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# Thyrsenols A and B, Two Unusual Polyether Squalene Derivatives

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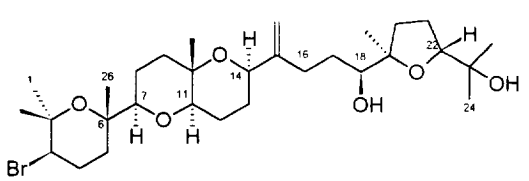
***Abstract***: Two new polyether squalene derivatives, thyrsenol A **2** and thyrsenol B **3**, have been isolated from the red alga *Laurencia viridis*. Their structures, which possess an unusual enol-ether moiety, were determined through the interpretation of 2D-NMR spectral data. The relative stereochemistry is proposed on the basis of ROESY and NOEDIFF data. Their cytotoxic activities were evaluated.

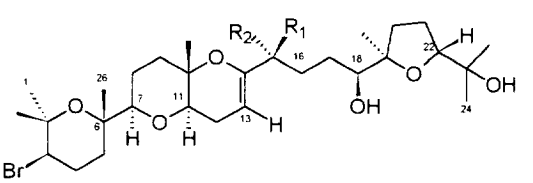

The chemical examination of the genus *Laurencia* has proved it to be a rich source of natural products yielding interesting bioactive metabolites such as sesquiterpenes, diterpenes and polyether squalene derivatives[1-5].

In a previous work with ether extracts of *Laurencia pinnatifida* (Gmal. Lamour), we have isolated a variety of compounds including a series of polyethers derived from dehydrothyrsiferol **1**[6]. Most of these compounds have shown a potent antitumoral activity and we wish to report here on the isolation, structural elucidation and the antitumoral activity of two new polyether derivatives isolated from the most polar fractions of the red alga *Laurencia viridis*, which possess an unusual enol-ether moiety.

The fresh material was collected near Callao Salvaje (Tenerife), dried and extracted with chloroform:methanol (1:1). The resultant extract was chromatographed through a series of silica gel medium pressure and Sephadex LH-20 columns, and the final purification was achieved by HPLC (μ-Porasil) column yielding pure thyrsenol A **2** (6.2 mg) and thyrsenol B **3** (2.3 mg).



