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Several new squalene-derived triterpenes from *Laurencia*

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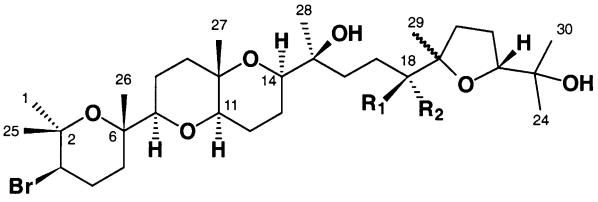
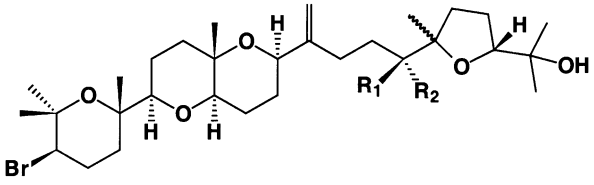
Abstract—Four new triterpenoids with a squalene carbon skeleton martiriol **7**, pseudodehydrothyrsiferol **8**, dioxepandehydrothyrsiferol **9** and 16-epihydroxydehydrothyrsiferol **10** have been isolated from the red alga *Laurencia viridis*. The structures were determined through the interpretation of their spectral data and the relative stereochemistries were proposed on the basis of ROESY and NOEDIFF data. Their cytotoxic activities were evaluated.

Marine polyethers are characterised, from the structural point of view, by the presence of rings of diverse size which form spiro or trans-fused systems. This type of active metabolites includes the marine polyether triterpenes isolated from red algae, sponges and mollusks.[1] The studies of these metabolites began with the isolation of thyrsiferol **1** 20 years ago,[2] and since that initial discovery, further examples have been isolated from the red algae of the genus *Laurencia*. This species has proved to be a rich source of natural products yielding interesting bioactive metabolites such as sesquiterpenes, diterpenes and polyether squalene derivatives.[3] The third group, although they are not the major constituents of this genus, had generated a high degree of interest with reports of thyrsiferol **1** and its congeners showing potent cytotoxic and inhibitory effects.[1,4–6]

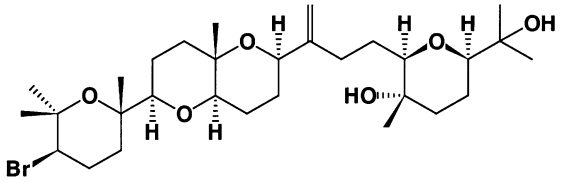


1 Thyrsiferol; R_1=OH, R_2=H, C-29= αCH_3
2 Venustatriol; R_1=H, R_2=OH, C-29= βCH_3

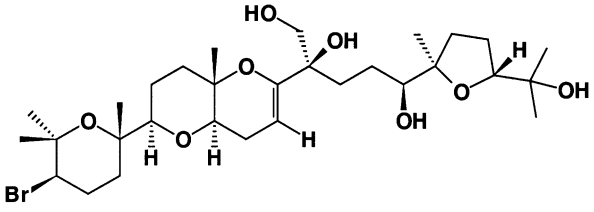
L. viridis, a new species described from specimens collected around the Canary Islands, in Macaronesia, is the most prolific source of this type of metabolites.[7–9] From this alga have been isolated new congeners of thyrsiferol **1** and venustatriol **2** with strong cytotoxic activities against P-388 cells. As the most representative examples we cite dehydrothyrsiferol **3**, dehydrovenustatriol **4**, isodehydrothyrsiferol **5** and thyrsenol B **6**, all of them showing IC_{50}=0.01 μg/mL.[8]



3 Dehydrothyrsiferol; R_1=OH, R_2=H, C-29= αCH_3
4 Dehydrovenustatriol; R_1=H, R_2=OH, C-29= βCH_3



5 Isodehydrothyrsiferol



6 Thyrsenol B

From selected fractions of the chloroform/methanol extract of this alga we have now isolated four new compounds martiriol **7**, pseudodehydrothyrsiferol **8**, dioxepandehydrothyrsiferol **9**

