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Metabolites from the Sponge *Plakortis simplex*. Part 3:[1] Isolation and Stereostructure of Novel Bioactive Cycloperoxides and Diol Analogues

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Dedicated to the dearest memory of Professor Francesco Cafieri

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Abstract—The known plakortide H (**1**), the novel cycloperoxide polyketides plakortide I (**2**) and J (**3**), and the diol analogues *seco*-plakortide H (**4**) and *seco*-plakortide I (**5**), have been isolated from the Caribbean marine sponge *Plakortis simplex* as their methyl esters **1a**–**5a**, and their structures fully characterized by spectroscopic and chemical means. The absolute stereochemistry at the three chiral sites C-3, C-4, and C-6 of plakortide H (**1**) and of the novel compounds **2**–**5** have been determined by applying the modified Mosher method on certain reaction products. The isolated compounds exhibited good cytotoxic activity against WEHI 164, a murine fibrosarcoma cell line. Cycloperoxide esters and carboxylic acids were found considerably more active than the corresponding acyclic diol analogues.

Stable cycloperoxides are commonly found as secondary metabolites of marine sponges belonging to the Plakinidae family. Most of the isolated compounds exhibit a 1,2 dioxane ring, substituted at position 3 with an acetate residue and at positions 4,6,6 with alkyl chains, respectively.[2] Plakortin, the first of such compounds, was reported in 1978 from the sponge *Plakortis halichondrioides*.[3] After its isolation, several related polyketides have appeared in the literature, and the nature of the aliphatic chains at position 6, together with the stereochemistry of the asymmetric centers belonging to the cycloperoxide ring represent the main sites of structural variation.[2] These compounds often exhibit antibacterial,[4] antifungal,[5] and cytotoxic[6] activities and some peroxides have also been shown to be active against the protozoan *Leishmania mexicana*.[7] Finally, plakortides F–H, recently isolated from *P. halichondrioides*,[8] were found to significantly enhance Ca^{2+} uptake by the cardiac sarcoplasmic reticulum. However, in spite of this richness of bioactivities, the biological role and the whole pharmacological potential of *Plakortis* cycloperoxides still need to be completely disclosed.

Encouraged by these data, we have recently begun the chemical investigation of the Caribbean sponge *Plakortis simplex*. We have previously reported the isolation of novel cyclic peroxides and related furano esters, in addition to the elucidation of the absolute stereochemistry of plakortin.[9] Further investigation of the apolar organic extract has now led to the isolation of the known plakortide H (**1**),[8] of two novel cycloperoxides named plakortide I (**2**) and J (**3**), and of the ring-opened analogues *seco*-plakortide H (**4**) and *seco*-plakortide I (**5**), which we have shown to be cytotoxic in vitro against murine fibrosarcoma, WEHI-164. Although a great number of *Plakortis* species have been examined so far, to the best of our knowledge, diol analogues of plakortin or plakortide cycloperoxides have never been reported before.

The sponge *Plakortis simplex* (order Homosclerophorida, family Plakinidae) was collected by hand along the coast of Berry Island (Bahamas) and immediately frozen. After homogenization, the organism (57 g, dry weight) was exhaustively extracted, in sequence, with methanol and 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 repeated chromatography over a column packed first with reversed phase (RP18) and then with normal (SI60) silica gel, affording two mixtures of carboxylic acids. In spite of our efforts, we were not able to further purify them by HPLC, using various conditions. These mixtures were therefore methylated with CH_2N_2 and then purified by direct-phase HPLC leading to the methyl esters (**2a**–**5a**) of four novel compounds (**2**–**5**), together with the known methyl ester

