values between H-22 with the diastereotopic protons H₂-21 (actual values observed: $J_{22-21\alpha}$ = 6.5 Hz and $J_{22-21\beta}$ = 6.8 Hz) in the most stable conformation of compound **2** (Table 2). The NOE connectivities observed between H-22 with H-30 β , H-24 α , and, most importantly, H-16, and the observation that in diacetate derivative **4**, wherein such H-bonds are no longer attainable, the H₂-30 protons appeared as broad singlets centered near δ 4.56.^[15] Thus, the relative stereochemistry at C22 in this compound was established as *R**. Since these compounds share the same biogenetic pathway, it is highly reasonable that 3*R**, 6*S**, 7*R**, 10*S**, 11*R**, 14*S**, 15*R**, 16*R**, 18*R**, 19*S**, 22*R** is the most likely configuration for aplysqualenol B (**2**).

With the aim at strengthening the relative stereochemistry at the 11 stereogenic centers (C3, C6, C7, C10, C11, C14, C15, C16, C18, C19 and C22) of aplysqualenol A (**1**), we undertook a configurational study by an integrated QM-NMR approach based on DFT calculations of ¹³C NMR chemical shifts and the analysis of the experimental NMR data. Critically, ¹³C chemical shifts were used to validate the theoretical models, and dipolar coupling correlations derived from 2D-NOESY NMR experiments were used to corroborate the arrangements suggested by QM methods, and to determine the relative configuration of the molecule. As all the proton and carbon values were assigned confidently by 2D-NMR experiments (Table 1), which were in agreement with the proposed structure **1**, we confined our study to the four possible diastereomers bearing on C15 and C22 (see stereoisomer models **1a–1d** in Figure 3), the two most flexible and thus compromising stereocenters of aplysqualenol A. To determine the relative configuration of these centers the ¹³C NMR isotropic shifts of each diastereomer (Figure 3) at its lowest energy configuration were calculated. The minimum energy configuration for each isomer was identified using a Monte Carlo conformational search with the MMFF force field as implemented in the Spartan 04 software package.^[16] All diastereomers were further optimized using HF/6-31G(2d,p) level of theory using the Gaussian 03 program.^[17] NMR chemical shifts were calculated using the GIAO method at the mPW1PW91/6-31G(2d,p) level of theory. Recently, Bifulco and coworkers reported this level of theory to give reasonable results for the ¹³C shift of organic compounds.^[7,18] The experimental values were plotted against the calculated shift, and a least-squares fit line was determined. The calculated shifts for each isomer were corrected using the slope and intercept to give scaled ¹³C shifts. The difference plots were determined by subtracting the corrected shifts from the experimental chemical shifts.

Diastereomer **1b** presented the best ¹³C-NMR chemical shift correlation with respect to the theoretical values (Figure 4) with an average of $\Delta\delta$ = 2.9 (Table 3). It also resulted in the conformation with the lowest energy compared with the other three isomers (**1a**, **1c**, and **1d**).

These theoretical results for diastereomer **1b** ($15R^*,22R^*$) are in agreement with other previously detailed experimental observations, including the observation that the coupling constants of H-22 with the diastereotopic protons H₂-21 are similar suggesting that the C21–C22 bond experiences free rotation and thus the coupling constants are averaged. Had the 18-O H been fixed with the methoxy-ether oxygen at C22 (diastereomers **1c** and **1d**), two significantly different coupling constant values (one much larger than the other) would have been anticipated. The $\Delta\delta$ values observed for the signals of carbons nearby the methoxy group at C22 also indicated the R^* configuration for this centre, as depicted in formula **1**. To summarize, neither diastereomer **1a** ($15R^*,22S^*$), **1c** ($15S^*,22R^*$), nor **1d** ($15S^*,22S^*$) is likely to be the correct structure for aplysqualenol A because of their inherently higher relative energies/ $\Delta\delta$ values. These theoretical calculations not only provided information on the R^* configuration of C15 and C22, but also corroborated the configurational arrangement of C14 depicted in **1** and determined as described above.