Keywords: polyether; terpenoids; squalene; marine metabolites.
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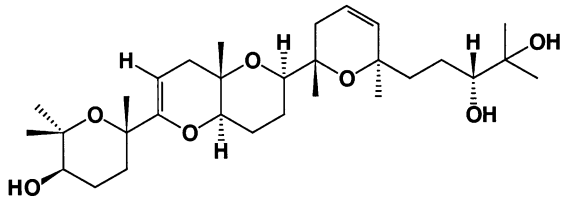
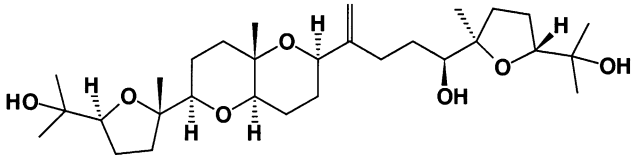
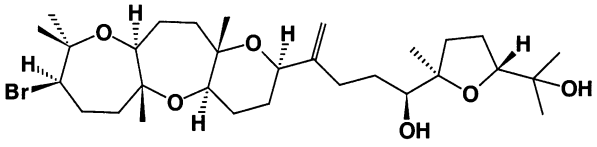
and 16-epihydroxydehydrothyrsiferol **10** using chromatography on Sephadex LH-20, Lobar LiChropred RP-8 and HPLC μ-Bondapak C-18 reversed phase columns. Their structures were determined through the interpretation of spectral data and the relative stereochemistry was proposed on the basis of ROESY and NOEDIFF data.



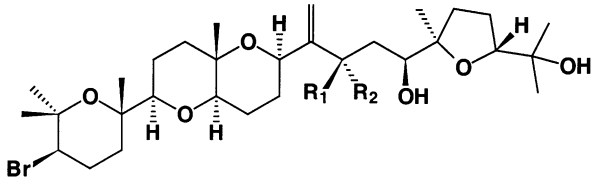
7 Martiriol



8 Pseudodehydrothyrsiferol



9 Dioxepandehydrothyrsiferol



10 16-Epihydroxydehydrothyrsiferol; R_1=OH; R_2=H
11 16-Hydroxydehydrothyrsiferol; R_1=H; R_2=OH

Martiriol **7** was isolated as an amorphous solid $[\alpha]^{25}{}_D=+4$ (*c* 0.03, $CHCl_3$) and the molecular formula determined as $C_{30}H_{50}O_7$ by mass measurement. The ^{13}C NMR spectrum confirmed the molecular formula and shows the presence of eight methyl, eight methylene and seven methine groups, together with seven quaternary carbon centres, which are α to oxygen (Table 1). The ^1H NMR spectrum exhibits in the low field region four methine signals at δ_H 3.85, 3.96, 3.99 and 4.06 identified as H-22, H-11, H-3 and H-14, respectively, by comparison with the ^1H NMR spectrum of another metabolite of this series. Besides these signals were observed three olefinic protons centred at δ_H 5.34 (H-8), 5.64 (H-18) and 5.66 (H-17), and two pairs of diastereotopic methylene signals, one centred at δ_H 1.90 and 2.05 corresponding to the protons H_2-9, and the other at δ_H 2.07 and 2.23 allocated to protons H_2-16 (Table 2).

A detailed analysis of the phase sensitive DQF-COSY and TOCSY spectra of **7** allowed five partial structures,

Table 1. ^{13}C NMR data for compounds **7**, **8**, **9** and **10**

No.	Compounds			
	7	**8**	**9**	**10**
1	29.7	24.0	24.6	31.0
2	85.7	70.6	77.8	75.0
3	82.8	86.7	59.5	59.0
4	28.1	26.3	31.6	28.2
5	35.0	35.2	40.1	37.1
6	85.2	84.0	78.9	74.4
7	170.5	84.0	76.0	86.8
8	98.8	24.5	28.4	22.9
9	35.2	38.7	41.1	38.6
10	85.9	72.8	78.0	73.5
11	84.5	78.9	71.2	79.3
12	21.2	21.8	24.3	21.7
13	29.1	26.4	26.8	25.9
14	85.7	72.5	70.2	70.8
15	73.8	151.3	150.5	152.9
16	41.5	29.7	29.8	70.3
17	122.2	29.9	30.0	35.9
18	141.1	76.2	76.1	73.8
19	70.6	86.1	86.1	85.6
20	31.4	31.6	31.6	31.9
21	25.8	26.5	26.6	26.5
22	84.3	87.6	87.6	87.6
23	70.6	70.4	70.5	70.4
24	24.0	23.9	24.0	24.0
25	24.9	27.5	25.4	23.6
26	21.2	22.7	20.6	20.1
27	29.8	19.4	19.0	19.1
28	24.8	109.9	109.8	110.4
29	29.7	23.7	23.8	23.5
30	28.0	27.7	27.7	27.7

