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TETRAHEDRON

Metabolites From The Sponge *Plakortis simplex*. Determination of Absolute Stereochemistry of Plakortin. Isolation and Stereostructure of Three Plakortin Related Compounds

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Abstract: The polyketides dihydroplakortin (**3**), and the dodecanoic acid derivatives **4** and **5**, were isolated from the Caribbean marine sponge *Plakortis simplex*, and their structures fully characterized by spectroscopic and chemical means. The absolute stereochemistries of the known plakortin (**1**) and of compounds **3-5** were determined by applying Mosher's and Kusumi's methods on opportune degradation products. The isolated compounds exhibited cytotoxic activity tested in vitro on WEHI 164, murine fibrosarcoma cell line. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Biologically active compounds; Marine metabolites; Peroxides; Stereochemistry

INTRODUCTION

Sponges of the genus *Plakortis* are well known among marine chemists as prolific producers of biologically active secondary metabolites. Apart from few classes of alkaloids, such as the pyrroloacridine plakinidines[1] or the pyrrolidine containing plakoridines,[2,3] almost all the isolated molecules are assumed to derive from the polyketide pathway,[4] and most of them contain stable cyclic peroxides. The first of these compounds to be reported was plakortin (**1**), a six membrered ring cycloperoxide found in 1978 by Faulkner's group in a *P. halicondrioides*.[5] Subsequently, a series of related bioactive metabolites have been isolated. They include plakinic acids,[6,7] the strongly antifungal peroxyketals named peroxyplakoric acids,[8] and the recently reported activators of cardiac SR-Ca^{2+} ATPase plakortones A-D.[9] In addition, an unusual furano α,β-unsaturated ester (**2**)[4,10] has also been described.

As a part of our screening for biologically active products from marine sources, we have recently begun the chemical investigation of the sponge *Plakortis simplex*.[11,12] In this paper we report the isolation, structure elucidation, and absolute stereochemistry of three bioactive polyketides from this organism, namely dihydroplakortin (**3**), the α,β-unsaturated ester **4**, and its 9,10-dihydro derivative **5**. Furthermore, the absolute stereochemistry of plakortin (**1**) has been determined.

Although the isolated metabolites possess two different backbone frameworks, they appear closely related from a biogenetic point of view, and a branched dodecanoic acid derivative could be identified as their common precursor.

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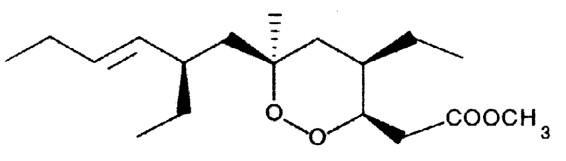

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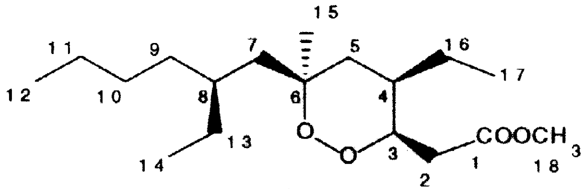

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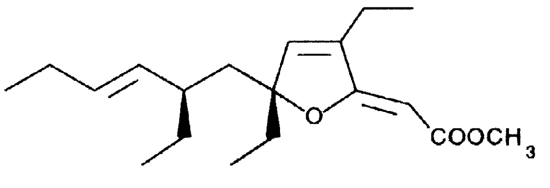

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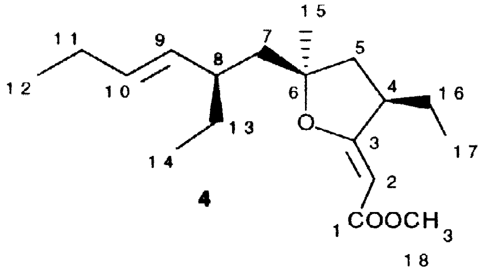

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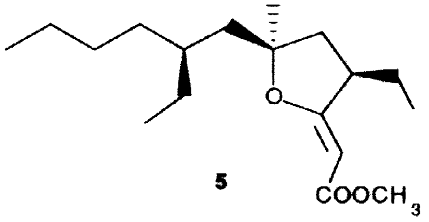

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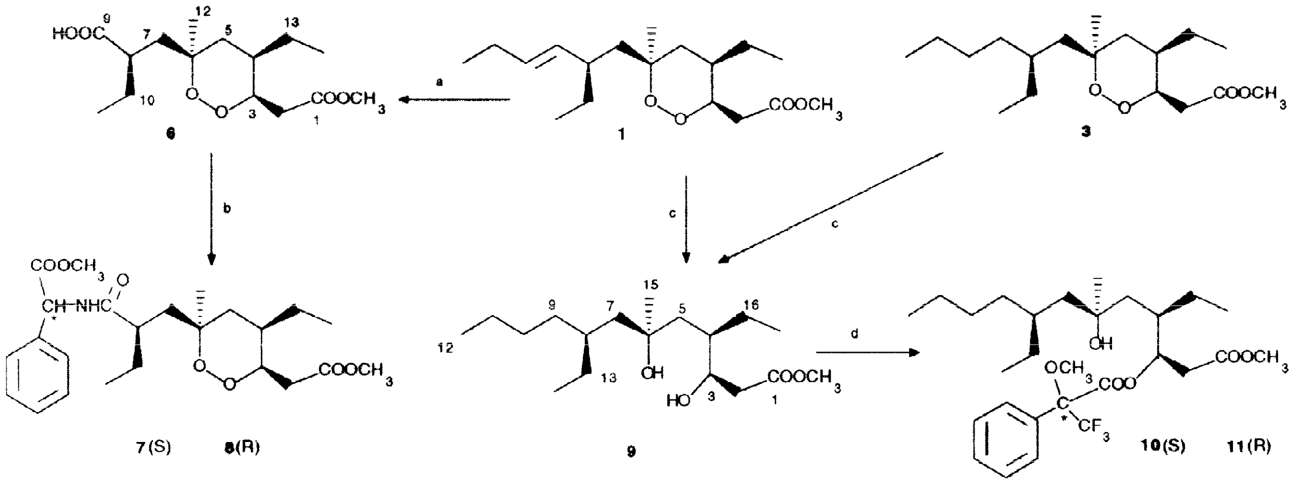
RESULTS AND DISCUSSION

A specimen of *P. simplex* (order Homosclerophorida, family Plakinidae) was collected by hand during an expedition to the Caribbean Sea, and immediately frozen. After homogenization, the organism (57 g dry wt.) was exhaustively extracted first with methanol and then with chloroform. The methanol extract was partitioned between *n*-BuOH and water, and then the organic phase, combined with the $CHCl_3$ extract, was subjected to chromatography over a column packed with reversed phase silica gel (RP18) and eluted with a system of solvents of decreasing polarity form H_2O/MeOH 9:1 to H_2O/MeOH 1:9. The less polar fractions were further purified by a medium pressure liquid chromatography (MPLC) over silica gel, and a preliminary spectroscopic analysis revealed that fractions eluted with *n*-hexane/EtOAc 9:1 and 8:2 were mainly composed of apolar polyketides. Repeated HPLC purifications of these fractions allowed us to obtain plakortin (**1**), and the novel metabolites dihydroplakortin (**3**), compounds **4** and **5** in a pure state, as colorless oils.

