Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 2237–2243

BIOORGANIC & MEDICINAL CHEMISTRY

Evaluation of the Cytotoxic Activity of Polyethers Isolated from *Laurencia*

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Received 28 January 1998; accepted 30 March 1998

Abstract—In this paper, we report on the conformational analysis of several polyether triterpenes with a squalene carbon skeleton which exhibited significant cytotoxic activity using a Monte Carlo conformational search and spectroscopical data. These studies indicate that the conformation of the side chain C-14/C-19 and the arrangement and direction of this chain may be among the fundamental factors related to the activity of this type of metabolites.

Introduction

Over the last fifty years a great number of new therapeutic agents have been discovered. However, in spite of the unquestionable advance of chemotherapy, it is calculated that a satisfactory treatment has yet to be developed for two out of every three diseases. This is the case of numerous forms of cancer and viral or heart diseases. The need for new, more specific and better adapted medicines is greater each day. The marine environment constitutes a vast potential source of new bioactive molecules.

One of the most interesting groups of substances of marine origin, from the pharmacological point of view, is that of the cyclic polyethers, which generally present a great diversity in size and potent biological activities. Red seaweed of genus *Laurencia* is known to produce the interesting active polyether squalene-derived metabolites thyrsiferol **1**, venustatriol **2** and dehydrothyrsiferol **3**,[1–5] which possess strong cytotoxic and antiviral properties.

Recently, we have reported on the isolation,[6–8] structural determination and cytotoxic effects of several new polyether triterpenes with a squalene carbon skeleton which exhibited significant pharmacological activity. These compounds were isolated from *Laurencia viridis sp. nov* (Ceramiales, Rhodomelaceae), a new species described from specimens collected around the Canary Islands.[9] The alga grows in the lower intertidal zone, intermingled with other turf algae. It occurs on exposed, overhanging rocks subject to strong wave-action. *Laurencia viridis* is an annual plant that grows rapidly during winter–spring months and decays in late summer. This species would therefore have an ephemerophycean life-form.

Results

The alga was collected in April 1994 in the intertidal zone at Paraíso Floral, Callao Salvaje and El Palmar, all located in South Tenerife (Canary Islands). It was air-dried, ground in a Wiley mill to 1 mm particle size and extracted with $CHCl_3$/MeOH (1/1) at room temperature.

The crude extract was successively chromatographed on a silica gel column and a Sephadex LH-20 column, the final purification being achieved on HPLC chromatography. This chromatographic study afforded, in addition to thyrsiferol **1** and dehydrothyrsiferol **3**, nine new compounds which showed remarkable cytotoxic activities: dehydrovenustatriol **4**, 15–16 dehydrovenustatriol **5**, predehydrovenustatriol acetate **6**, isodehydrovenustatriol **7**, 16-hydroxydehydrovenustatriol **8**, 10-epi-15, 16-dehydrothyrsiferol **9**, 10-epidehydrothyrsiferol **10**, thyrsenol A **11** and B **12**.[6–8]

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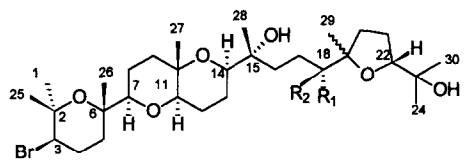
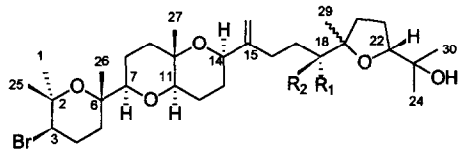
0968-0896/98/$—see front matter
PII: S0968-0896(98)00178-3

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 them to possess a potent and selective activity against P-388 cells, although for the compounds thyrsenol **1**, dehydrothyrsiferol **3**, dehydrovenustatriol **4**, isodehydrothyrsiferol **7**, thyrsenol B **12**, (IC_{50} = 0.01 μg/mL), it was significantly higher than for compounds 15-16-dehydrovenustatriol **5**, thyrsenol A **11** (IC_{50} = 0.25 μg/mL), 16-hydroxydehydrothyrsiferol **8**, 10-epi-15-16-dehydrothyrsiferol **9** (IC_{50} = 0.50 μg/mL), 10-epidehydrothyrsiferol **10** (IC_{50} = 1.00 μg/mL) and predehydrovenustatriol acetate **6** (IC_{50} = 1.20 μg/mL), thus establishing that small chemical changes in the molecule greatly condition the cytotoxic activities. The results of biological assays are summarized in Table 1.