From a biogenetic viewpoint the polyoxygenated squalene-derived ethers isolated from *A. dactylomela* could arise from a common precursor, (10*R*, 11*R*)-(+)-squalene-10,11-epoxide (**9**) isolated from *Laurencia okamura*.^[19] Upon further oxidation, this compound could give rise to (6*S*, 7*S*, 10*R*, 11*R*, 14*S*, 15*S*, 16*R*, 18*R*, 19*R*)-16*R*-hydroxy-squalene tetraepoxide (**10**) (such intermediate has not been reported yet). Biogenesis of the A–B–C–D ring system could stem from the concerted cyclizations of four epoxides after formation of a bromonium ion at C2–C3, thus forming the framework of these metabolites. In point of fact, the aberrant S^* stereochemistry at carbon C14 in compounds **1** and **2** (indicating overall retention of configuration at that center with respect to **10**) indicates that the proposed biogenesis through the cyclization of the squalene tetraepoxide precursor should in fact be concerted. Upon further oxidative metabolism of the remaining olefin followed by *O*-methylation, aplysqualenol A (**1**) could be obtained (Scheme 1).^[20] Compound **1** would in turn evolve to aplysqualenol B (**2**) following enzymatic hydroxylation at C30.

Conclusions

Aplysqualenol A (**1**) and B (**2**) are novel bromotriterpenes structurally related to thyriferol (**5**) which suggests **1** and **2** to be of dietary origin. Often, opisthobranch mollusks belonging to the order *Anaspidea*, feed on red and brown algae from which they sequester selected bioactive metabolites that are stored in the digestive gland and secreted in the mucus for defensive purposes.^[21] Thus, the presence of aplysqualenol A and B in *A. dactylomela* suggests a likely involvement of these molecules in the chemical defensive mechanism of the sea slug.^[7] Polyether squalene-derived of the type represented by **1** and **2** are currently quite rare, in contrast to thyriferol types.^[4] Thus, the aplysqualenols represent the only examples of this small family of marine metabolites to display the S^* configuration at C14 and to possess an ether linkage between C16 and C19. The unprecedented S^* stereochemistry at C14 leads, not only to a conformationally more stable 2,7-dioxabicyclo[4.4.0]decane system (chair/chair B–C rings system) than that present in thyriferol (**5**) and its congeners (chair/twist-boat B–C rings system), but also changes the arrangement and direction of the flexible side chain from a less favored axial position to a more stable equatorial orientation.^[22,23]

Upon screening in the NCI's in vitro antitumor assay consisting of 60 human tumor cell lines aplysqualenol A (**1**) exhibited inhibitory activity against SNB-19 CNS cancer and T-47D breast cancer with IC₅₀ values of 0.4 and 0.3 $\mu\text{g/mL}$, respectively. To explore its antiviral properties aplysqualenol A was evaluated against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2), varicella zoster virus (VZV), human cytomegalovirus (HCMV), and Epstein-Barr virus (EBV). At concentrations above 4 $\mu\text{g/mL}$ compound **1** showed a 90% maximal response (EC₉₀) against HSV-1, HSV-2, HCMV, and VZV viruses

(acyclovir was used as a control with EC₅₀ values of 0.95, 0.95, 0.22, and 0.14 µg/mL, respectively). Remarkably, aplysqualenol A was very toxic against the Epstein-Barr virus in VCA Elisa assay (EC₉₀ < 0.08 µg/mL; acyclovir EC₅₀ = 1.1 µg/mL) with no accompanying toxicity seen in the host Daudi cells.^[24] Compounds **1** and **2** showed moderate antiplasmodial activity against *Plasmodium falciparum* with IC₅₀ values of 11 and 18 µg/mL, respectively. In vitro antituberculosis screening of aplysqualenol A (**1**) against *Mycobacterium tuberculosis* H₃₇Rv at a concentration of 6.25 µg/mL showed no inhibitory activity.