The major product, which amounted to more than 90% of the total yield of polyketide fraction, was easily identified as the known plakortin (**1**) by comparison of its $[\alpha]_D$ value and NMR data with those previously reported.[5]

As stated above, plakortin (**1**) was the first polyketide peroxide isolated from *Plakortis* sponges; nevertheless, this molecule, its 3-epimer, and the free acid (named plakortic acid) have been isolated by several research groups as the major bioactive metabolites of many species of the genus *Plakortis*.[4,6,13-15] However, in spite of the wide occurrence of plakortin, the absolute configuration of its four stereogenic centers C-3, C-4, C-6 and C-8 has never been defined. Only the relative stereochemistry of the peroxide ring is known, being determined in the original work of Faulkner *et al.*[5] by a lanthanide-induced shift study.



Scheme 1. *a.* $KMnO_4/NaIO_4$ in *t*-BuOH. *b.* (*S*) or (*R*) PGME, benzotriazolyloxytri(pyrrolidinyl) phosphonium hexafluorophosphate (PyBoP) (1.5 eq.), 1-hydroxybenzotriazole (HOBT) (1.5eq), *N*-methylmorpholine (4 eq.), in DMF. *c.* H_2 on 10% Pd *d.* (R) or (S) MTPA chloride in dry pyridine.

Our strategy for the assignment of absolute stereochemistry of plakortin is based on application of the Mosher's modified method[16] and of the recently reported Kusumi's method[17] on opportune degradation products (Scheme 1). Oxidative cleavage ($KMnO_4/NaIO_4$ in *t*-BuOH) of the double bond $\Delta^{9,10}$ of plakortin (**1**) afforded the carboxylic acid **6** in high yield (80%). This was then analyzed with the chiral amide Kusumi method,[17] which is based on the reaction between a carboxylic acid and (*S*) and (*R*) phenylglycine methyl ester (PGME). The Δδ (*S*-*R*) values obtained by analysis of ^1H NMR spectra of the PGME amides **7** and **8** are reported in Fig.1. As pointed out by Nagai and Kusumi in their original paper,[17] the differences are not very marked; however, they are completely consistent and indicate the *R* configuration at the C-8 of compound **6**, and thus of plakortin.

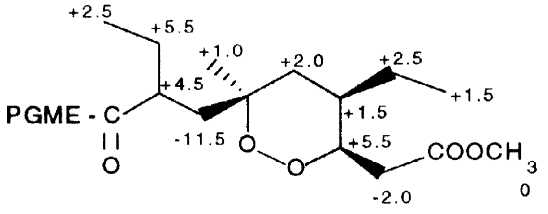


Fig.1. Δδ (*S*-*R*) values (in Hz) for PGME amide derivatives of compound **6**

In order to determine the absolute stereochemistry of the peroxide ring, plakortin was initially reduced to the acyclic diol **9** (H_2/10% Pd, on charcoal catalyst, 88% yield). ^1H and ^{13}C NMR resonances of compound **9** have been fully assigned by 2D methods (COSY, HMQC, HMBC) and are reported in the Experimental Section. Treatment of two aliquots of **9** with (-) and (+) MTPA chloride in dry pyridine provided mono ester derivatives **10** and **11**, respectively, whose Δδ (*S*-*R*) values (fig. 2), according to the modified Mosher method[16] for secondary alcohols, assigned the *R* configuration at C-3.

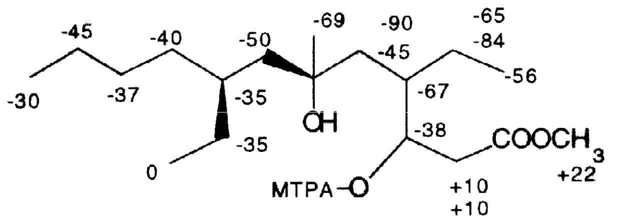


Fig. 2. Δδ (*S*-*R*) values (in Hz) for MTPA ester derivatives of compound **9**

Therefore, on the basis of the already reported relative geometry, the absolute configuration of the C-4 (*R*) and C-6 (*S*) centers could be assigned, and this allowed us to establish the absolute stereostructure of plakortin as shown in **1**.

Together with plakortin, a closely related compound was isolated (**3**; $[\alpha]_D$ +49), and it was characterized as 9,10-dihydroplakortin taking into account the following evidence: i) the EIMS (40 eV) spectrum of **3** exhibited a molecular ion peak at *m/z* 314, two mass units higher than that of **1**. This peak was very small due to a facile loss of the side chain and, accordingly, the base peak was detected at *m/z* 201 (M^+ - C_8H_{17}). The molecular formula $C_{18}H_{34}O_4$ of **3** has been confirmed by high resolution EIMS (found *m/z* 314.2474, calculated *m/z* 324.2457). ii) The ^1H NMR spectrum of **3** appeared almost identical to that of plakortin, the main differences being confined to the lack of the two olefin proton signals, substituted by two overlapping methylene signals between δ 1.22 and 1.24, and to the consistent upfield shift of the H_2-11, H-8 and H_3-12 signals. The structure of this novel metabolite was confirmed by 2D NMR spectroscopy and, in particular, COSY, HMQC, and HMBC spectra were used to assign all the ^1H and ^{13}C NMR resonances of **3** (Table 1).

Absolute stereochemistry of compound **3** has been readily defined by connection with the above determined configuration of plakortin (Scheme 1). In particular, catalytic hydrogenation of dihydroplakortin (H_2/10% Pd, on charcoal catalyst) afforded compound **9** (identified by $[\alpha]_D$ value, ^1H and ^{13}C NMR data), thus indicating that the absolute configuration of the chiral centers of **3** must be assigned as that of the corresponding carbons in plakortin (**1**).