C-3→C-5, C-8→C-9, C-11→C-14, C-16→C-18 and C-22→C-20 to be constructed as shown in Fig. 1A. The HMBC data permitted the foregoing partial structures to be connected to the quaternary carbons and the methyl groups. Thus, the HMBC analysis may be conveniently started from the quaternary carbon C-10 (δ_C 85.9), which was correlated with the protons at δ_H 5.34 (H-8, dd, *J*=2 and 6 Hz), 3.96 (H-11, dd, *J*=6 and 10 Hz), 4.06 (H-14, dd, *J*=5.7 and 10.3 Hz) and 1.12 (H_3-27, s). Furthermore, the correlations of the carbon C-7 (δ_C 170.5) with the protons H_2-9 and H-11 permitted it to be established that this compound possesses an unusual enol-ether system in ring B at carbons C-7–C-8. On the other hand, the HMBC correlations of the allylic carbon C-16 (δ_C 41.5) with the protons H-14 and H_3-28 (δ_H 1.19, s), and the correlations of carbon C-19 (δ_C 70.6) with H-17, H-18 and H_3-29 (δ_H 1.30, s) permitted the fourth ring system to be located. This ring D is formed by an ether linkage between the quaternary carbons C-15 and C-19.

The important feature, from the biogenetic point of view, was the absence of the bromine atom at C-3, substituted by a hydroxyl group. This datum was obtained through the HSQC experiment and the long-range correlations observed in HMBC experiments. Finally, the remaining correlations observed in these experiments permitted completion of the carbon skeleton assignment for martiriol **7** (Fig. 1A, Tables 1 and 2).

A plausible relative stereochemistry of this compound was proposed based on selected NOESY correlations and ^1H–^1H coupling constants. The magnitude of $J_{3-4\alpha}$=6 Hz and

Table 2. [1]H NMR data for compounds **7**, **8**, **9** and **10**

Carbon	Martiriol **7**			Pseudodehydrothyrsiferol **8**			Dioxepandehydrothyrsiferol **9**			16-Epi-hydroxydehydrothyrsiferol **10**		
1	1.21	s		1.11	s	–	1.32	s	–	1.26	s	–
2	–	–	–	–	–	–	–	–	–	–	–	–
3	3.99	dd	6.0; 10.0	3.76	dd	5.8; 9.1	4.16	dd	2.0; 10.2	3.89	dd	4.1; 12.3
4	1.52/1.93			1.84			2.10/2.20			2.11/2.24		
5	1.78/2.06			1.66/2.04			1.60/1.76			1.54/1.83		
6	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	3.32	dd	2.6; 11.4	3.53	dd	1.0; 10.3	3.08	dd	1.6; 10.6
8	5.34	dd	2.0; 6.0	1.51/1.66			1.43/1.83			1.47/1.74		
9	1.90/2.05	dd/dd	2.0; 12.0/6.0; 12.0	1.57/1.81			1.50/1.69			1.51/1.75		
10	–	–	–	–	–	–	–	–	–	–	–	–
11	3.96	dd	6; 10	3.46	dd	5.6; 11.7	3.49	dd	5.1; 11.5	3.38	dd	5.6; 11.5
12	1.78/2.03			1.65/1.84			1.51/1.73			1.65/1.76		
13	1.55/1.98			1.85/2.08			1.84/2.04			1.93/2.18		
14	4.06	dd	5.7; 10.3	4.29	dd	4.2; 7.1	4.10	dd	5.5; 5.7	4.35	dd	5.5; 5.7
15	–	–	–	–	–	–	–	–	–	–	–	–
16	2.23/2.07		2.7; 13.0/5.3; 13.0	2.20/2.46			2.23/2.44			4.70	ddd	1.6; 6.3; 10.0
17	5.66	ddd	5.3; 2.7; 11.0	1.48/1.64			1.40/1.63			1.73/1.86		
18	5.64	d	11.0	3.53	dd	1.5; 10.8	3.50	dd	1.7; 9.9	3.77	dd	1.6; 10.0
19	–	–	–	–	–	–	–	–	–	–	–	–
20	1.56/2.07			1.58/2.10			1.81/2.10			1.58/2.13		
21	1.92/2.06			1.83 (2H)			1.57/1.86			1.84/2.07		
22	3.85	dd	4.5; 7.5	3.76	dd	6.5; 9.8	3.73	dd	5.8; 10.2	3.74	dd	6.0; 9.9
23	–	–	–	–	–	–	–	–	–	–	–	–
24	1.12	s	–	1.13	s	–	1.11	s	–	1.12	s	–
25	1.16	s	–	1.19	s	–	1.34	s	–	1.39	s	–
26	1.20	s	–	1.14	s	–	1.10	s	–	1.19	s	–
27	1.12	s	–	1.25	s	–	1.15	s	–	1.24	s	–
28	1.19	s	–	4.89/5.05	bs/bs	–	4.86/4.98	bs/bs	–	5.17/5.30	bs/bs	–
29	1.30	s	–	1.14	s	–	1.12	s	–	1.12	s	–
30	1.18	s	–	1.21	s	–	1.20	s	–	1.20	s	–
OH-16										3.30	d	6.3
OH-18				2.38	s	–	2.35	s	–	2.66	s	–