In order to obtain more data to establish a possible structure–activity relationship between these compounds, we decided to start a process of molecular simplification. Thus compounds **3** and **14** were treated with $VO(acac)_2$/hv and $RuCl_3/NaIO_4$, respectively, yielding the compounds of oxidative fragmentation **13** and **15** (Scheme 1). None of them showed significant cytotoxic activity, which together with the above comments about the activity of compounds **1, 3–12**, suggests to us that a part of the explanation to these facts may be related to the different conformations of the analogues. Thus, as we had in all cases detailed spectroscopical data, we decided to approach a conformational study to try to find a possible explanation to the variations observed in the activity. Stable conformations of these compounds were calculated by a multiconformer search with the Monte Carlo program in Macromodel[11] version 4.5 and MM2 force fields,[12] using approximate distance constraints (2–4 Å), derived from the observed intense NOESY correlations. For each search, 5000 starting structures were generated and minimized until the gradient was less than 0.05 (kj/mol)/Å using the truncated Newton–Raphson method implemented in Macromodel. Conformations with an energy greater than 50 kJ/mol above the global minimum were discarded.



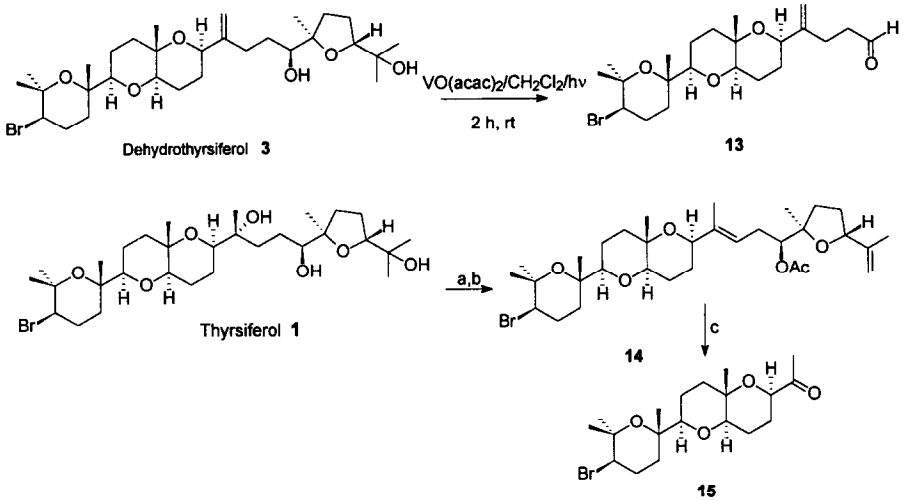
1 Thyrsiferol, R_1= H, R_2=OH, C-29 = α CH_3
2 Venustatriol, R_1= OH, R_2=H, C-29 = β CH_3



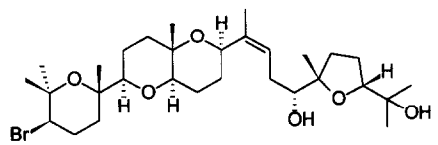
3 Dehydrothyrsiferol, R_1= H, R_2=OH, C-29 = α CH_3
4 Dehydrovenustatriol, R_1= OH, R_2=H, C-29 = β CH_3

Table 1. IC_{50} of biological assays of the pure polyether squalene derivatives of *Laurencia viridis*

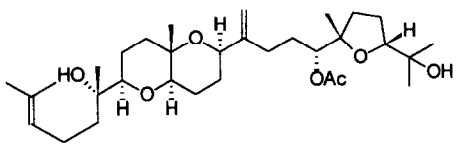
n°	Compounds	IC_{50}							
		P388		A549		HT29		MEL28	
		μg/mL	μM	μg/mL	μM	μg/mL	μM	μg/mL	μM
1	Thyrsiferol	0.01	0.016	10.0	16.53	10.0	16.53		
3	Dehydrothyrsiferol	0.01	0.017	2.5	4.26	2.5	4.26	5	8.52
4	Dehydrovenustatriol	0.01	0.017	2.5	4.26	2.5	4.26	2.5	4.26
5	15,16-Dehydrovenustatriol	0.25	0.430	2.5	4.26	2.5	4.26	2.5	4.26
6	Predehydrovenustatriol Acetate	1.20	2.180	2.5	4.54	5.0	9.09	2.5	4.54
7	Isodehydrothyrsiferol	0.01	0.017	2.5	4.26	2.5	4.26	2.5	4.26
8	16-Hydroxydehydrothyrsiferol	0.50	0.830	1.2	1.99	1.2	1.99	1.2	1.99
9	10-Epi-15,16-dehydrothyrsiferol	0.50	0.850	2.5	4.26	1.2	2.04	2.5	4.26
10	10-Epidehydrothyrsiferol	1.00	1.700	5.0	8.52	5.0	8.52	5.0	8.52
11	Thyrsenol A	0.25	0.400	> 1.0	> 1.62	> 1.0	> 1.62	> 1.0	> 1.62
12	Thyrsenol B	0.01	0.016	> 1.0	> 1.62	> 1.0	> 1.62	> 1.0	> 1.62
13		> 1.0	> 2.26	> 1.0	> 2.26	> 1.0	> 2.26	> 1.0	> 2.26
15		> 2.0	> 4.96	> 2.0	> 4.96	> 2.0	> 4.98		