Compound **4** was isolated as a colorless oil, $[\alpha]_D$ + 20. The molecular formula $C_{18}H_{30}O_3$ was deduced by high resolution EIMS (found *m/z* 294.2189, calculated *m/z* 214.2194) and it was in accordance with NMR data. Two of the four degrees of unsaturation required by this formula were attributed to a β-oxy-α,β-unsaturated ester group by analysis of the FT-IR (KBr) spectrum, which showed absorption bands at ν_{max} 1720 and 1690 cm^{-1}. The presence of such functionality was confirmed by ^{13}C NMR resonances of C-1 (δ 166.7), C-2 (δ 86.5), and C-3 (δ 160.5), which are similar to those already reported for compound **2**.[4]

The ^1H NMR spectrum of **4** contained a methyl ester signal at δ 3.66, a singlet at δ 4.71, due to the proton attached to C-2, a methyl singlet at δ 1.30, two olefinic multiplets centered at δ 5.11 and 5.38, respectively, and a series of signals between δ 0.8 and 3.0, which the 2D NMR COSY and HOHAHA spectra allowed us to assign to two distinct spin systems. All the proton resonances observed in the ^1H NMR spectrum were unambiguously associated with the relevant carbon signals in the ^{13}C NMR spectrum using a 2D HMQC experiment (assignment shown in Table 1). A -CH_2CH-CH_2CH_3 moiety was deduced for the first spin system, and, in particular, its CH_2CH- portion must be a part of a tetrahydrofurano ring, as suggested by the key HMBC cross peaks (^2J and ^3J) of both H_2-5 and H-4 with a quaternary oxygen-bearing carbon at δ 90.3 (C-6) and with the enolic carbon at δ 160.5 (C-3), belonging to the α,β-unsaturated ester.

Table 1. ^1H and ^{13}C NMR Spectral Data of Dihydroplakortin (**3**), Compounds **4** and **5** (in $CDCl_3$).

Pos.	**3**		**4**		**5**	
	δC, mult.	δH, mult., *J* in Hz	δC, mult.	δH, mult., *J* in Hz	δC, mult.	δH, mult., *J* in Hz
1	170.9, C		166.7, C		166.7, C	
2a	31.2, CH_2	3.01, dd, 15.9, 9.7	86.5, CH	4.71, s	86.6, CH	4.70, s
b		2.38, dd, 15.9, 3.5				
3	78.9, CH	4.51, m	160.5, C		161.2, C	
4	34.8, CH	2.20, m	45.0, CH	2.91, m	45.2, CH	2.94, m
5a	35.1, CH_2	1.45, dd, 13.5, 4.8	41.5, CH_2	1.97, dd, 12.4, 8.3	41.6, CH_2	2.00, dd, 12.5, 8.0
b		1.30, dd, 13.5, 9.0		1.43, dd, 12.4, 2.0		1.51, dd, 12.5, 2.5
6	80.6, C		90.3, C		90.1, C	
7a	45.2, CH_2	1.37[a]	47.3, CH_2	1.99, dd, 11.8, 5.9	46.2, CH_2	1.68, dd, 13.8, 5.1
b		1.33[a]		1.69, dd, 11.8, 9.9		1.46[a]
8	30.1, CH	1.47, m	42.0, CH	2.00, m	30.8, CH	1.47[a]
9	34.2, CH_2	1.22[a]	134.0, CH	5.11, dd, 15.3, 9.7	34.1, CH_2	1.23[a]
10	29.5, CH_2	1.23[a]	132.5, CH	5.38, dt, 15.3, 6.9	29.4, CH_2	1.24[a]
11	22.7, CH_2	1.27[a]	25.3, CH_2	2.02, m	22.5, CH_2	1.28[a]
12	13.2, CH_3	0.89, t, 6.9	14.3, CH_3	0.96, t, 7.6	13.0, CH_3	0.89, t, 7.3
13a	27.0, CH_2	1.35[a]	29.6, CH_2	1.48, m	26.8, CH_2	1.36[a]
b		1.28[a]		1.22, m		1.45[a]
14	10.1, CH_3	0.83, t, 6.9	12.0, CH_3	0.84, t, 7.6	10.9, CH_3	0.87, t, 7.3
15a	21.5, CH_3	1.38, s	22.5, CH_3	1.30, s	22.3, CH_3	1.32, s
b						
16a	25.0, CH_2	1.21[a]	25.5, CH_2	1.77, m	25.6, CH_2	1.78, m
b		1.13, dq, 13.9, 6.9		1.33, m		1.36[a]
17a	10.8, CH_3	0.92, t, 6.9	11.8, CH_3	0.93, t, 7.6	11.8, CH_3	0.95, t, 7.3
b						
18	52.0, CH_3	3.70, s	50.5, CH_3	3.66, s	50.5, CH_3	3.65, s

[a] Overlapped with other signals.

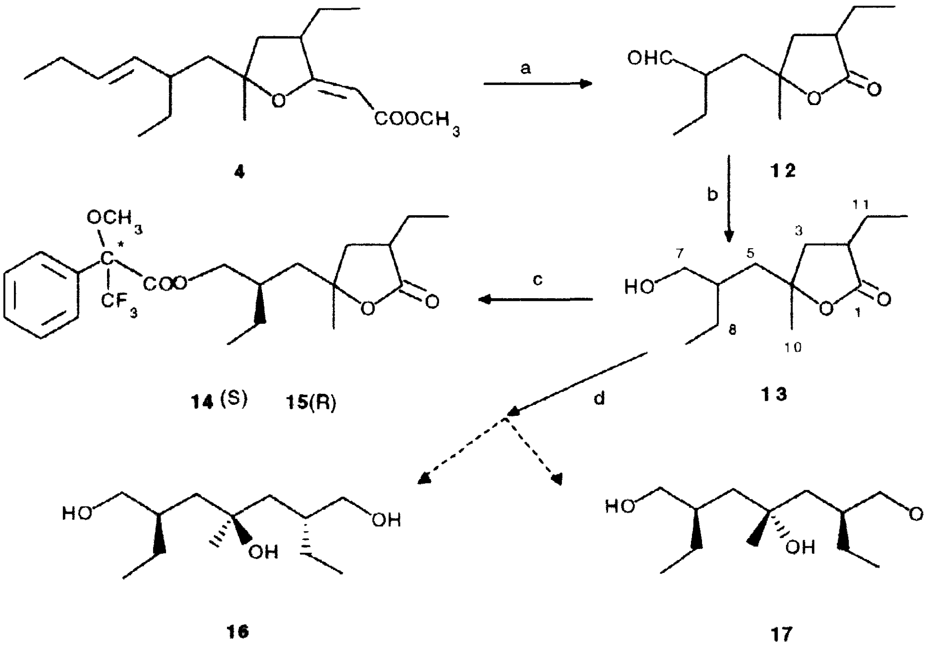
In addition, the HMBC correlation peaks of C-3 with H_2-16 and of C-5 with H_3-15 further confirmed the proposed C-1 to C-6 substructure, which must possess ethyl and methyl ramifications at C-4 and C-6, respectively. The remaining fragment of **4** was easily assigned because its ^1H NMR values appeared very similar to those of the alkyl chain of plakortin, the comparison being guided by inspection of cross-peaks in the COSY spectrum of **4**. The *E* geometry of the $\Delta^{9,10}$ double bond was inferred by H-9/H-10 coupling constant (J = 15.3 Hz). Once again the HMBC spectrum was used to confirm the connection of this side chain with the furano ring; diagnostic cross-peaks were detected between H_2-7 and C-6 and C-15 and between H-8 and C-6. Based on these data, the gross structure of compound **4** was assigned as the methyl ester of 3,6-epoxy-4,8-diethyl-6-methyl-2,9-dodecadienoic acid.