$J_{3-4\beta}$=10 Hz and the NOE correlation between H-3 and the protons H_3-1 suggested that the relative stereochemistry at C-3 was R^*. Furthermore, the NOE interaction between the H-11 and H-14 confirmed the characteristic chair/twist-boat BC rings system in this type of metabolites, thus implying that it must be C-6 S^*, C-10 S^*, C-11 R^* and C-14 R^*. The absence of a NOE interaction between H_3-28 and H_3-29 established the relative stereochemistry in ring D linkage

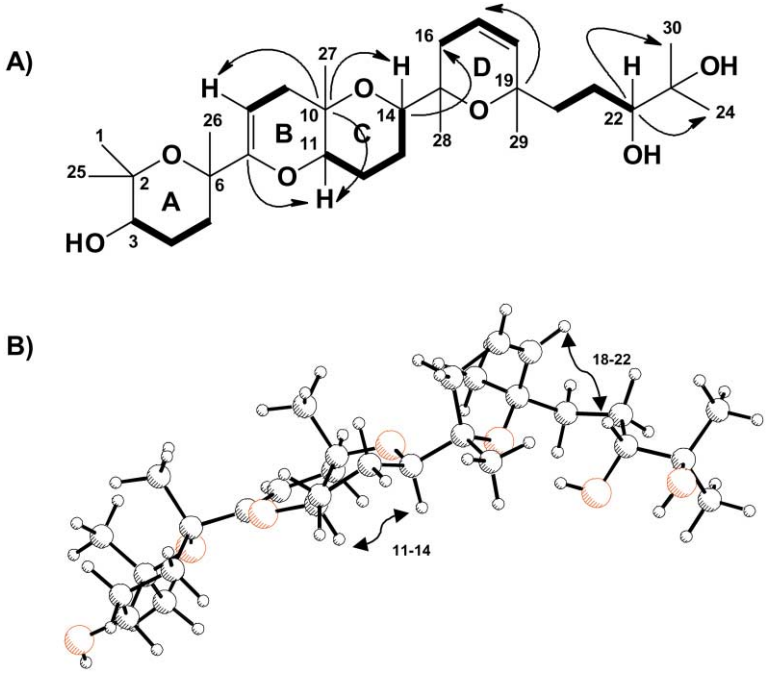


Figure 1. (A) Partial structures of martiriol **7**, based on phase-sensitive DQF-COSY and TOCSY spectrum, and selected HMBC correlations. (B) Lowest energy conformation and significant NOE correlations.

as *trans*. The facts that the coupling constants of H-22 were $J_{22-21\alpha}$=4.5 Hz and $J_{22-21\beta}$=7.5 Hz with the diastereotopic protons H_2-21, and the NOE correlation between H-18 and H-22 inferred that the secondary alcohol was fixed with the pyrane oxygen in a H-bond as shown in Fig. 1B.