Experimental Section

General Experimental Procedures

Optical rotations were measured as the sodium line (589 nm) with a Perkin-Elmer Polarimeter Model 243B. FT-IR spectra were measured with a Nicolet Magna 750 FT-IR spectrophotometer as a thin film on a NaCl disc. ¹H and ¹³C NMR spectral data and ¹H–¹H COSY, NOESY, APT, HMQC, and HMBC experiments were measured in CDCl₃ with a Bruker Avance DRX-500 FT-NMR spectrometer. ¹H–¹H COSY, NOESY, HMQC, and HMBC spectra were measured using standard Bruker pulse sequences. Chemical shifts are given on a δ (ppm) scale with CHCl₃ (1H, 7.26 ppm) and CDCl₃ (¹³C, 77.0 ppm) as the internal standard. Mass spectra were taken at the Mass Spectrometry Laboratory of the University of Illinois at Urbana–Champaign. Column chromatography (CC) was performed on silica gel (35–75 mesh). TLC analyses were carried out using glass silica gel plates and spots were visualized by exposure to I₂ vapors or heating silica gel plates sprayed with 5% H₂SO₄ in EtOH. All solvents were either spectral grade or distilled from glass prior to use. The percentage yield of each compound is based on the weight of the crude MeOH–CHCl₃ extract.

Animal Material

Two large specimens of *Aplysia dactylomela* (Rang, 1828) (order Anaspidea, superfamily Aplysioidea, family Aplysiidae) were collected on April 28, 2001 in Bahia Salinas, Cabo Rojo, Puerto Rico by hand at 2 feet deep. Each individual was found grazing upon the red alga *Laurencia obtusa*. A voucher specimen has been deposited at the Department of Chemistry, University of Puerto Rico, Río Piedras, Puerto Rico (deposit number ADPR01-1).

Extraction and Isolation of Aplysqualenol A (**1**) and B (**2**) and the Known Compound Elatol (**11**)

The organisms were freeze-dried and the dry animal (54 g dry weight) was cut into small pieces and extracted with 1:1 CHCl₃–MeOH (6 × 1L). The combined organic extracts were concentrated and the residue obtained (39 g) was suspended in water (500 mL) and partitioned between *n*-hexane, CHCl₃, EtOAc and *n*-butanol. The *n*-hexane extract (11 g) was chromatographed over silica gel (30 g) using *n*-hexane→CHCl₃→EtOAc→MeOH in increasing polarity as eluant. The 100% CHCl₃ eluate (980 mg) was chromatographed over silica gel (30 g) with 15% CHCl₃ in *n*-hexane yielding 16 fractions. Subfraction 8 (55 mg) was subsequently chromatographed over silica gel (3 g) with 25% CHCl₃ in *n*-hexane to afford known elatol (**11**) (37 mg; 0.1% yield).^[25] The fraction (1.2 g) that eluted with CHCl₃–EtOAc (8:2) was chromatographed over silica gel (31 g) with 10% *n*-hexane in CHCl₃ to afford 9 fractions. Subfraction 8 (101 mg) was further purified over silica gel (5 g dry-packed) eluting with 2% EtOAc in CHCl₃. The least polar fraction (66 mg) was purified further by CC over silica gel (5 g) using 10% EtOAc in *n*-hexane to yield 8 fractions. Subfraction 7 (44 mg) was subsequently purified by CC over silica gel (3 g) with 10% EtOAc in *n*-hexane followed by NP-HPLC [Ultrasphere-Cyano 250 × 10 mm, flow rate: 2.0

mL/min, UV detection set at $\lambda = 254$ nm] using a combination of 95:5 *n*-hexane–2-propanol to afford pure aplysqualenol A (**1**) (24 mg). The portion (1.5 g) eluting with EtOAc–MeOH (8:2) was filtered and purified over a Bio-Beads SX-3 column (toluene) to yield 3 fractions. The second fraction was chromatographed over silica gel (4 g) using 0.5% MeOH in CHCl_3 to afford pure aplysqualenol B (**2**) (7.1 mg).

Aplysqualenol A (**1**)

colorless oil; $[\alpha]_{\text{D}}^{20} +48.1$ (*c* 1.6, CHCl_3); IR (film) ν_{max} 3418, 3072, 2984, 2954, 2875, 1738, 1648, 1456, 1379, 1323, 1127, 1098, 905, 761 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3 , 500MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 125MHz), see Table 1; EIMS m/z $[\text{M} - \text{HBr}]^+$ 536 (12), 505 (4), 503 (4), 423 (2), 405 (15), 403 (14), 387 (12), 385 (10), 361 (7), 359 (6), 323 (12), 305 (8), 291 (3), 289 (4), 279 (8), 247 (10), 245 (9), 225 (15), 214 (17), 207 (44), 205 (41), 182 (24), 153 (100), 125 (59), 109 (64), 85 (36); HRFABMS (3-NBA) m/z $[\text{M} + \text{Na}]^+$ calcd for $\text{C}_{31}\text{H}_{53}^{79}\text{BrO}_7\text{Na}$ 639.2872, found 639.2858 (Δ 1.4 mDa).