The ROESY spectrum of **4** showed correlation peaks indicating the spatial proximity of H-4 with H-5a and H_3-15, which allowed us to infer the relative stereochemistry around the tetrahydrofurano ring. In addition, coupling of H-2 with H-4 and H_2-16, was indicative of the *Z* configuration of the $\Delta^{2,3}$ double bond.

With the gross structure of **4** in our hands, the following task was to establish its absolute stereochemistry. The strategy used is summarized in Scheme 2. Treatment of **4** with osmium tetroxide/sodium periodate produced the oxidative cleavage of the two double bonds affording the lactone **12**, which contains an aldehyde function. Compound **12** was initially oxidized to the corresponding carboxylic acid, but, unfortunately, in this case, application of the above used Kusumi method furnished very small ^1H NMR differences between the two diastereomeric amides, which were judged not sufficient to unambiguously deduce the stereochemistry.

In order to apply a recently proposed MTPA method[15,18,19] for the assignment of absolute stereochemistry at C-2 of primary β-substituted alcohols, compound **12** was reduced to the corresponding alcohol **13** by reaction with sodium borohydride in MeOH. Compound **13** was so treated with (-) and (+) MTPA chloride in dry pyridine, and afforded quantitatively the esters **14** and **15**, respectively. Comparison of the C-7 methylene proton chemical shifts in the ^{1}H NMR spectra of **14** (δ 4.23 and 4.21) and **15** (δ 4.35 and 4.13), according to the reported models, suggested *R* configuration at C-6 of **13**, corresponding to C-8 of compound **4**.

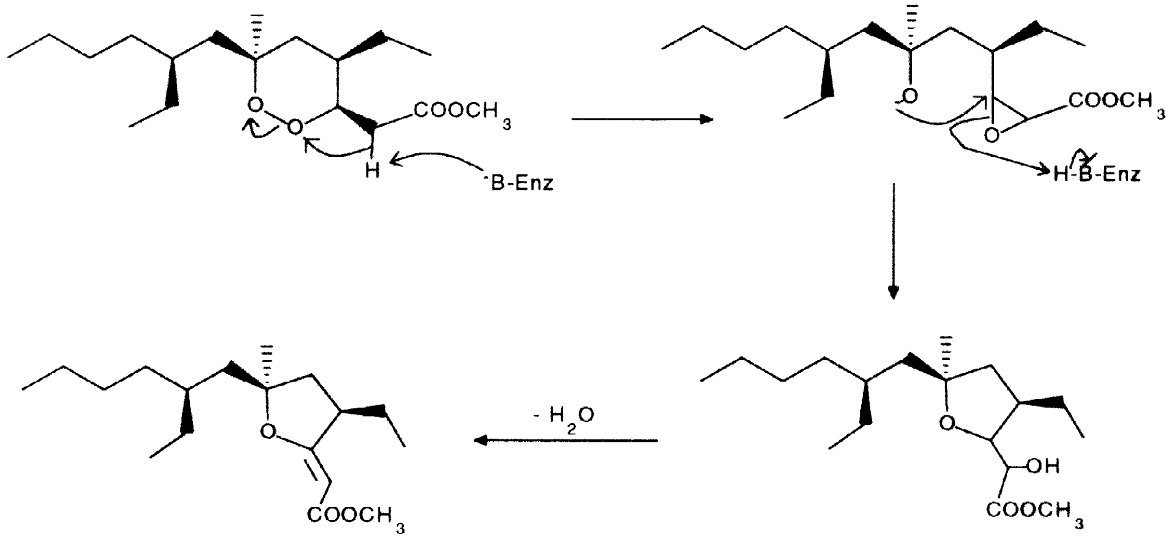
The absolute configuration of the two remaining asymmetric centers of compound **4** was assigned on the basis of the properties of the product obtained by reduction of lactone **13** with $LiAlH_4$. Taking into account the above determined stereochemistry of C-8 and the reported relative geometry around the tetrahydrofurano ring, one of the two triols **16** and **17** could be produced (Scheme 2). Triol **17**, possessing a C_S symmetry, is an optically inactive *meso* form, while triol **16** is a chiral molecule. Therefore only measurement of the $[\alpha]_D$ value and examination of ^{1}H NMR spectrum were needed for the assignment of their structures. Considering that the molecule obtained by reduction of lactone **13** showed $[\alpha]_D$ + 15 and a quite complex ^{1}H NMR spectrum, both indicative of chirality, it must possess structure **16**. Consequently, we assign to compound **4** the 4*R*,6*S* configuration.



Scheme 2. *a.* $OsO_4/NaIO_4$ in phosphate buffer/CH_3CN *b.* $NaBH_4$ in MeOH *c.* (R) or (S) MTPA chloride in dry pyridine *d.* $LiAlH_4$ in dry ether.

The gross structure of the last metabolite, methyl-3,6-epoxy-4,8-diethyl-6-methyl-2-dodecenoate (**5**, colorless oil, $[\alpha]_D$ +5), was determined using arguments which parallel those above employed for dihydroplakortin. In particular, the molecular formula, established as $C_{18}H_{32}O_3$ by high resolution EIMS, is two mass units higher than that of **4**. Moreover, both ^{1}H and ^{13}C NMR spectra of **5**, lacking the two olefin resonances, substituted by signals in the alkyl region, were in perfect agreement with the structure of a hydrogenated derivative of compound **4**. Full assignment of the ^{1}H and ^{13}C NMR resonances of **5** was achieved by 2D NMR spectroscopy and is reported in Table 1. The relative stereochemistry around the tetrahydrofurano ring has been deduced by the strong correlation peak of H-4 with H_3-15 evident in the ROESY spectrum. Furthermore, as in **4**, coupling of H-2 with H-4 and H_2-16 was indicative of the *Z* configuration of the $\Delta^{2,3}$ double bond.