Compound **8**, pseudodehydrothyrsiferol, was also isolated as an amorphous white solid, $[\alpha]^{25}_{D}=-13.1$ (*c* 0.13, $CHCl_3$), and proved to be an isomer of martiriol **7** as established by HRMS. Its spectroscopic data resembled those of dehydrothyrsiferol **3** which aided significantly in the structure assignment for this molecule. Compound **8** was nearly identical with **3** except for the protons H-3 (δ_H 3.76), H_2-4 (δ_H 1.84), H_2-5 (δ_H 2.04 and 1.66), H_3-1 (δ_H 1.11) and H_3-25 (δ_H 1.19) that fixed the modification at ring A. The HSQC experiment for this moiety showed the correlations δ_C: 24.0 (C-1), 86.7 (C-3), 26.3 (C-4), 35.2 (C-5) and 27.5 (C-25). The quaternary carbons C-2 and C-6 were assigned on the basis of its HMBC correlations at δ_C 70.6 and 84.0, respectively. All these data suggested that the usual tetrahydropyran ring system A has changed in this compound to a tetrahydrofuran ring system resulting from the ether link between carbons C-3 and C-6. ROE correlations between H-3 (δ_H 3.76) and H_3-1 (δ_H 1.11), H_3-25 (δ_H 1.19), H-7 (δ_H 3.32) and not with H_3-26 (δ_H 1.14) established the opposite orientation between the methyl group H_3-26 and the methine proton H-3, indicating that the relative configuration at C-3 was S^*.

Dioxepandehydrothyrsiferol **9** was obtained as a white solid. Mass spectral analysis of this metabolite showed a mass ion consistent with the molecular formula $C_{30}H_{51}O_6{}^{81}Br$ (obs. 588.282410; Calcd 588.284855). Assignment of the structure for this metabolite was again aided by the considerable spectroscopical analogy to dehydrothyrsiferol **3**. The only significant variations in the ^1H NMR spectrum between compound **9** and **3** were the downfield shifts in the protons H-3 (δ_H 4.16, dd, *J*=2.0 and 10.2 Hz) and H-7 (δ_H 3.53, dd, *J*=1.0 and 10.3 Hz), as well as the remarkable change in the H-3 coupling constants with respect to other products of this series with a tetrahydropyran ring system A. In addition, the protons of methyl groups C-1, C-25 and C-26 were centred at δ_H 1.32, 1.34 and 1.10, respectively. These data suggested that the modifications in this compound were mainly located in rings A and B. The connectivities observed in COSY and HMQC experiments made it possible to assign the fragment C-1→C-11 as follows: δ_C: 24.6 (C-1), 59.5 (C-3), 31.6 (C-4), 40.1 (C-5), 76.0 (C-7), 28.4 (C-8), 41.1 (C-9) and 71.2 (C-11). The C–O quaternary centres at C-2, C-6 and C-10 were identified at δ_C 77.8, 78.9 and 78.0 by their correlations with H-3, H-7 and H-11, respectively, in the HMBC experiment. Furthermore, in this experiment it was possible to observe the correlations between the carbon C-2 (δ_C 77.8) and the proton H-7 (δ_H 3.53), and between the carbon C-6 (δ_C 78.9) and the proton H-11 (δ_H 3.49). These facts can only be explained by the presence of three fused cyclic ethereal groups (rings A, B, C) in compound **9** as shown in Fig. 2, that resembled a fragment observed in the brevetoxins, hemibrevetoxin and ciguatoxin. Moreover, in this Fig. 2 is shown a plausible conformation of these A, B, C rings based on the coupling constants and ROE correlations observed that indicated the relative stereochemistry at C-3, C-6, C-7, C-10 and C-11 as R^*, R^*, S^*, S^* and R^*, respectively.

The last compound isolated, 16-epi-hydroxydehydrothyrsiferol **10**, was an isomer of 16-hydroxydehydrothyrsiferol **11** published in 1997.[9] Interpretation of COSY, HMQC, HMBC and NOESY data allowed assignments of ^1H and ^{13}C signals, which readily demonstrated that the sole difference between them was the stereochemistry of the hydroxyl group at C-16. Thus, the multiplicity of proton H-16 (δ_H 4.70, ddd, *J*=1.6, 6.3, 10 Hz) inferred that this alcohol was fixed with a H-bond at the hydroxyl group in C-18, which was supported by the constant coupling and the ROE connectivities observed between H-17α (δ_H 1.73) and both H-16 (δ_H 4.70) and H-18 (δ_H 3.77), and those