**1**



**2** $R_1$= OH $R_2$= $CH_2$-OH

**3** $R_1$= $CH_2$-OH $R_2$=OH

| | Thyrsenol A (2) | | | | Thyrsenol B (3) | | | |
|---|---|---|---|---|---|---|---|---|
| C | $\delta^{13}C$ | $\delta^{1}H$ | | Coupling constants (Hz) | $\delta^{13}C$ | $\delta^{1}H$ | | Coupling constants (Hz) |
| 1 | 31.39 | 1.27 | s | | 31.01 | 1.28 | s | |
| 2 | 75.38 | | | | 74.98 | | | |
| 3 | 59.35 | 3.89 | dd | 4; 12.4 | 58.94 | 3.90 | dd | 4.4; 12.3 |
| 4 | 28.59 | 2.12<br>2.24 | | | 28.21 | 2.11<br>2.24 | | |
| 5 | 37.50 | 1.50<br>1.81 | | | 37.15 | 1.50<br>1.83 | | |
| 6 | 74.70 | | | | 74.33 | | | |
| 7 | 86.70 | 3.08 | dd | 2.3; 11.2 | 86.36 | 3.09 | dd | 2.3; 11.3 |
| 8 | 23.25 | 1.48<br>1.83 | | | 22.84 | 1.47<br>1.80 | | |
| 9 | 36.86 | 1.59<br>1.87 | | | 36.45 | 1.57<br>1.84 | | |
| 10 | 74.02 | | | | 73.66 | | | |
| 11 | 77.31 | 3.31 | dd | 6; 10.6 | 77.20 | 3.29 | dd | 5.9; 10.6 |
| 12 | 24.63 | α 1.82<br>β 2.13 | ddd<br>ddd | 5.5; 6; 14.8<br>2;10.6; 14.8 | 24.17 | α 1.85<br>β 2.09 | ddd<br>ddd | 5.4;5.9;14.8<br>2;10.6;14.8 |
| 13 | 94.03 | 5.02 | dd | 2; 5.5 | 91.60 | 4.85 | dd | 2; 5.4 |
| 14 | 152.11 | | | | 152.44 | | | |
| 15 | 85.52 | | | | 85.83 | | | |
| 16 | 32.04 | α 1.81<br>β 1.93 | | | 31.75 | α 2.01<br>β 2.12 | | |
| 17 | 26.85 | β 1.71<br>α 1.84 | | | 27.10 | 1.88<br>1.89 | | |
| 18 | 86.59 | 3.92 | dd | 7; 7 | 85.67 | 4.03 | dd | 7; 7 |
| 19 | 84.20 | | | | 84.94 | | | |
| 20 | 34.93 | 1.66<br>1.98 | | | 34.61 | 1.68<br>1.84 | | |
| 21 | 26.58 | 1.85<br>1.95 | | | 25.88 | 1.84<br>1.94 | | |
| 22 | 87.56 | 3.77 | dd | 6; 9.5 | 87.83 | 3.79 | dd | 4.9; 10.3 |
| 23 | 70.84 | | | | 70.29 | | | |
| 24 | 24.50 | 1.12 | s | | 24.22 | 1.12 | s | |
| 25 | 24.12 | 1.40 | s | | 23.65 | 1.41 | s | |
| 26 | 20.42 | 1.21 | s | | 19.99 | 1.22 | s | |
| 27 | 15.50 | 1.07 | s | | 15.29 | 1.08 | s | |
| 28 | 66.97 | β 3.49<br>α 3.63 | d<br>d | 11.1<br>11.1 | 67.92 | 3.56 | bs | |
| 29 | 24.95 | 1.23 | s | | 25.15 | 1.30 | s | |
| 30 | 28.10 | 1.21 | s | | 27.75 | 1.24 | s | |

**Table 1.**- NMR Data for Thyrsenols A and B in $CDCl_3$.

Thyrsenol A **2**[8] was isolated as an amorphous solid $[\alpha]^{25}_D$ = +12.9 (*c* 0.69, $CHCl_3$). The molecular formula was determined as $C_{30}H_{51}O_8Br$ by mass measurement. It showed, in the FAB mass spectrum, NBA being the most suitable matrix, an ion at m/z 625/623 due to a fragment formed by the loss of water ($M^+$+ Na - $H_2O$). The $^{13}C$-NMR spectrum confirms the molecular formula and shows the presence of seven methyl, ten methylene and six methine groups, together with seven quaternary carbon centres, which are α to oxygens. The $^{1}H$-NMR spectrum exhibits in the lowfield region five methine signals at δ 3.92, 3.89, 3.77, 3.31 and 3.08 identified as H-18, H-3, H-22, H-11 and H-7, respectively, by comparison with the $^{1}H$-NMR spectrum of

compound **1**. Together with these were observed a diastereotopic methylene signal centred at δ 3.63 and 3.49, an olefinic proton centred at δ 5.02 and an allylic methylene group at δ 2.13 and 1.82, these last proton signals being the most remarkable differences between the proton NMR spectra of compounds **1** and **2**, and which through the COSY experiment were located in the zone of carbons C-11/C-28.

Thus, the COSY analysis in this region may be conveniently started from the methine H-11 (δ 3.31), which is coupled to the methylene H-12 (δ 2.13 and 1.82) and these protons proved to be connected with the H-13 (δ 5.02), the system of coupled protons in this region terminating with the presence of two quaternary centres. This was confirmed by the combined 2D-HMQC-TOCSY[7] experiment which showed the correlation between the proton signal H-13 (δ 5.02) and the carbon signals at δ 24.63 (C-12) and 77.31 (C-11) and, furthermore, permits the chemical shifts of all methyl, methylene and methine carbons to be unequivocally assigned. The quaternary carbon signal C-14 was centred at δ 152.11 through its HMBC correlations with H-12 and H-13, while those observed between H-11 and H-12 with the signal at δ 74.02 identified this last carbon signal as C-10. Moreover, C-14 was correlated with H-28, which appeared in the $^1$H-NMR spectrum as an isolated AB system centred at δ 3.63 and 3.49. These protons were also correlated in the HMBC experiment with the quaternary carbon centred at δ 85.82 (C-15), which in turn was correlated with the olefinic proton H-13 and the methylene protons H-16. These correlations established the presence of an unusual enol-ether system at C-13/C-14 together with a diol system at C-15/C-28 instead of the double bond observed in compound **1**. The above spectral data together with all other correlations observed in the 2D-NMR experiments supported the planar structure for this compound as **2**.