Aplysqualenol B (**2**)

colorless oil, $[\alpha]_{\text{D}}^{20} +27.1$ (*c* 1.4, CHCl_3); IR (film) ν_{max} 3408, 2978, 2952, 2862, 1726, 1453, 1381, 1130, 1095, 1059, 1032, 911 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3 , 500MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 125MHz), see Table 2; HRFABMS (3-NBA) m/z $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{31}\text{H}_{54}^{79}\text{BrO}_8$ 633.3002, found 633.3003 (Δ -0.1 mDa).

Elatol (**11**)

The $[\alpha]_{\text{D}}^{20}$, UV (MeOH), $^1\text{H-}$ and $^{13}\text{C-NMR}$, and HREIMS data were identical in all respects to those previously reported.^[25]

Acetylation of Aplysqualenol A (**1**)

Compound **1** (1.7 mg, 0.003 mmol) was dissolved in a mixture of dry pyridine (500 μL) and acetic anhydride (500 μL) and heated to 55 $^{\circ}\text{C}$ for 5h. The cooled reaction mixture was concentrated in vacuo and the oily residue obtained was purified by CC over silica gel (1 g) using 1% MeOH in CHCl_3 to yield pure aplysqualenol A acetate (**3**) (1.7 mg, quantitative yield). Compound **3**: $[\alpha]_{\text{D}}^{20} +56.0$ (*c* 1.0, CHCl_3); IR (film) ν_{max} 3549, 3071, 2983, 2951, 2858, 2819, 1740, 1454, 1380, 1242, 1125, 1099, 1029, 921 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3 , 500MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 125MHz), see Table 4 as Supporting Information; HREIMS m/z $[\text{M} - \text{HBr}]^+$ calcd for $\text{C}_{33}\text{H}_{54}\text{O}_8$ 578.3819, found 578.3826 (Δ -0.7 mDa), 578 (18), 405 (7), 403 (7), 387 (5), 385 (4), 361 (5), 359 (5), 323 (8), 256 (11), 224 (26), 207 (42), 205 (42), 153 (59), 125 (72), 120 (94), 118 (100), 117 (57).

Acetylation of Aplysqualenol B (**2**)

Aplysqualenol B (3.0 mg; 0.005 mmol) was dissolved in a mixture of dry pyridine (500 μL) and acetic anhydride (500 μL) and then stirred at 25 $^{\circ}\text{C}$ overnight. The reaction mixture was concentrated in vacuo and the oil obtained was purified by CC over silica gel (1.0 g) with 0.5% MeOH in CHCl_3 to afford aplysqualenol B diacetate (**4**) (3.0 mg; 88% yield). Compound **4**: colorless oil; $[\alpha]_{\text{D}}^{20} +44.0$ (*c* 0.5, CHCl_3); IR (film) ν_{max} 3541, 3467, 3087, 2987, 2951, 2869, 2859, 2821, 1740, 1655, 1459, 1440, 1380, 1242, 1121, 1097, 1054, 1029, 922 cm^{-1} ; $^1\text{H-NMR}$ (CDCl_3 , 300MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 75MHz), see Table 5

as Supporting Information; HRESIMS m/z $[M + H]^+$ calcd for $C_{35}H_{58}^{79}BrO_{10}$ 717.3213, found 717.3216 (Δ -0.3 mDa).

Computational Method

Initially, a Monte Carlo (MC) multiple-minimum search with the MMFFs force field was conducted to make a full exploration of the conformational space for all the four possible stereoisomers involving the C15 and C22 stereocenters of aplysqualenol A (**1**). The molecular mechanics MC conformational search employs an algorithm as implemented in Spartan 04 software package. All the stereoisomers thus obtained were further subjected to *ab initio* quantum chemical geometry optimizations at the HF/6-31G(2d,p) level of theory. The *ab initio* calculations were carried out on a Linux AMD64 1.8 GHz using the Gaussian 03 program package.^[17] The Gaussian 03 uses an expansion of molecular orbitals in atomic-centered Gaussian basis sets such as the double-zeta plus polarization 6-31G(2d,p). NMR shielding tensors were computed with the gauge-independent atomic orbital (GIAO) method at the hybrid density-functional mPW1PW91/6-31G(2d,p) level of theory.