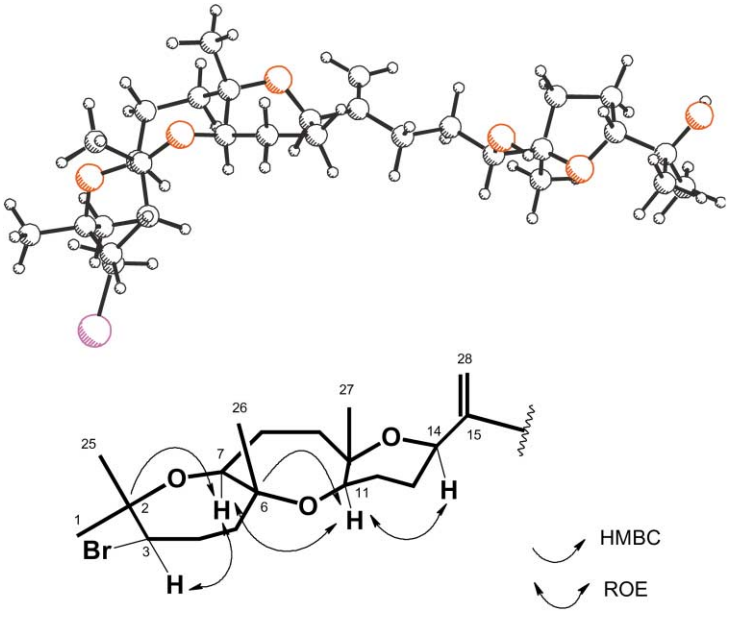


Figure 2. Lowest energy conformation and significant ROE and HMBC correlations for compound **9**.

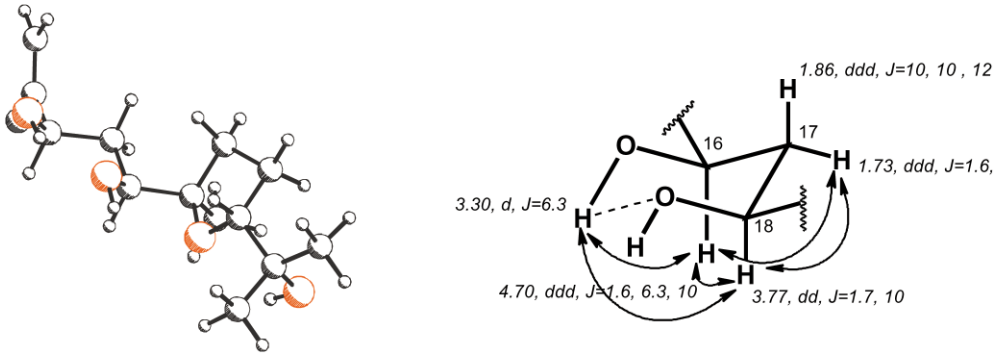
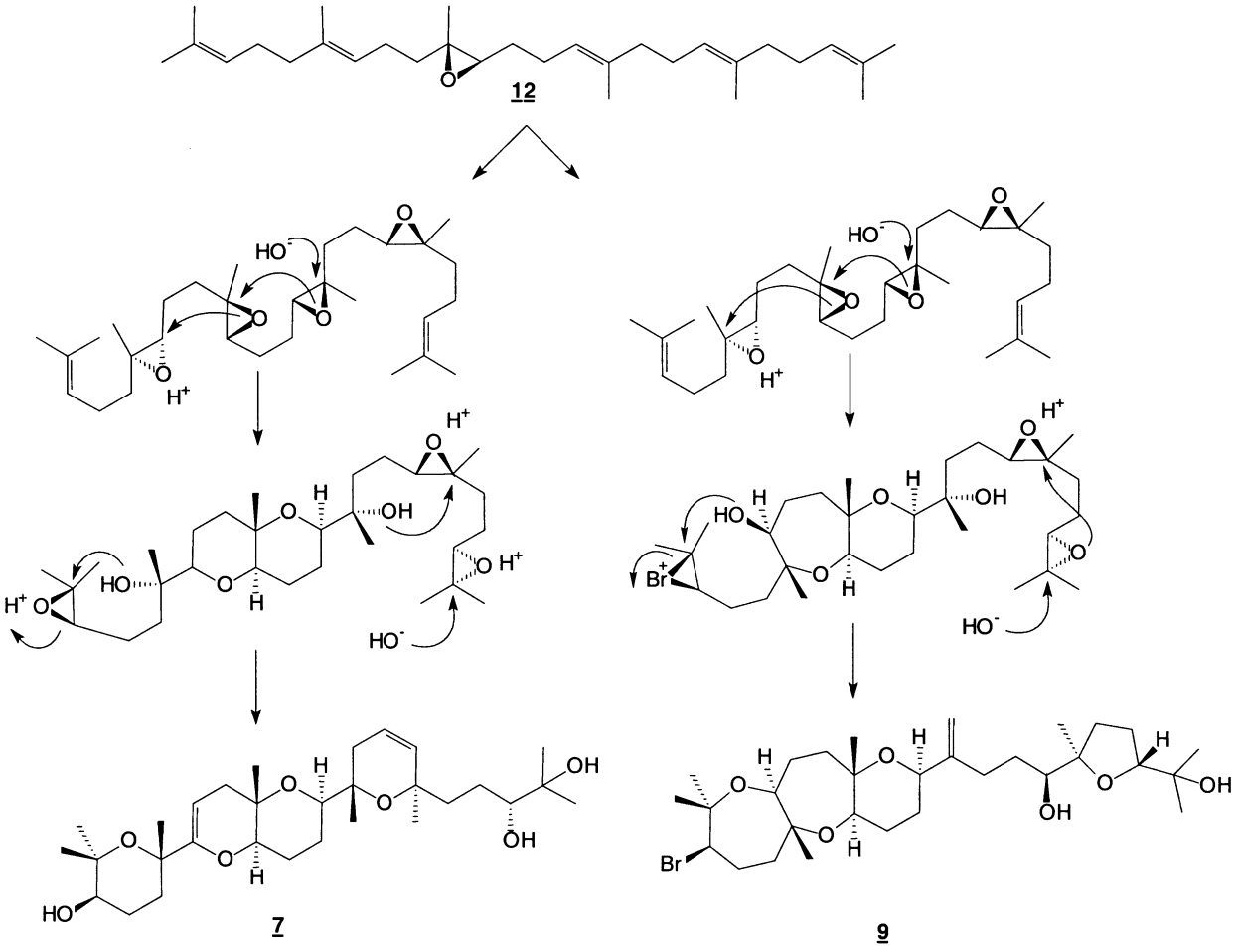