Thyrsenol B **3**[9] $[\alpha]^{25}_D$ = -1.1 (*c* 0.26, $CHCl_3$) proved to be an isomer of compound **2** on the basis of its mass spectrum, and its spectroscopical data showed that the chemical structure was closely related to that of thyrsenol A **2**. Once the COSY, HMQC, HMQC-TOCSY and HMBC experiments of compounds **3** were accomplished and compared with those from compound **2**, the most remarkable differences were observed in the methine proton signals H-13, H-18 and the methylene H-28 with slightly displaced chemical shift values. Moreover, the methylene protons H-28 appeared as an isolated AB system in compound **2** while in compound **3** they were a broad singlet (Table **1**). Taking these data into account, we arrived at the conclusion that these compounds must be differentiated in their stereochemistry around C-15.

The relative stereochemistry of carbons C-3, C-6, C-7, C-10, C-11, C-18, C-19, and C-22 in compounds **2** and **3** were established as identical on the basis of the observed ROE data in the ROESY experiments in $CDCl_3$, and identical with that observed for dehydrothyrsiferol, **1**. The relative stereochemistry of carbon C-15 in compounds **2** and **3** was established as follows. The fact that in compound **2** the H-28 protons were diastereotopic, inferred that the primary alcohol was fixed with the enol-ether oxygen in a H-bond, which was supported by the ROE connectivities observed between H-13 with H-16(β); between H-28(β) with H-17(β) and between H-28(α) with H-16(α). Thus, the relative stereochemistry at C-15 in this compound was established as R*. The same strategy was adapted to thyrsenol B, **3**, the 15 S* stereochemistry being in agreement with the cross peaks observed in the ROESY experiment between H-13 with H-18 and H-16(α).

Biological assays of the pure isolates were undertaken, making use of *in vitro* bioassays and focusing on cytotoxic activities[10]. Cytotoxic effects were evaluated with a battery of cultured tumor cells lines:P-388 (ATCC CCL-46), suspension culture of a lymphoid neoplasm from a DBA/2 mouse; A-549 (ATCC CCL-185), monolayer culture of a human lung carcinoma; HT-29 (ATCC HTB-38), monolayer culture of a human colon

carcinoma; MEL-28 (ATCC HTB-72), monolayer culture of a human melanoma. Cells were maintained, in logarithmic growth in EMEM/neaa, supplemented with 5% Fetal Calf Serum (FCS), $10^{-2}$M sodium bicarbonate and 0.1 g/l penicillin G + 0.1 g/l streptomycin sulfate. This screening procedure to establish the cytotoxic activity of these compounds showed that both compounds possess a potent activity against P-388 cells, although for compound **3** ($IC_{50}$= 0.01 μg/ml, 0.016.μM) it was significantly higher than for compound **2** ($IC_{50}$= 0.25 μg/ml, 0.40 μM). Moreover, dehydrothyrsiferol **1** showed the same activity as compound **3**, Table 2.