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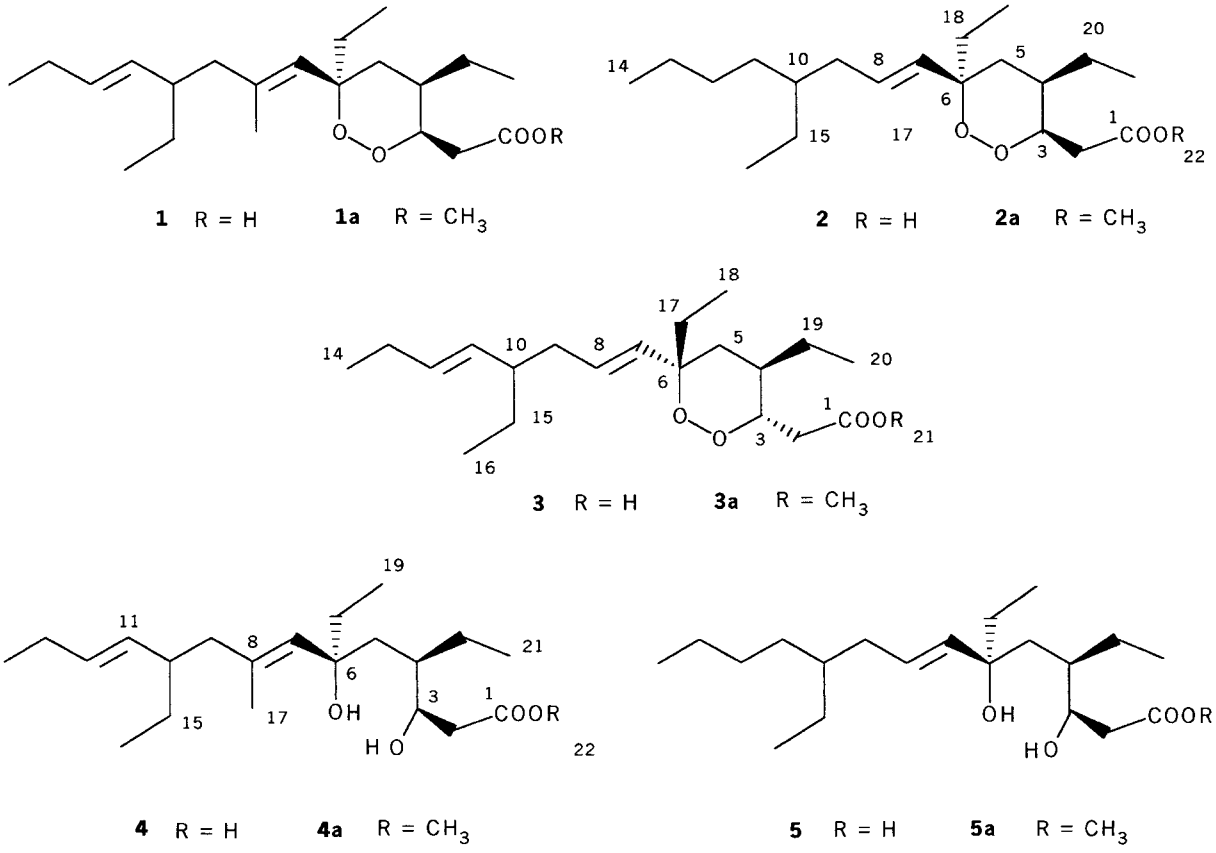
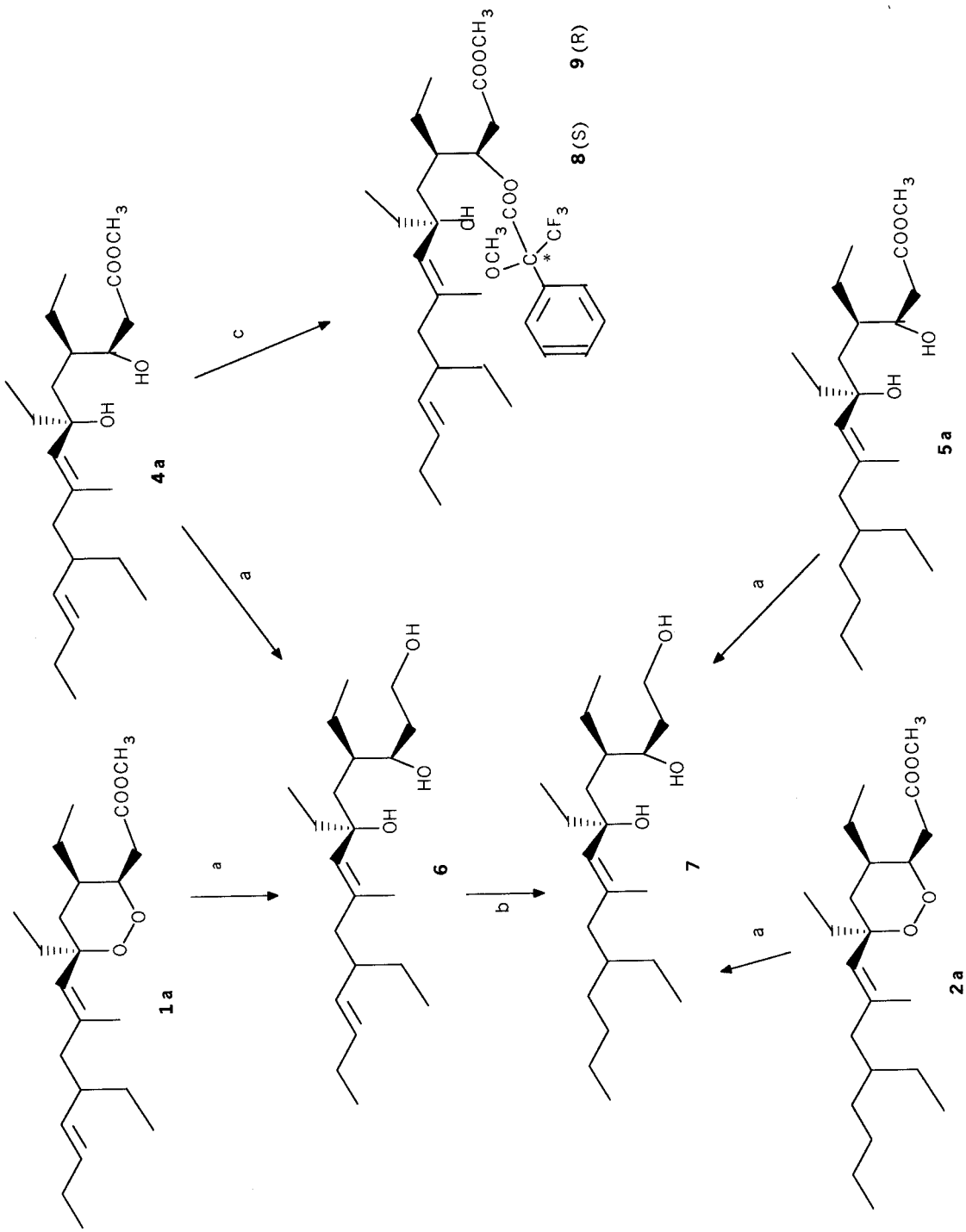