Biological Screening Assays

For a general description of the approach used by the NIAID's Antimicrobial Acquisition and Coordinating Facility (AACF) for determining antiviral activity and toxicity for herpes viruses, visit: <http://niaid-aacf.org/protocols/Herpes.htm>. Anticancer activity screening by the Developmental Therapeutics Program (DTP) of the National Cancer Institute is conducted following this general protocol: most of the compounds screened have no antiproliferative activity (up to 85%). In order to avoid screening inactive compounds across all the cell lines, a prescreen is done using 3 highly sensitive cell lines (breast MCF-7, lung NCI-H640, CNS SF-268). Antiproliferative activity must be seen in these cell lines in order to continue to the 60 cell line panel. The 60 different human tumor lines are incubated with 5 different doses of compound and a sulforhodamine blue (SRB) assay is performed after 48 hours to determine cytotoxicity. From the 5 point curve, the following concentrations are extrapolated: GI₅₀ (inhibits growth by 50%), TGI (totally inhibits growth), LC₅₀ (kills 50% of cells). For the specific screening methods from the DTP website, visit: <http://www.dtp.nci.nih.gov/branches/btb/ivclsp.html>. Compounds shown to have anticancer activity in cell lines within the NCI 60 panel may then move on to animal trials and if successful, may eventually move on to be tested in clinical trials. Additional experimental details for our primary *in vitro* antimicrobial assays against *Mycobacterium tuberculosis* and *Plasmodium falciparum* have been previously described.^[26,27]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Mark W. Miller (UPR Institute of Neurobiology) and Dr. Ileana I. Rodríguez (UPR-RP) for providing logistic support during the collection of *A. dactyloides*. The assistance of Juan A. Santana during molecular modeling studies is gratefully acknowledged. The National Cancer Institute (NCI), the National Institute of Allergy and Infectious Diseases (NIAID), the Tuberculosis/Antimicrobial Acquisition & Coordinating Facility (TAACF), and the Institute for Tropical Medicine and Health Sciences (Panama) provided *in vitro* cytotoxicity, antiviral, antituberculosis, and antimalarial activity data, respectively. High-resolution EI, ESI, and FAB mass spectral determinations were provided by the Mass Spectroscopy Laboratory of the University of Illinois at Urbana-Champaign. B. V. thanks the UPR-RISE Fellowship Program and the Puerto Rico Cancer Center for financial support. This work was partially supported by the NIH-SCORE Program (Grant S06GM08102) of the University of Puerto Rico.

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Figure 1.
Diagnostic fragment ions (m/z) of aplysqualenol A (**1**) detected in the EI mass spectrum.

Figure 2.
Plausible conformations of rings A–B–C–D in **1** showing selected NOESY correlations.

Figure 3.

Minimum energy configurations of the four stereoisomer models **1a–1d** considered for quantum mechanical calculations of ^{13}C NMR isotropic shifts. The hydrogen atoms have been omitted for clarity. Oxygen atoms are indicated in red. Hydrogen bonds are represented as dashed lines between the donor hydrogen and the acceptor atom. Bonds with less than ideal geometry are displayed with a blue tint. The intensity of the colour increases as the bond becomes less ideal.

Figure 4.

Deviations from the average of the carbon chemical shifts of distereomers **1a-1d**. The *x* and *y* axes represent position number and $\Delta\delta$ in parts per million, respectively. To discriminate between stereoisomers **1a** and **1b** (the two most likely structures for aplysqualenol A on the basis of the calculation results shown in Table 3), a careful analysis was done on individually calculated ^{13}C chemical shifts for carbons C22 and C30, which were expected to experience larger variations upon inversion of configuration at C22. As shown here, very large differences in the $\Delta\delta^{13}\text{C}$ values of **1a** and **1b** were observed for C22 and C30 (+11.9 vs +4.0 and -7.8 vs -3.0, respectively), suggesting again the exclusion of stereoisomer **1a**.