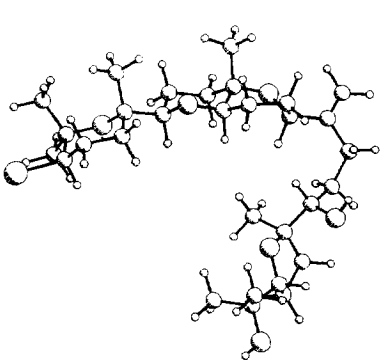
| Compounds | $IC_{50}$ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | P388 | | A549 | | HT29 | | MEL28 | |
| | μg/ml | μM | μg/ml | μM | μg/ml | μM | μg/ml | μM |
| Dehydrothyrsiferol **1** | 0.01 | 0.017 | 2.5 | 4.26 | 2.5 | 4.26 | 5 | 8.52 |
| Thyrsenol A **2** | 0.25 | 0.40 | > 1.0 | >1.62 | > 1.0 | >1.62 | > 1.0 | >1.62 |
| Thyrsenol B **3** | 0.01 | 0.016 | > 1.0 | >1.62 | > 1.0 | >1.62 | > 1.0 | >1.62 |

**Table 2**.- $IC_{50}$ of biological assays of the pure thyrsenols

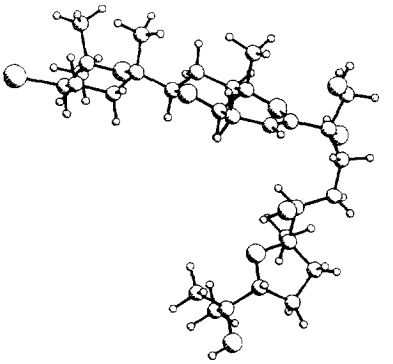
This difference in activity might be related to the different conformations observed in the side chain of these compounds. For that reason, we decided to establish the stable conformations of compounds **1-3** by molecular mechanics calculations. A multiconformer search was carried out for dehydrothyrsiferol **1**, thyrsenol A **2** and thyrsenol B **3**, using the Monte Carlo program in Macromodel. Approximate distance constraints (2-4 Å), derived from the observed intense NOESY correlations, were used in this search. Structures generated with this method are shown in Figure **1**.

The results of the conformational analysis confirmed that the presence of the H-bond through the C-28 hydroxyl group in thyrsenol A **2** introduces in the side chain a remarkably different conformation compared with that observed for thyrsenol B **3**, which, in turn, was similar to that obtained for dehydrothyrsiferol **1**, indicating that the arrangement and direction of the flexible chain around the carbon C-14 to C-19 were identical. On the basis of these findings and taking into account that both compounds showed the same degree of cytotoxic activity, we concluded that the arrangement and direction of this chain may be among the fundamental factors to evalute the activity of this type of metabolites.

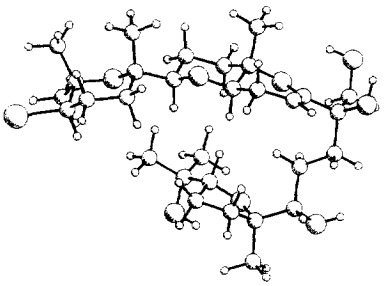
As far as we know, all the examples isolated with a common squalene polyether skeleton belong to the thyrsiferol or venustatriol series, the differences between them being established in the configuration of the C-18 and C-19 atoms, which are opposite. This promted the authors to propose a common two-step biogenesis with a concerted cyclization of three epoxides C6/C7, C10/C11 and C14/C15 as a first step and the formation of the furan ring as second step, which established the differences between the two series. However, the isolation of compounds **2** and **3** reinforces the hypothesis we have put forward after the isolation of the recently published 10-epidehydrothyrsiferol[11], that the biosynthesis of these squalene-polyether derivatives through the cyclization of the squalene tetraepoxide precursor may not be concerted.



Dehydrothyrsiferol **1**



Thyrsenol B **3**



Thyrsenol A **2**

**Figure 1**. Stereo view of proposed 3D structure for dehydrothyrsiferol and thyrenols

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## EXPERIMENTAL

***General methods***. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. IR spectra were measured on a Bruker IFS55 spectrometer. The NMR spectra were obtained with a Bruker AMX-400 instrument. Chemical shifts are reported relative to TMS and coupling constants are given in Hz. HRMS were performed on a Kratos MS-80RFA spectrometer. HPLC was carried out with a LKB 2248 system equipped with a differential diffractometer detector. Silica gel CC and TLC were performed on Silica gel Merck 60 G. TLC plates were visualised by spraying with $H_2SO_4/H_2O$/AcOH (1:4:20) and heating. All solvents were purified by standard techniques.