Figure 3. Partial structure of compound **10**.

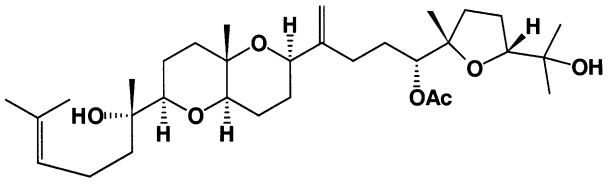
methines and the OH proton at C-16 (δ_H 3.30, d, J=6.3 Hz) (Fig. 3).

From a biogenetic viewpoint the polyoxygenated squalene-derived ethers isolated from *Laurencia* species may arise from a common precursor, the (10*R*, 11*R*)-squalene 10,11 epoxide **12** isolated from *Laurencia okamurai*.[11] This compound could evolve to 6*S*, 7*S*, 10*R*, 11*R*, 14*R*, 15*R*, 18*S*, 19*S* squalene tetraepoxide as common intermediate for all metabolites hitherto isolated. However, this intermediate has not yet been found. Initially, the biogenesis of the A–B–C ring system had been proposed as a result of the concerted cyclisations of three epoxides after formation of a bromonium ion at C-2–C-3, thus forming the framework of these metabolites. But, as a consequence of the identification on the one hand of predehydrovenustatriol acetate **13** that lacks the bromine at ring A and possesses a double bond between the carbons C-2–C-3; and on the other hand martiriol **7** and pseudodehydrothyrsiferol **8** with a hydroxyl group and an ether link at C-3, respectively, it would be more plausible to assume that it occurs through a sequential process, rather than as the classic biogenetic proposal for these metabolites. In these compounds, the process could start with an enzymatic protonation at the 6*S*, 7*S*-epoxide



Scheme 1. Possible biogenesis for compounds **7** and **9**.