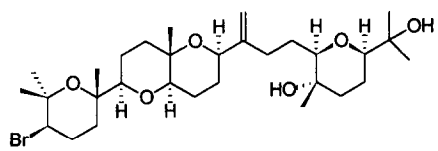
Scheme 1. *Reagents and conditions*: (a) Ac_2O/py, rt, 10 h; (b) $SOCl_2$/py, −20 °C, 10 h; (c) $RuCL_3$-$NaIO_4$-CCl_4/CH_3CN/H_2O (2/2/3), rt, 2 h.



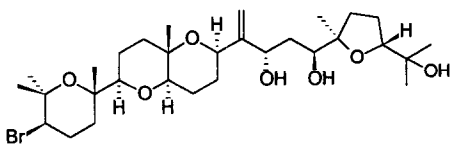
5 15,16-Dehydrovenustatriol



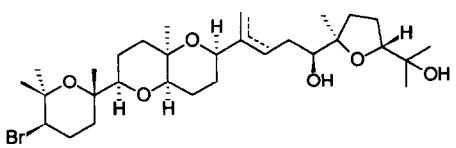
6 Predehydrovenustatriol acetate



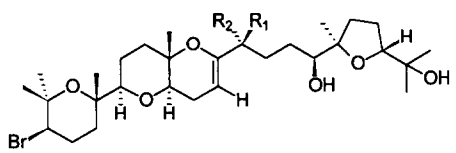
7 Isodehydrothyrsiferol



8 16-Hydroxydehydrothyrsiferol



9 10-Epi-15,16-dehydrothyrsiferol, $\Delta^{15\text{-}16}$
10 10-Epidehydrothyrsiferol, $\Delta^{15\text{-}28}$



11 Thyrsenol A, R_1=OH, R_2=CH_2-OH
12 Thyrsenol B, R_1=CH_2-OH, R_2=OH

Discussion

Structures generated by this method are shown in Figures 1–4, associating the spatial arrangement of the flexible chain between carbons C-14/C-19 with the value of IC_{50} presented by each one. Thus, we observe that compounds **11** and **12**, that vary in a chiral centre and have different IC_{50} values, adopt different spatial conformations. For metabolites that do not alter their relative cytotoxic power either upon varying the chirality of the carbons C-18 and C-22 (compounds **3** and **4**) or upon substituting the tetrahydrofuran ring for a tetrahydropyran ring (compounds **3** and **7**) we can prove that the arrangement of the chains is similar. If we analyze the changes produced in the direction of the chains, the isomerization of the double bond between carbons C-15/C-28 to C-15/C-16, we find a reasonable justification of the different cytotoxic activity changes on the basis of the different conformations on the flexible chain.

Conclusion

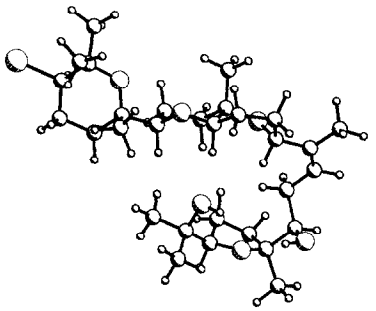
In conclusion, this study confirms that the presence of the flexible chain around carbon C-14 to C-19 is one of the fundamental factors related to the cytotoxic activity of this type of metabolites isolated from *Laurencia*

viridis against P-388 cells of a murine lymphoid neoplasm. Also, we can conclude that the most potent metabolites thyrsiferol **1**, dehydrothyrsiferol **3**, dehydrovenustatriol **4**, isodehydrothyrsiferol **7**, thyrsenol B **12**, present a very similar arrangement of the flexible chain, while if the direction of the chain is diverted in both senses, the power of the cytotoxic activity is reduced progressively until arriving at 10-epidehydrothyrsiferol **10**, the least active of the series, which possesses the flexible chain in the opposite direction to that of compounds such as dehydrothyrsiferol **3**, one hundred times more potent.