***Plant material***. Seeds of *Laurencia viridis* were collected in April 1994 in the intertidal zone at Callao Salvaje (Tenerife, Canary Islands). Dried material of sterile plants, sporophytes and gametophytes is deposited at TFC Phyc. (Herbario de la Universidad de La Laguna, Departamento de Biología Vegetal, Botánica, Tenerife).

***Extraction and isolation*** The alga was air-dried and ground in a Wiley mill to 1 mm particle size. The dried alga (4 Kg) was extracted with $CHCl_3$:MeOH (1:1) at room temperature. The solvent was evaporated *in vacuo* and the crude extract was chromatographed on a silica gel column using *n*-hexane-EtOAc mixtures of increasing polarity. The *n*-hexane:EtOAc (3:2) eluate, after solvent evaporation, was successively chromatographed with the following columns and solvents: a Sephadex LH-20, *n*-hexane:$CHCl_3$:MeOH (2:1:1); a medium pressure silica gel chromatography, *n*-hexane:EtOAc (7:3). For selected fractions, its final purification was carried out by HPLC employing μ-Porasil prepacked column and using *n*-hexane:EtOAc (7:3) and *n*-hexane:EtOAc (3:2) as eluent, affording pure **2** (6.2 mg) and **3** (2.3 mg), respectively.

*Cytotoxicity assay:* A simple screening procedure has been carried out to determine and compare the antitumor activity of these compounds, using an adapted version of the method described by Bergeron et al. (1984)[10]. P-388 cell were seeded into 16 mm wells at $1x10^4$ cells per well in 1 mL aliquots of MEN 5FCS containing the indicated concentration of drug. A separate set of cultures without drug was seeded as control growth to ensure that cells remained in exponential phase growth. All determinations were carried out in duplicate. After three days of incubation at 37° C, 10% $CO_2$ in a 98% humid atmosphere, an approximately $IC_{50}$ was determined by comparing the growth in wells with drug to the growth in control wells. A-549, HT-29 and MEL-28 cell were seeded into 16 mm wells at $2x10^4$ cells per well in 1 mL aliquots of MEN 10FCS containing the indicated concentration of drug. A separate set of cultures without drug was seeded as control growth to ensure that cells remained in exponential phase growth. All determinations were carried out in duplicate. After three days of incubation at 37° C, 10% $CO_2$ in a 98% humide atmosphere, the wells were stained with 0.1% Crystal Violet. An approximately $IC_{50}$ was determined by comparing the growth in wells with drug to the growth in control wells. The $IC_{50}$ values were used as a parameter for cytotoxicity.

**References and notes**


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8. **Compound 2**: amorphous solid; $[\alpha]^{25}_D$ = + 12.9 (*c* 0.69, $CHCl_3$); IR $\upsilon_{max}$ ($CHCl_3$): 3855, 3688, 3442, 3020, 2956, 2928, 2857, 1718, 1671, 1603, 1460, and 1376 $cm^{-1}$; FAB MS m/z: 625/623 [$M^+$+Na-$H_2O$], 603/601 [$MH^+$-$H_2O$], 585/583 [$MH^+$+Na-$C_3H_7O$], 571/569 [$M^+$-$CH_2OH$-$H_2O$], 543/541 [$M^+$-$H_2O$-$C_3H_7O$]; $^1H$ and $^{13}C$-NMR (Table 1).
9. **Compound 3:** amorphous solid; $[\alpha]^{25}_D$ = - 1.1 (*c* 0.26, $CHCl_3$); IR $\upsilon_{max}$ ($CHCl_3$): 3855, 3689, 3568, 3442, 3021, 2957, 2928, 2856, 1717, 1671, 1602, 1459, and 1377 $cm^{-1}$; FAB MS m/z: 625/623 [$M^+$+Na-$H_2O$], 603/601 [$MH^+$-$H_2O$], 585/583 [$MH^+$+Na-$C_3H_7O$], 571/569 [$M^+$-$CH_2OH$-$H_2O$], 543/541 [$M^+$-$H_2O$-$C_3H_7O$]; $^1H$ and $^{13}C$-NMR (Table 1).
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