followed by cyclisation and hydroxylation at C-15. Then, the intermediate would evolve following the pathway in Scheme 1 towards the proposed product with *trans*-relationship between the oxane ether linkages in rings BC. This proposal is consistent with the NOE correlation observed between H-11 and H-14 in all metabolites isolated from this alga. The other ether rings A and D in martiriol **7** must be formed by protonation of 2, 3*R* and 18*S*, 19*S* epoxides and reaction with the hydroxyl groups at C-6 and C-15, respectively. Subsequent hydroxylation at C-23 could then afford the relative configuration proposed as 15*S**, 19*R** and 22*R** on the basis of these biogenetic considerations. In compound **8**, ring A must be formed by protonation of 2, 3*R* epoxide and reaction with the hydroxyl group at C-6 on the C-3 position with the formation of a tetrahydrofuran system present in this metabolite. When the cyclisation reaction of the hydroxyl group present at C-10 evolves on carbon C-7, as shown in Scheme 1, dioxepandehydrothyrsiferol **9** can be obtained. The biogenetic proposal for the formation of the ring D in compound **8**–**10** in a similar manner to that of dehydrothyrsiferol **3** has been recently published.[1]



13 **Predehydrovenustatriol acetate**

Biological assays of the pure compounds were undertaken, making use of in vitro bioassays and focusing on cytotoxic activity. 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.[10,12] The results of this screening procedure established that martiriol **7** was inactive at concentrations lower than 10 μg/mL and pseudodehydrothyrsiferol **8**, dioxepandehydrothyrsiferol **9**, 16-epi-hydroxydehydrothyrsiferol **10** were inactive at concentrations lower than 1 μg/mL.

1. Experimental

1.1. 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 400 Advance and a 500 MHz instruments. Chemical shifts are reported relative to TMS and coupling constants are given in Hz. HRMS were performed on a VG AutoSpec FISON 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.

1.2. Plant material

Seeds of *L. viridis* were collected in April 1998 in the intertidal zone at Callao Salvaje, Paraiso Floral, El Palmar (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).

1.3. Extraction

The dried alga (5 kg) was extracted with $CHCl_3$/MeOH (1:1) at room temperature. The combined extracts were evaporated in vacuo to leave a dark-green viscous oil (65.0 g, 1.3% dry weight).

1.4. Chromatographic separation

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 Sephadex LH-20 (600×70 mm ∅) using *n*-hexane/$CHCl_3$/MeOH (2:1:1) as eluent and a medium pressure silica gel chromatography LiChropred-Si with *n*-hexane/EtOAc (3:2). Selected fractions were combined and rechromatographed on a medium pressure reverse-phase Lobar LiChropred RP-18 (310×25 mm ∅) using MeOH/H_2O (4:1) as eluent. Each compound was finally chromatographed using a μ-Bondapack C-18 (150×9 mm ∅) column HPLC reverse phase chromatography with acetonitrile/H_2O (9:1) as eluent yielding pure **7** (2.3 mg), **8** (2.3 mg), **9** (2.3 mg) and **10** (2.3 mg).

1.4.1. Compound 7. Amorphous white solid; $[\alpha]^{25}_D=+4$ (*c* 0.03, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3688, 3456, 3022, 2929, 2855, 1731, 1602, 1459, 1374 and 1240 cm^{-1}; HRMS: 524.37147 (Calcd 524.371304 $C_{30}H_{52}O_7$ [M^+]); MS at *m/z*: 524, 436, 365, 322, 277, 237; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) See Tables 1 and 2.

1.4.2. Compound 8. Amorphous white solid; $[\alpha]^{25}_D=-13.1$ (*c* 0.13, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3427, 2971, 2870, 1723, 1461, 1375 and 1217 cm^{-1}; HRMS: 524.37107 (Calcd 524.37130 $C_{30}H_{52}O_7$ [M^+]); MS at *m/z*: 524, 506, 488, 470, 447, 429, 403, 381, 363; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) See Tables 1 and 2.

1.4.3. Compound 9. Amorphous white solid; $[\alpha]^{25}_D=+39$ (*c* 0.07, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3439, 2972, 2855, 1644, 1455, 1377 and 1085 cm^{-1}; HRMS: 588.28241 (Calcd 588.28485 $C_{30}H_{51}O_6{}^{81}Br$ [M^+]); MS at *m/z*: 588, 586, 571, 569, 529, 527, 445, 443, 291, 289; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) See Tables 1 and 2.

1.4.4. Compound 10. Amorphous white solid; $[\alpha]^{25}_D=+12$ (*c* 0.07, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3417, 2928, 1644, 1455, 1377 and 1096 cm^{-1}; HRMS: 604.27967 (Calcd 604.27976 $C_{30}H_{51}O_6{}^{81}Br$ [M^+]); MS at *m/z*: 604, 602, 568, 566, 529,

527, 469, 451; ^{1}H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) See Tables 1 and 2.

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