Unfortunately, the very small amount obtained for **5** prevented us from determining its absolute configuration. We confidently propose the stereochemistry reported in the figure, by assuming that compound **5** could biosynthetically originate from dihydroplakortin following the enzyme-mediated pathway illustrated in Scheme 3. This mechanism parallels that recently hypothesized by Faulkner *et al.*[20] for the biosynthetic origin of compound **2**. Probably, in a quite similar manner the novel compound **4** could derive from plakortin (**1**). Our hypothesis appears strengthened by the absolute stereochemistries found for **1** and **4**.



Scheme 3. Proposed enzymatic mechanism for the biosynthesis of compound **5** from dihydroplakortin

Some cyclic peroxides of plakortin[5] and of chondrillin[21] families have been investigated for pharmacological activities and a general trend seems to emerge. Most cycloperoxide acids have exhibited antifungal[6-8,22] and/or antibacterial[5,22] activities which disappear[6,22] when the acids are converted to the corresponding esters. On the contrary, cytotoxic activities seem not to be so dramatically affected by esterification, and some cytotoxic cycloperoxide esters have also been reported.

We evaluated plakortin (**1**) and its 9,10-dihydro derivative (**3**) for cytotoxic activity against WEHI 164, murine fibrosarcoma cell line, at 72 h. Plakortin is cytotoxic with IC_{50} of 7.0 μg/mL, whereas, interestingly, its hydrogenated analogue is much less active ($IC_{50} > 20$ μg/mL).

Not much is known about the pharmacological activity of metabolites belonging to the structural class of compound **2**. Recently Faulkner *et al.*[20] have reported that two furans very strictly related to **2** were active against the proliferation of *Leishmania mexicana* promastigotes, whereas no data were available about their cytotoxic activity. In our test against WEHI 164 compounds **4** and **5** resulted moderately cytotoxic (IC_{50} of 10.0 and 15.5 μg/mL, respectively).

EXPERIMENTAL SECTION

General Methods. Optical rotations were measured in $CHCl_3$ on a Perkin-Elmer 192 polarimeter equipped with a sodium lamp (λ = 589 nm) and a 10-cm microcell. IR (KBr) spectra were recorded on a Bruker model IFS-48 spectrophotometer. UV spectra were obtained in CH_3CN using a Beckman DU70 spectrophotometer. Low and high resolution EI mass spectra (70 or 40 eV) were performed on a VG Prospec (FISONS) mass spectrometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were determined on a Bruker AMX-500 spectrometer; chemical shifts are referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26, δ_C = 77.0). Homonuclear ^1H connectivities were determined by using COSY experiments. One bond heteronuclear ^1H-^{13}C connectivities were determined with HMQC pulse sequence using a BIRD pulse of 0.50 s before each scan in order to suppress signals originating from protons not directly bound to ^{13}C (interpulse delay set for $^1J_{CH}$ = 130 Hz). During the acquisition time, ^{13}C broad band decoupling was performed using the GARP sequence. Two and three bond ^1H-^{13}C connectivities were determined by HMBC experiments optimized for a $^{2,3}J$ of 8.5 Hz. Medium-pressure liquid chromatographies (MPLC) were performed using a Büchi 861 apparatus with RP18 and SiO_2 (230-400 mesh) stationary phases. High performance liquid chromatography (HPLC) separations were achieved on a Beckman apparatus equipped with RI detector and LUNA Si60 (250 x 4 mm) columns. Solvents were provided by Carlo Erba; reagents by Sigma

Collection, extraction and purification. A specimen of *Plakortis simplex* was collected in Summer 1992 along the coasts of Little San Salvador Island, and identified by Prof. M. Pansini (Università di Genova). A voucher specimen has been deposited at the Istituto di Zoologia, Università di Genova, Italy with the ref. n° 2006. The organism was immediately frozen after collection and kept frozen until extraction, when the sponge (57 g, dry weight after extraction) was homogenized and extracted with methanol (4 x 500 mL) and with chloroform (4 x 500 mL). The methanol extract was initially partitioned between H_2O and *n*-BuOH and then the organic phases were combined and concentrated *in vacuo* affording 29.3 g of a brown-colored viscous oil. This was subjected to chromatography on a column packed with RP18 silica gel and eluted with a system of solvents of decreasing polarity from MeOH/H_2O 1:9 to 9:1. Fractions eluted with MeOH/H_2O 8:2 and 9:1 were combined (13.3 g) and further chromatographed by MPLC (SiO_2 230-400 mesh; solvent gradient system of increasing polarity from *n*-hexane to MeOH). Fractions eluted with *n*-hexane/EtOAc 9:1 and 8:2 were separately rechromatographed by HPLC (eluant *n*-hexane/EtOAc 94:6, flow 0.8 mL/min) affording plakortin (**1**, 1.08 g), dihydroplakortin (**3**, 3.5 mg), compound **4** (3.0 mg), and compound **5** (0.8 mg) in a pure state.

Oxidative cleavage of Plakortin. To a solution of plakortin (20 mg) in 10 mL of *t*-BuOH were added 4 mL of 0.04 M Na_2CO_3 and 22 mL of an aqueous solution 0.023 M in $KMnO_4$ and 0.09 M in $NaIO_4$. The reaction was allowed to proceed at 37 °C for 20 h with stirring. After acidification with 5 N H_2SO_4, the solution was decolorized with a saturated solution of oxalic acid and extracted with diethyl ether (30 mL, twice). Combined organic phases were dried over Na_2SO_4, filtered and then concentrated *in vacuo*. The obtained fraction appeared exclusively composed by compound **6** (15 mg, 80% yield), pure by TLC, MS and NMR.

Compound **6**. Amorphous solid. $[\alpha]_D^{25}$ +141 (c = 0.009 in $CHCl_3$). mp. 127-129 °C. IR (KBr) ν_{max} 2868, 1745, 1690, 1477, 1444, 1394 cm^{-1}. ^1H NMR ($CDCl_3$): δ 4.50 (H-3, m), 3.70 (H_3-15, s) 3.01 (H-2b, dd, J = 15.9, 9.7 Hz), 2.50 (H-8, m), 2.36 (H-2a, dd, J = 15.9, 3.5 Hz), 2.19 (H-4, m), 2.02 (H-7b, dd, J = 14.6, 9.7 Hz), 1.62 (H-10a, m), 1.54 (H-10b, m), 1.50 (H-7a, dd, J = 14.6, 2.0 Hz), 1.44 (H-5b, dd, J = 13.9, 4.2 Hz), 1.37 (H_3-12, s), 1.34, (H-5a, dd, J = 13.9, 2.0 Hz), 1.22 (H-13b, m), 1.14 (H-13a, m), 0.92 (H_3-11, t, J = 7.6 Hz), 0.90

(H_3-14, t, J = 7.6 Hz). EIMS (40 eV) *m/z* 302 (M^+), 201 (M^+ – side chain: $C_{10}H_{17}O_4$), 127 (M^+ – CH_2COOCH_3 - side chain: $C_7H_{11}O_2$). HREIMS: *m/z* 302.1733, calcd. for $C_{15}H_{26}O_6$ 302.1729.