Table 1. ^{1}H and ^{13}C NMR data of Plakortides I (**2a**) and J (**3a**) methyl esters (in $CDCl_3$)

Pos.	**2a**		**3a**	
	δC, mult.	δH, mult., *J* in Hz	δC, mult.	δH, mult., *J* in Hz
1	171.5, C		175.1, C	
2a	31.5, CH_2	3.04, dd, 14.7, 9.6	38.4, CH_2	2.62, dd,15.4, 3.5
b		2.38, dd, 14.7, 3.0		2.28, m
3	78.5, CH	4.48, m	82.5, CH	4.21, dt,10.3, 10.3, 3.5
4	35.3, CH	2.08, m	39.1, CH	1.59, m
5a	32.3, CH_2	1.72, dd, 10.5, 3.7	38.4, CH_2	2.03[a]
b		1.28[a]		1.21[a]
6	85.1, C		88.3, C	
7	127.5, CH	5.17, s	133.5, CH	5.48, d, 14.7
8	137.2, C		130.4, CH	5.46[a]
9a	42.3, CH_2	1.98, dd, 13.2, 7.4	40.6, CH_2	2.12, ddd, 14.0, 10.3, 5.9
b		1.91, dd, 13.2, 8.1		2.06[a]
10	32.1, CH	1.46, m	46.9, CH	1.92, m
11	33.1, CH_2	1.26[a]	133.4, CH	5.15, dd,15.4, 8.8
12	29.0, CH_2	1.27[a]	132.4, CH	5.42, dt, 15.4, 6.6
13a	22.5, CH_2	1.21[a]	27.9, CH_2	1.99[a]
b		1.13[a]		1.98[a]
14	13.0, CH_3	0.90, t, 7.3	16.1, CH_3	0.97, t, 7.3
15a	25.1, CH_2	1.26[a]	29.7, CH_2	1.45[a]
b		1.25[a]		1.21[a]
16	10.0, CH_3	0.88, t, 7.3	13.6, CH_3	0.82, t, 7.3
17	17.0, CH_3	1.70, s	35.4, CH_2	1.45[a]
18a	23.2, CH_2	1.97[a]	12.7, CH_3	0.82, t, 7.3
b		1.28[a]		
19a	8.0, CH_3	0.85, t, 7.3	26.3, CH_2	1.43[a]
b				1.10, dq, 7.3, 2.2
20a	25.2, CH_2	1.25[a]	12.5, CH_3	0.90, t, 7.3
b		1.24[a]		
21	10.2, CH_3	0.84, t, 7.3	52.5, CH_3	3.69, s
22	52.0, CH_3	3.70, s		