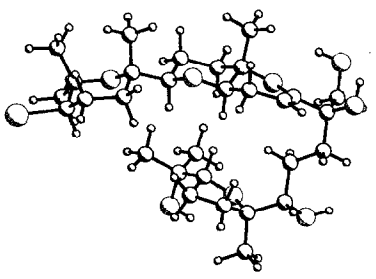
Experimental

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 visualized by spraying with H_2SO_4/H_2O/AcOH (1/4/20) and heating. All solvents were purified by standard techniques.

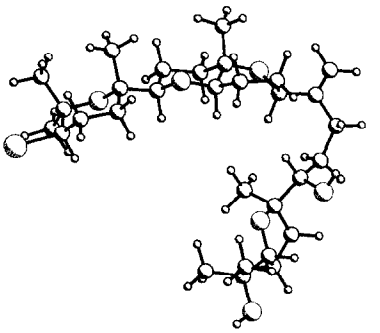


5 15-16 Dehydrovenustatrol

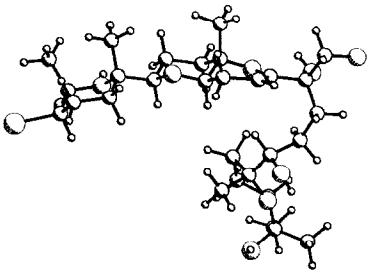


11 Thyrsenol A

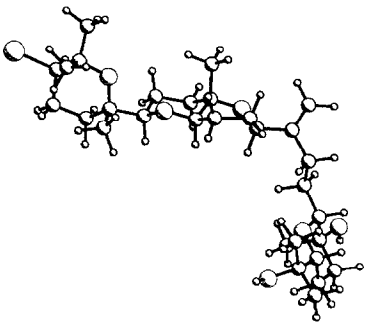
Figure 1. Stereo view of proposed 3-D structures for compounds **5** and **11** with $IC_{50} = 0.25\ \mu g/mL$.



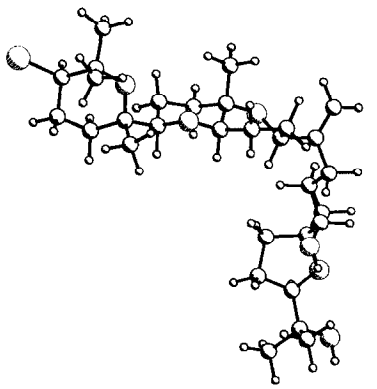
3 Dehydrothyrsiferol



12 Thyrsenol B

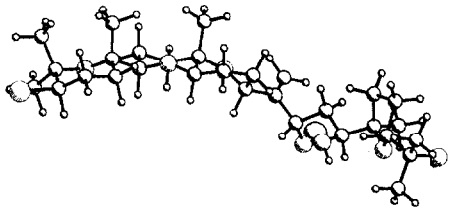


7 Isodehydrothyrsiferol

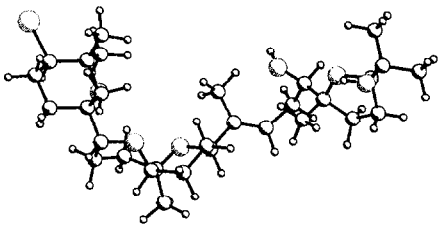


4 Dehydrovenustatriol

Figure 2. Stereo view of proposed 3D structures for compounds **3**, **12**, **7** and **4** with $IC_{50} = 0.01\ \mu g/mL$.

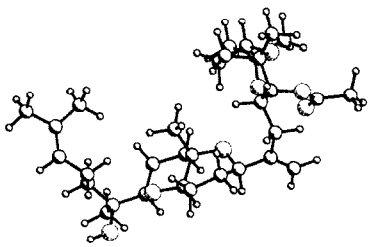


8 16-Hydroxydehydrothyrsiferol

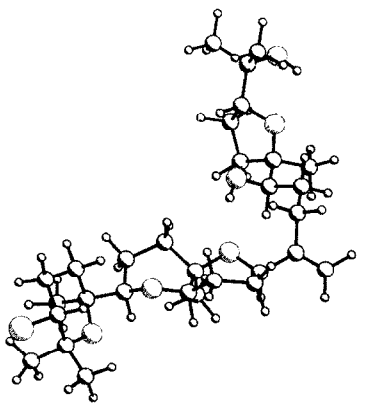


9 10-Epi-15,16-dehydrothyrsiferol

Figure 3. Stereo view of proposed 3-D structures for compounds **8** and **9** with $IC_{50} = 0.50\,\mu g/mL$.