Scheme 1. Proposed biogenetic pathway for aplysqualenol A (**1**).

Table 1

¹H NMR (500 MHz), ¹³C NMR (125 MHz), ¹H-¹H COSY, HMBC, and NOESY spectral data for aplysqualenol A (**1**) in CDCl₃.^[a]

Atom	δ _H , mult, integr (J in Hz)	δ _C , mult ^[b]	¹ H- ¹ H COSY	HMBC ^[c]	NOESY
1	1.40, s	23.6 (CH ₃)		H3, H25	H4α, H25, H26
2		75.0 (C)		H1, H3, H4αβ, H25	
3	3.88, dd, 1H (4.0, 12.3)	58.9 (CH)	H4αβ	H1, H4αβ, H5αβ, H25	H4α, H25
4α	2.10, dq, 1H (3.9, 4.0, 4.5, 13.5)	28.3 (CH ₂)	H3, H4β, H5αβ		H3, H4β
4β	2.22, dq, 1H (3.5, 12.3, 13.1, 13.5)		H3, H4α, H5αβ		H1, H4α, H26
5α	1.82, m, 1H	37.1 (CH ₂)	H4αβ, H5β	H26	H5β, H7
5β	1.53, m, 1H		H4αβ, H5α		H5α, H26
6		74.3 (C)		H4αβ, H7, H8α, H26	
7	3.09, dd, 1H (2.2, 10.9)	86.9 (CH)	H8αβ	H9β, H26	H5α, H8α, H9α, H11
8α	1.53, m, 1H	22.9 (CH ₂)	H7, H8β, H9αβ	H9α	H7, H8β
8β	1.78, m, 1H		H7, H8α, H9αβ		H8α, H26, H27
9α	1.53, m, 1H	37.5 (CH ₂)	H8αβ, H9β	H27	H7, H9β, H11
9β	1.73, m, 1H		H8αβ, H9α		H9α, H27
10		73.0 (C)		H8αβ, H9αβ, H27	
11	3.01, dd, 1H (3.8, 11.4)	80.8 (CH)	12αβ	H9β, H27	H7, H9α, H13α
12α	1.66, m, 1H	24.3 (CH ₂)	H11, H13αβ	H14	
12β	1.53, m, 1H		H11, H13αβ		H14, H27
13α	1.50, m, 1H	26.0 (CH ₂)	H12αβ, H14		H11, H28
13β	1.65, m, 1H		H12αβ, H14		
14	3.54, dd, 1H (2.5, 11.6)	72.5 (CH)	H13αβ	H13αβ, H28	H12β, H27, H29
15		74.9 (C)		H13αβ, H14, 15-OH, H16, H17αβ, H28	
16	3.85, dd, 1H (4.2, 9.1)	79.1 (CH)	H17αβ	H17α, H18, H28	H17α, H18, H28
17α	2.34, ddd, 1H (6.0, 8.9, 14.3)	35.3 (CH ₂)	H16, H17β, H18		H16, H17β, H18
17β	1.96, dd, 1H (3.0, 14.3)		H16, H17α, H18		H17α, 18-OH, H28
18	3.81, dd, 1H (2.0, 5.9)	75.8 (CH)	H17αβ, 18-OH	H17β, 18-OH, H20β, H29	H16, H17α, H20α, H30
19		85.7 (C)		H17β, H20αβ, H29	
20α	1.40, m, 1H	34.1 (CH ₂)	H20β, H21αβ	H21αβ, H22, H29	H18, H20β