Preparation of PGME amides of compound **6**. To a solution of compound **6** (4.0 mg, 0.013 mmol) in DMF (1 mL) at 0 °C, 3.0 mg (1.5 eq.) of (*S*) PGME hydrochloride, 8.1 mg of benzotriazolyloxytri (pyrrolidinyl) phosphonium hexafluorophosphate (PyBoP) (1.5 eq.), 2.0 mg of 1-hydroxybenzotriazole (HOBT) (1.5 eq), 5.5 μL of *N*-methylmorpholine (4 eq.) were added and the mixture was stirred at room temperature for 3 h. After addition of brine, the reaction mixture was extracted three times with EtOAc/benzene 2:1 and the obtained organic phase was washed in sequence with 50 mL of 1.2 N HCl, water, $NaHCO_3$ saturated aqueous solution, and water. The obtained organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressures affording the (*S*) PGME amide **7** (5.5 mg, 95 % yield). Using (*R*) PGME hydrochloride, the same procedure afforded the (*R*) PGME amide **8** in the same yield. No racemization occurred in the reaction.

Compound **7**. [(*S*) PGME amide]. Pale yellow amorphous solid. mp. 56-58 °C. IR (KBr) ν_{max} 2297, 1740, 1678, 1591, 1477, 1444, 1394 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.34 (phenyl protons, m), 6.52 (PGME N$\underline{H}$, d, J = 6.5 Hz), 5.56 (PGME C$\underline{H}$, d, J = 6.5 Hz), 4.50 (H-3, m), 3.70 (PGME OC$\underline{H}_3$, s), 3.68 (H_3-15, s), 3.00 (H-2b, dd, J = 14.0, 9.6 Hz), 2.37 (H-2a, dd, J = 14.0, 3.7 Hz), 2.31 (H-8, m), 2.19 (H-4, m), 2.05 (H-7b, dd, J = 14.7, 9.6 Hz), 1.72 (H-7a, dd, J = 14.7, 4.5 Hz), 1.61 (H-10b, m), 1.46 (H-10a, overlapped), 1.44 (H-5b, partially overlapped), 1.37 (H_3-12, s), 1.35, (H-5a, dd, J = 13.9, 2.0 Hz), 1.20 (H-13b, m), 1.14 (H-13a, m), 0.91 (H_3-11, t, J = 6.6 Hz), 0.89 (H_3-14, t, J = 7.4 Hz). FABMS (glycerol matrix, positive ions) *m/z* 450 $[M+H]^+$. HRFABMS: *m/z* 450.2423, calcd. for $C_{24}H_{35}NO_7$ 450.2414.

Compound **8**. [(*R*) PGME amide]. Pale yellow amorphous solid. IR (KBr) ν_{max} 2299, 1739, 1679, 1594, 1474, 1442, 1391 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.35 (PGME phenyl protons, m), 6.53 (PGME N$\underline{H}$, d, J = 6.4 Hz), 5.55 (PGME C$\underline{H}$, d, J = 6.4 Hz), 4.48 (H-3, m), 3.70 (PGME OC$\underline{H}_3$, s), 3.68 (H_3-15, s), 3.00 (H-2b, dd, J = 16.2, 9.6 Hz), 2.36 (H-2a, partially overlapped), 2.30 (H-8, m), 2.19 (H-4, m), 2.08 (H-7b, dd, J = 14.7, 9.6 Hz), 1.72 (H-7a, dd, J = 14.7, 4.5 Hz), 1.62 (H-10b, m), 1.44 (H-10a, overlapped), 1.43 (H-5b, partially overlapped), 1.37 (H_3-12, s), 1.34, (H-5a, dd, J = 13.9, 2.0 Hz), 1.19 (H-13b, m), 1.14 (H-13a, m), 0.91 (H_3-11, t, J = 7.6 Hz), 0.88 (H_3-14, t, J = 7.6 Hz). FABMS (glycerol matrix, positive ions) *m/z* 450 $[M+H]^+$. HRFABMS: *m/z* 450.2426, calcd. for $C_{24}H_{35}NO_7$ 450.2414

Reduction of plakortin and dihydroplakortin. Palladium on charcoal catalyst (10%, 20 mg) was added to 180 mg of plakortin in dry EtOH. The solution was stirred at r.t. under an atmosphere of hydrogen for 5 h. The catalyst was then removed by filtration and the solvent evaporated to obtain a mixture, which, purified by HPLC on SI60 column (eluent *n*-hexane/EtOAc 8:2), afforded the diol **9** in a pure state (160 mg, 88% yield). The same compound **9** was obtained when 2 mg of dihydroplakortin (**3**) were subjected to the same procedure.

Compound **9**. Colorless oil. $[\alpha]_D^{25}$ +13 (c = 0.024 in $CHCl_3$). ^1H NMR ($CDCl_3$): δ 4.15 (H-3, dt, J = 10.4, 2.8 Hz), 3.67 (H_3-18, s) 2.50 (H-2b, dd, J = 16.6, 10.4 Hz), 2.32 (H-2a, dd, J = 16.6, 2.8 Hz), 1.90 (H-4, m), 1.59 (H-5b, dd, J = 15.3, 9.0 Hz), 1.39 (H-8, overlapped), 1.38 (H-7b, overlapped), 1.34 (H-13b, overlapped), 1.31 (H-7a, overlapped), 1.28 (H-13a, H_2-11, H-5a, H-16b overlapped),1.24 (H_2-10, m), 1.22 (H_2-9, m), 1.14 (H_3-15, s), 1.11 (H-16a, m), 0.90 (H_3-17, t, J = 7.6 Hz), 0.85 (H_3-12, t, J = 6.9 Hz), 0.81 (H_3-14, t, J = 7.6 Hz). ^{13}C NMR ($CDCl_3$): δ 170.5 (C-1), 72.4 (C-6), 70.3 (C-3), 51.7 (C-18), 48.6 (C-7), 41.2 (C-5), 40.3 (C-4), 40.0 (C-8), 34.7 (C-9), 34.6 (C-2), 28.8 (C-10), 27.6 (C-13), 26.5 (C-16), 26.0 (C-11), 23.0 (C-15), 14.1 (C-12), 12.3 (C-17), 10.3 (C-14). EIMS (40 eV) *m/z* 316 (M^+), 283 (M^+ - H_2O - CH_3). HREIMS: *m/z* 316.2606, calcd. for $C_{18}H_{36}O_4$ 316.2614.