6 Predehydrovenustatriol acetate



10 10-Epidehydrothyrsiferol

Figure 4. Stereo view of proposed 3-D structure for compounds **6** and **10** with $IC_{50} \geq 1.0\,\mu g/mL$.

Plant material

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

Extraction

The dried alga (4 kg) was extracted with CH_2Cl_2 in a Soxhlet apparatus for 24 h each and after with $CHCl_3$/MeOH (1/1) at room temperature. The combined extracts were evaporated in vacuo to leave a dark-green viscous oil (52.0 g, 1.3% dry weight).

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 a Sephadex LH-20 (600×70 mm∅), with *n*-hexane/$CHCl_3$/MeOH (2/1/1) as eluent; and a medium pressure silica gel chromatography, collecting 25 mL fractions: *n*-hexane/EtOAc (7/3), fractions 1–70; *n*-hexane/EtOAc (1/1), fractions 71–100; EtOAc, fractions 101–150. Fractions exhibiting similar tlc profiles were combined and each one was rechromatographed on a medium pressure reverse-phase Lobar LiChropred RP-8 (310×25 mm∅), with MeOH/H_2O (9/1) as eluent and later on a μ-Bondapack C-18 (150×19 mm∅) column HPLC reverse-phase chromatography with acetonitrile/H_2O (9/1) as eluent. Fractions 40–51 (816 mg) yielded pure **6** (3.3 mg) and impure **4** and **5**; final purification was carried out by HPLC employing μ-Porasil (150×19 mm) prepacked column and using *n*-hexane/EtOAc (7/3), affording pure **4** (6.5 mg) and **5** (26.2 mg). Fractions 52–72 (750.7 mg) yielded pure **3** (219.8 mg) and 73–82 compound **1** (36 mg). Fractions 83–107 (344 mg) yielded pure **8** (2.3 mg) and impure **7** and **10**, which was rechromatographed by HPLC on μ-Porasil, *n*-hexane/EtOAc (3/2), affording pure **7** (2.3 mg) and **10** (23.2 mg). Fractions 108–130 (1.235 g) gave, after final purification by HPLC employing Spherisorb Silica 5μ (300×6.5 mm∅) prepacked column in n-hexane/EtOAc (1/1), pure **9** (2.2 mg).

Cell cultures

Cells were maintained in logarithmic phase of growth in Eagle's Minimum Essential Medium, with Earle's Balanced Salts, with 2.0 mM L-glutamine, with non-essential amino acids, without sodium bicarbonate

(EMEM/neaa); supplemented with 10% Fetal Calf Serum (FCS), 10^{-2} M sodium bicarbonate and 0.1 g/L penicillin G + streptomycin sulfate. The antitumor cell employed have been P-388 (ATCC CCL 46, suspension culture of a lymphoid neoplasm from 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) and MEL-28 (ATCC HTB-72, monolayer culture of a human melanoma).

Cytotoxicity assay

A simple screening procedure has been carried out to determine and compare the antitumor activity of these compounds, using an adapted from the method described by Bergeron et al. (1984),[10] adriamycin being used as a positive control. P-388 cells were seeded into 16 mm wells at 1×10^4 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, and approximately IC_{50} was determined by comparing the growth in wells with drug to the growth in wells control. A-549, HT-29 and MEL-28 cell were seeded into 16 mm wells at 2×10^4 cells per well in 1 mL aliquots of MEN 10FCS containing the indicated concentration of drug. A separate set of cultures without drugs were 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, 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 wells control. The IC_{50} values were used as a parameter for cytotoxicity.