Atom	δ_H , mult, integr (J in Hz)	δ_C , mult ^[b]	1H - 1H COSY	HMB ^[c]	NOESY
20 β	1.30, dd, 1H (4.8, 12.3)		H20 α , H21 $\alpha\beta$		H20 α
21 $\alpha\beta$	1.64, m, 1H; 1.56, m, 1H	28.2 (CH ₂)	H20 $\alpha\beta$, H22		H29
22	3.44, dd, 1H (6.5, 6.8)	86.1 (CH)	H21 $\alpha\beta$	H20 $\alpha\beta$, H21 $\alpha\beta$, H24 $\alpha\beta$, H30, OCH ₃	H24 α , H30, OCH ₃
23		144.4 (C)		H30	
24 α	4.87, br s, 1H	113.8 (CH ₂)	H24 β , H30	H22, H30	H22, H24 β
24 β	4.93, br s, 1H		H24 α , H30		H24 α , H30
25	1.27, s, 3H	31.0 (CH ₃)		H1, H3	H1, H3,
26	1.20, s, 3H	20.1 (CH ₃)			H1, H4 β , H5 β , H8 β
27	1.15, s, 3H	14.8 (CH ₃)		H11	H8 β , H9 β , H12 β , H14
28	1.17, s, 3H	18.1 (CH ₃)			H13 α , 15-OH, H16, H17 β
29	1.22, s, 3H	19.4 (CH ₃)			H14, 18-OH, H21 β
30	1.63, s, 3H	16.3 (CH ₃)	H24 $\alpha\beta$	H22, H24 $\alpha\beta$	H18, H22, H24 β , OCH ₃
OCH ₃	3.19, s, 3H	56.0 (CH ₃)		H22	H22, H30
15-OH	2.97, br s, 1H				H28
18-OH	3.47, br d, 1H (7.3)		H18		H17 β , H29

^[a] Spectra were recorded at 25 °C. Chemical shifts are in ppm relative to TMS.

^[b] ¹³C NMR multiplicities were obtained from APT experiments.

^[c] Protons correlated to carbon resonances in the ¹³C column. Parameters were optimized for ^{2,3}J_{CH} = 6 and 8 Hz.

¹H NMR (500 MHz), ¹³C NMR (125 MHz), ¹H-¹H COSY, HMBC, and NOESY spectral data for aplysqualenol B (2) in CDCl₃.^[a]

Table 2

Atom	δ_{H} , mult, integr (J in Hz)	δ_{C} , mult ^[b]	¹ H- ¹ H COSY	HMBC ^[c]	NOESY
1	1.39, s	23.6 (CH ₃)		H25	H4 β , H25, H26
2		75.0 (C)		H1, H3, H25	
3	3.88, dd, 1H (4.0, 12.3)	58.9 (CH)	H4 $\alpha\beta$	H1, H4 β , H5 β , H25	H4 α , H5 α , H25
4 α	2.09, dq, 1H (3.8, 3.9, 4.1, 13.5)	28.2 (CH ₂)	H3, H4 β , H5 $\alpha\beta$		H3, H4 β , H5 α
4 β	2.24, dq, 1H (3.7, 12.8, 13.1, 13.5)		H3, H4 α , H5 $\alpha\beta$		H1, H4 α , H26
5 α	1.81, m, 1H	37.1 (CH ₂)	H4 $\alpha\beta$, H5 β	H26	H4 α , H5 β , H7, H26
5 β	1.52, m, 1H		H4 $\alpha\beta$, H5 α		H5 α , H26
6		74.3 (C)		H26	
7	3.10, dd, 1H (2.2, 10.9)	86.9 (CH)	H8 $\alpha\beta$	H8 α , H9 α , H26	H5 α , H8 α , H9 α , H11
8 α	1.52, m, 1H	23.0 (CH ₂)	H7, H8 β , H9 $\alpha\beta$		H7, H8 β
8 β	1.78, m, 1H		H7, H8 α , H9 $\alpha\beta$		H8 α , H26, H27
9 α	1.50, m, 1H	37.5 (CH ₂)	H8 $\alpha\beta$, H9 β	H27	H7, H9 β , H11
9 β	1.72, m, 1H		H8 $\alpha\beta$, H9 α		H9 α , H27
10		73.0 (C)		H8 α , H9 β , H27	
11	3.01, dd, 1H (3.9, 11.4)	80.7 (CH)	12 $\alpha\beta$	H9 β , H13 β , H27	H7, H9 α , H13 α
12 α	1.66, m, 1H	24.3 (CH ₂)	H11, H12 β , H13 $\alpha\beta$		H11
12 β	1.53, m, 1H		H11, H12 α , H13 $\alpha\beta$		H27
13 α	1.48, m, 1H	26.0 (CH ₂)	H12 $\alpha\beta$, H13 β , H14		H11, H28
13 β	1.61, m, 1H		H12 $\alpha\beta$, H13 α , H14		
14	3.54, dd, 1H (2.5, 11.7)	72.5 (CH)	H13 $\alpha\beta$	H13 α , H28	H27
15		75.0 (C)		H14, H17 α , H28	
16	3.84, dd, 1H (4.2, 9.1)	79.2 (CH)	H17 $\alpha\beta$	H17 α , H28	H17 α , H22, H28
17 α	2.33, ddd, 1H (5.9, 9.1, 14.4)	35.2 (CH ₂) ^[d]	H16, H17 β , H18		H16, H17 β , H18
17 β	1.97, ddd, 1H (1.3, 4.0, 14.4)		H16, H17 α , H18		H17 α , H28
18	3.80, br m, 1H	75.9 (CH) ^[d]	H17 $\alpha\beta$, 18-OH	H17 β , H29	H17 α , H20 α
19		85.7 (C)		H17 β , H20 α , H29	
20 α	1.42, m, 1H	34.2 (CH ₂)	H20 β , H21 $\alpha\beta$	H22, H29	H18, H20 β