Preparation of MTPA esters of compound **9**. Diol **9** (2.6 mg) was dissolved in 2 mL of dry pyridine, treated with (-) MTPA chloride (90 μL) and then maintained at r.t., with stirring, overnight. After removal of the solvent, the reaction mixture was purified by HPLC on SI60 column (eluent *n*-hexane/EtOAc 85:15), affording (*S*) MTPA ester **10** in a pure state (3.8 mg, 85% yield). Using (+) MTPA chloride, the same procedure afforded the (*R*) MTPA ester **11** in the same yield.

Compound **10**. [(*S*) MTPA ester]. Amorphous solid. mp. 45-47 °C. IR (KBr) ν_{max} 3354, 2868, 1740, 1687, 1571, 1541, 1468, 1387, 1186, 1128 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.35 and 7.45 (MTPA phenyl protons, m), 5.63 (H-3, m), 3.59 (MTPA $OC\underline{H}_3$, s), 3.35 (H_3-18, s), 2.52 (H-2b, dd, *J* = 16.2, 8.1 Hz), 2.44 (H-2a, dd, *J* = 16.2, 4.0 Hz), 1.60 (H-4, m), 1.18 (H-5b, overlapped), 1.15 (H-8, m), 1.12 (H-16b, overlapped), 1.11 (H-7b, overlapped), 1.11 (H-11b, overlapped), 1.05 (H_2-13, m), 1.03 (H-7a, overlapped), 0.98 (H-9b, overlapped), 0.98 (H-5a, overlapped) 0.97 (H-9a, overlapped), 0.97 (H_2-10, overlapped), 0.96 (H-11a, overlapped), 0.95 (H-16a, overlapped), 0.86 (H_3-15, s), 0.62 (H_3-17, t, *J* = 7.3 Hz), 0.59 (H_3-12, t, *J* = 7.0 Hz), 0.55 (H_3-14, t, *J* = 7.0 Hz). FABMS (glycerol matrix, positive ions) *m/z* 533 $[M+H]^+$. HRFABMS: *m/z* 533.3080, calcd. for $C_{28}H_{44}O_6F_3$ 533.3090.

Compound **11**. [(*R*) MTPA ester]. Amorphous solid. IR (KBr) ν_{max} 3350, 2869, 1740, 1687, 1573, 1541, 1468, 1388, 1186, 1129 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.32 and 7.55 (MTPA phenyl protons, m), 5.71 (H-3, m), 3.64 (MTPA $OC\underline{H}_3$, s), 3.30 (H_3-18, s), 2.51 (H-2b, dd, *J* = 16.2, 8.6 Hz), 2.43 (H-2a, dd, *J* = 16.2, 4.7 Hz), 1.73 (H-4, m), 1.36 (H-5b, dd, *J* = 14.3, 6.7 Hz), 1.25 (H-16b, overlapped), 1.22 (H-8, m), 1.21 (H-7b, overlapped), 1.13 (H-7a, overlapped), 1.12 (H-11b, overlapped), 1.12 (H-16a, overlapped), 1.12 (H_2-13, overlapped), 1.11 (H-5a, overlapped), 1.06 (H-9b, overlapped), 1.06 (H_2-10, overlapped), 1.06 (H-11a, overlapped), 1.05 (H-9a, overlapped), 1.00 (H_3-15, s), 0.73 (H_3-17, t, *J* = 7.3 Hz), 0.66 (H_3-12, t, *J* = 7.0 Hz), 0.55 (H_3-14, t, *J* = 7.3 Hz). FABMS (glycerol matrix, positive ions) *m/z* 533 $[M+H]^+$. HRFABMS: *m/z* 533.3083, calcd. for $C_{28}H_{44}O_6F_3$ 533.3090.

Dihydroplakortin (**3**). Colorless oil. $[\alpha]_D^{25}$ +49 (c = 0.002 in $CHCl_3$); IR (KBr) ν_{max} 1737, 1480, 1450, 1386, 1000 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (40 eV) *m/z* 314 (M^+), 241 ($M^+ - CH_2COOCH_3$), 201 (base peak, M^+ – side chain: $C_{10}H_{17}O_4$); HREIMS: *m/z* 314.2474, calcd. for $C_{18}H_{34}O_4$ 314.2457; *m/z* 201.1138, calcd. for $C_{10}H_{17}O_4$ *m/z* 201.1127.

*Methyl 3,6-epoxy-4*R*,8*R*-diethyl-6*S*-methyl-2*Z*,9*E*-dodecadienoate* (**4**). Colorless oil. $[\alpha]_D^{25}$ +20 (c = 0.001 in $CHCl_3$); IR (KBr) ν_{max} 1720, 1690, 1630, 1286 cm^{-1}. ^1H and ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV): *m/z* 294 (M^+), 263 ($M^+ - OCH_3$), 183 (M^+ – side chain: C_8H_{15}); HREIMS: *m/z* 294.2189, calcd. for $C_{18}H_{30}O_3$ *m/z* 294.2194; *m/z* 183.1034, calcd. for $C_{10}H_{15}O_3$ *m/z* 183.1021.

Oxidative cleavage of compound **4**. A suspension of compound **4** (2.2 mg) and $NaIO_4$ (25 mg) in phosphate buffer (pH 7, 350 μL), H_2O (350 μL) and CH_3CN (700 μL) was stirred with OsO_4 (200 μL of 0.02 M solution in t-BuOH) at room temperature for 2 h. The reaction mixture was diluted with ether and the organic layer was separated and concentrated *in vacuo*. The obtained residue, containing aldehyde **12**, was dissolved in MeOH (1 mL), and treated with an excess of $NaBH_4$ (8.0 mg) at 0°C. After 1h, the reaction mixture was quenched with aqueous saturated NH_4Cl (500 μL) and extracted three times with EtOAc. The extracts were washed with $NaHCO_3$ aqueous saturated solution, dried over Na_2SO_4, filtered and concentrated. Practically pure compound **13** (1.4 mg) was so obtained.