Compound 13

Dehydrothyrsiferol **3** (25 mg, 0.044 mmol) in CH_2Cl_2 was photo-oxygenated by passing a slow stream of dry oxygen gas while externally irradiation with a street lamp at 15 °C in the presence of catalytic $VO(acac)_2$ for 2 h. Then, the solvent was evaporated in vacuo and the residue was filtered over silica gel with ether as eluent. The aldehyde **13** was isolated as amorphous white solid; $[\alpha]_D^{25} = +5.3$ (*c* 0.36, $CHCl_3$); IR ν_{max} ($CHCl_3$): 3567, 2955, 2929, 2856, 2360, 2341, 1717, 1558, 1508, 1457 and 1230 cm^{-1}; HRMS: M^+ no observed, 363.25354 (calcd $C_{22}H_{35}O_4$ 363.25353 [M^+-HBr]); 291.07991 (calcd $C_{13}H_{22}O_2{}^{81}Br$ 291.07827 [$M^+-C_9H_{13}O_2$]); 289.07969 (calcd $C_{13}H_{22}O_2{}^{79}Br$ 289.08032 [$M^+-C_9H_{13}O_2$]); MS: *m/z* 424, 363, 291, 289, 278, 277, 274, 205, 125; ^1H NMR (400 MHz, $CDCl_3$) δ 1.20 (s, 3H), 1.21 (s, 3H), 1.27 (s, 3H), 1.40 (s, 3H), 1.50 (m, 1H), 1.53 (m, 1H), 1.55 (m, 1H), 1.60 (m, 1H), 1.75 (m, 1H), 1.76 (m, 1H), 1.80 (m, 1H), 1.81 (m, 1H), 1.82 (m, 1H), 2.08 (m, 1H), 2.13 (m, 1H), 2.26 (m, 1H), 2.48 (m, 2H), 2.61 (m, 2H), 3.08 (dd, $J=11.0$, 2.5 Hz, 1H), 3.42 (dd, $J=11.3$, 5.7 Hz, 1H), 3.89 (dd, $J=12.3$, 4.1 Hz, 1H), 4.28 (dd, $J=6.7$, 5.0 Hz, 1H), 4.83 (bs, 1H), 5.06 (bs, 1H), 9.78 (dd, $J=1.4$, 1.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.36 (q), 20.06 (q), 21.71 (t), 22.93 (t), 23.63 (q), 24.72 (t), 26.17 (t), 28.23 (t), 31.01 (q), 37.08 (t), 38.66 (t), 42.16 (t), 58.99 (d), 72.60 (d), 72.97 (s), 74.37 (s), 74.96 (s), 78.90 (d), 86.69 (d), 110.26 (t), 149.84 (s), 202.20 (d).

Compound 15

Thyrsiferol **1** (20 mg, 0.034 mmol) was acetylated using the general procedure (Ac_2O/Py) to obtain thyrsiferol-18-acetate. It was treated with thionyl chloride in pyridine at −20 °C for 15 h afforded **14**, the major dehydrated product in C_{15}–C_{16} and C_{23}–C_{24} positions. This product was dissolved in a round bottom flask in 2 mL of carbon tetrachloride, 2 mL of acetonitrile and 3 mL of water. Sodium metaperiodate (4.1 equiv) and ruthenium trichloride hydrate (2.2 mol%) was added and stirred vigorously for 2 h at rt. Then 5 mL of CH_2Cl_2 was added and the phases were separated. After extraction and chromatographic separation, yielded **15** as colorless oil. IR ν_{max} ($CHCl_3$): 2970, 2870, 1725, 1465, 1385, 1100, 900 and 745 cm^{-1}; HRMS: 403.1464 (calcd $C_{19}H_{32}O_4{}^{79}Br$ 403.1484); MS: *m/z* 405, 404, 403, 402, 361, 359, 207, 205; ^1H NMR (400 MHz, $CDCl_3$) δ 1.06 (s, 3H), 1.19 (s, 3H), 1.27 (s, 3H), 1.39 (s, 3H), 2.10 (dd, $J=13.2$, 3.9 Hz, 1H), 2.23 (dd, $J=13.2$, 3.4 Hz, 1H), 2.28 (s, 3H), 2.44 (dd, $J=13.5$, 2.5 Hz, 1H), 3.10 (dd, $J=11.2$, 2.4 Hz, 1H), 3.16 (dd, $J=11.3$, 4.4 Hz, 1H), 3.88 (dd, $J=12.2$, 3.9 Hz, 1H), 3.98 (dd, $J=6.8$, 2.0 Hz, 1H).

Acknowledgements

We gratefully acknowledge financial support from the Programas Nacionales de Tecnología de Alimentos, Ref. ALI95-1012-C05-02 and Ciencia y Tecnología Marina MAR95-1981-C04-02. The authors also gratefully acknowledge discussions with E. Quintana at the Centro de Investigaciones Químicas, Isla de la Cartuja and M.D. García-Gravalos at Pharma Mar.

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