Atom	δ_H , mult, intrgt (<i>J</i> in Hz)	δ_C , mult ^[b]	¹ H- ¹ H COSY	HMBC ^[c]	NOESY
20 β	1.31, m, 1H		H20 α , H21 $\alpha\beta$		H20 α
21 $\alpha\beta$	1.65, m, 2H	28.6 (CH ₂)	H20 $\alpha\beta$, H22	H22	
22	3.62, dd, 1H (6.5, 6.8)	85.0 (CH)	H21 $\alpha\beta$	H21 $\alpha\beta$, H24 $\alpha\beta$, OCH ₃	H16, H24 α , H30 β , OCH ₃
23		146.9 (C)		H24 $\alpha\beta$, H30 $\alpha\beta$	
24 α	5.06, br s, 1H	114.0 (CH ₂)	H24 β	H22, H30 $\alpha\beta$	H22, H24 β , OCH ₃
24 β	5.24, br s, 1H		H24 α		H24 α , H30 α
25	1.27, s, 3H	31.0 (CH ₃)		H1	H1, H3
26	1.20, s, 3H	20.1 (CH ₃)		H5 α , H8 β	H4 β , H5 β , H8 β
27	1.15, s, 3H	14.8 (CH ₃)		H9 β , H11	H8 β , H12 β , H14
28	1.17, s, 3H	18.1 (CH ₃)			H13 α , H16, H17 β
29	1.22, s, 3H	19.4 (CH ₃)			
30 α	4.09, br d, 1H (14.3)	62.9 (CH ₂)	H30 β	H22, H24 $\alpha\beta$	H24 β , H30 β
30 β	4.21, br d, 1H (13.5)		H30 α		H22, H30 α
OCH ₃	3.26, s, 3H	56.4 (CH ₃)		H22	H22, H24 α
15-OH	3.11, br s, 1H				
18-OH	3.55, br s, 1H		H18		

^[a] Spectra were recorded at 25 °C. Chemical shifts are in ppm relative to TMS.

^[b] ¹³C NMR multiplicities were obtained from APT experiments.

^[c] Protons correlated to carbon resonances in the ¹³C column. Parameters were optimized for ^{2,3}*J*_{CH} = 6 and 8 Hz.

^[d] Signal recorded as a broad low-intensity resonance line.

Table 3Calculated parameters for the four diastereomers **1a–1d**.

Properties	1a	1b	1c	1d
MAE ^[a]	3.1	2.9	3.9	3.5
Energy ^[b]	−1741.8144	−1741.8191	−1741.8057	−1741.8125
δE ^[c]	2.98	0	8.42	4.17
R ² ^[d]	0.9909	0.9982	0.9897	0.9953

^[a] Mean absolute error in ppm found for calculated ¹³C NMR chemical shifts versus ¹³C experimental values: $MAE = \Sigma [|(\delta_{exp} - \delta_{calcd})|] / n$.

^[b] Data in atomic units.

^[c] Data in kcal/mol.

^[d] Correlation coefficient obtained by a linear fit of the calculated, δ_{calcd} , versus experimental, δ_{exp} , ¹³C NMR chemical shifts.