Compound **13**. Amorphous solid. mp. 40-42 °C. IR (KBr) ν_{max} 3395, 2925, 1732, 1450, 1376, 1250 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.66 (H_2-7, m), 2.95 (H-2, m), 2.02 (H-5a, dd, *J* = 11.8, 6.5 Hz), 1.95 (H-3a, dd, *J* = 12.5, 8.5 Hz), 1.88 (H-6, m), 1.85 (H-11a, m), 1.70 (H-5b, dd, J = 11.8, 10.1 Hz), 1.41 (H-8a, overlapped), 1.40 (H-3b, overlapped), 1.38 (H_3-10, s), 1.32 (H-11b, m), 1.28 (H-8b, m), 0.96 (H_3-12, t, J = 7.2 Hz), 0.92 (H_3-9, t, J = 7.2 Hz). EIMS (70 eV): *m/z* 214 (M^+). HREIMS: *m/z* 214.1586, calcd. for $C_{12}H_{22}O_3$ 214.1569.

Preparation of MTPA esters of compound **13**. Compound **13** (0.3 mg) was dissolved in 300 μL of dry pyridine, treated with (-) MTPA chloride (10 μL) and then stirred overnight at r.t. After removal of the solvent, the obtained solid contained the (*S*) MTPA ester **14**. Using (+) MTPA chloride, the same procedure afforded the (*R*) MTPA ester **15**.

Compound **14**. [(*S*) MTPA ester]. IR (KBr) ν_{max} 1732, 1687, 1460, 1375, 1184, 1132 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.37 and 7.47 (MTPA phenyl protons, m), 4.23 (H-7a, dd, J = 10.5, 4.5), 4.21 (H-7b, dd, J = 10.5, 3.9), 3.57 (MTPA OCH_3, s), 2.96 (H-2, m), 2.05 (H-5a, dd, J = 11.5, 6.5 Hz), 1.97 (H-3a, dd, J = 12.5, 8.5 Hz), 1.92 (H-6, m), 1.85 (H-11a, m), 1.73 (H-5b, dd, J = 11.5, 10.3 Hz), 1.41 (H-8a, overlapped), 1.40 (H-3b, overlapped), 1.38 (H_3-10, s), 1.32 (H-11b, m), 1.28 (H-8b, m), 0.96 (H_3-12, t, J = 7.2 Hz), 0.90 (H_3-9, t, J = 7.2 Hz). EIMS (70 eV): *m/z* 430 (M^+). HREIMS: *m/z* 430.1979, calcd. for $C_{22}H_{29}O_5F_3$ 430.1967.

Compound **15**. [(*R*) MTPA ester]. IR (KBr) ν_{max} 1734, 1689, 1462, 1372, 1184, 1130 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.33 and 7.54 (MTPA phenyl protons, m), 4.35 (H-7a, dd, J = 10.8, 4.5), 4.13 (H-7b, dd, J = 10.8, 5.9), 3.63 (MTPA $OC\underline{H}_3$, s), 2.95 (H-2, m), 2.07 (H-5a, dd, J = 11.7, 6.5 Hz), 1.96 (H-3a, dd, J = 12.5, 8.5 Hz), 1.94 (H-6, m), 1.86 (H-11a, m), 1.72 (H-5b, dd, J = 11.7, 10.3 Hz), 1.42 (H-8a, overlapped), 1.41 (H-3b, overlapped), 1.37 (H_3-10, s), 1.32 (H-11b, m), 1.28 (H-8b, m), 0.96 (H_3-12, t, J = 7.6 Hz), 0.90 (H_3-9, t, J = 7.6 Hz). EIMS (70 eV): *m/z* 430 (M^+). HREIMS: *m/z* 430.1969, calcd. for $C_{22}H_{29}O_5F_3$ 430.1967.

Reduction of compound **13**. Lithium aluminium hydride (1.5 mg) was added to a stirred solution of compound **13** (0.8 mg, 0.0037 mmol) in dry ether at 0°C. After stirring for 20 min, the excess reagent was destroyed with EtOAc and the product was partitioned between ether and dilute HCl solution. The ether layer was dried over Na_2SO_4, filtered, and the solvent evaporated to yield the triol **16** (0.6 mg), in the pure state.

Compound **16**. Amorphous solid. $[\alpha]_D^{25}$ +15. ^{1}H NMR ($CDCl_3$): δ 3.68 (1H, overlapped), 3.67 (1H, overlapped), 3.66 (2H, overlapped), 1.89 (1H, m), 1.87 (1H, m), 1.74 (1H, overlapped), 1.73 (1H, overlapped), 1.58 (1H, m), 1.57 (1H, m), 1.36 (1H, m), 1.34 (1H, m), 1.26 (3H, s), 1.19 (1H, m), 1.17 (1H, m), 0.92 (3H, t, J = 7.3 Hz), 0.91 (3H, t, J = 7.3 Hz). EIMS (60 eV): *m/z* 218 (M^+).

*Methyl-3,6-epoxy-4*R*,8*R*-diethyl-6*S*-methyl-2*Z*-dodecenoate* (**9**). Colorless oil. $[\alpha]_D^{25}$ +5 (c = 0.001 in $CHCl_3$); IR (KBr) ν_{max} 1725, 1687 cm^{-1}. ^{1}H and ^{13}C NMR ($CDCl_3$): see Table 1. EIMS (70 eV): *m/z* 296 (M^+), 265 ($M^+ - OCH_3$); HREIMS: *m/z* 296.2359, calcd. for $C_{18}H_{32}O_3$ *m/z* 296.2353.

Cytotoxicity assay. WEHI 164 (1 × 10^4 cells) were plated on 96-well plates in 50 μL and allowed to adhere at 37°C in 5% CO_2/95% air for 2 h in Dulbecco's modified Eagle's medium (DMEM, from Biowhittaker, Boehringer Ingelhneim). Thereafter, the medium was replaced with 50 μL of fresh medium, and 50 μL of 1:4 v/v serial dilutions of test compounds **1**, **3**, **4**, and **5** were added and the cells were incubated for 72 h. The cell viability was assessed through the MTT conversion assay.[23] Briefly, after incubation, 25 μL of MTT (from Sigma; 5 mg/mL) were added to each cell, and the cells were incubated for additional 3 h. After this time, the cells were lyzed and the dark blue crystals solubilized with 100 μL of a solution containing 50% v/v SDS with an adjusted pH of 4.5.[24] The optical density (OD) of each cell was measured with a microplate spectrophotometer equipped with a 620-nm filter. The viability of cell line in response to treatment with each compounds **1**, **3**, **4**, and **5** was calculated as % dead cells = 100 - (OD treated/OD control) × 100. The results are expressed as IC_{50} (the concentration that inhibited the cell growth by 50%).

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