[a] Overlapped with other signals.



Scheme 1. (a) $LiAlH_4$ in dry ether; (b) H_2/Pd; (c) (*R*) and (*S*) MTPA chloride in dry pyridine.

1a. This was easily assigned as plakortide H methyl ester, considering that its $[\alpha]_D$ value, ^1H and ^{13}C NMR spectra were identical with those reported in the literature.[8]

Compound **2a**, named plakortide I methyl ester, was isolated as a colorless oil, $[\alpha]_D=-42$. Its structure was attributed to the 11,12 dihydroderivative of **1a**, by interpretation of the following spectral evidence. First of all, HREIMS of **2a** accounted for the molecular formula $C_{22}H_{40}O_4$, containing two H more than **1a**. Decisive information on structure **2a** was obtained by inspection of the ^1H and ^{13}C NMR spectra ($CDCl_3$, Table 1), and through the analysis of 2D experiments (COSY, HMQC, and HMBC). Interpretation of these data was greatly aided by comparison with parallel spectra obtained for plakortide H (**1**). In particular, the ^1H NMR spectrum of **2a** appeared quite similar to that of **1a**, with the key exception of the lack of H-11 and H-12 olefinic signals (substituted by two overlapped 2H multiplets at δ 1.26 and 1.27, respectively) and of an upfield shift of the surrounding protons in the ^1H NMR spectrum of **2a**. In accordance, the ^{13}C NMR spectrum of **2a** exhibited the resonances of only two olefinic carbons at δ 127.5 (C-7) and 137.2 (C-8). Also the relative stereochemistry around the cycloperoxide ring was shown to be the same as that of plakortide H. In particular, the ^1H NMR resonances of H-4 (δ 2.08), H-3 (δ 4.48), and H_2-2 (δ 3.04 and 2.38) were very close to those of the corresponding protons in **1a**, and the NOE couplings between H_2-2 and H-5ax (δ 1.28), H-3 and H-4, and between H-4 and H_2-18 (δ 1.97 and 1.28), observed for **2a**, were all indicative of the same relative geometry reported for plakortide H. Moreover, the ROESY cross peak of H-7 (δ 5.17) with H_2-9 (δ 1.98 and 1.91) indicated the *E* geometry of the double bond, defining the structure of plakortide I methyl ester (**2a**).

By separation of a more polar fraction obtained from the same extract, *seco*-plakortide H methyl ester (**4a**) and *seco*-plakortide I methyl ester (**5a**) were isolated and their structures attributed to those of the diol analogues of the cycloperoxides plakortides H and I, respectively.

Compound **4a**, an amorphous solid, $[\alpha]_D=+13$, showed, based on HREIMS data, the molecular formula $C_{22}H_{40}O_4$ (the same as compound **2a**). This formula implies two H atoms more than plakortide H. However, unlike **2a**, a preliminary inspection of the ^1H and ^{13}C NMR spectra of **4a** (assignments reported in Table 2) indicated that two double bonds were still present. On the other hand, a detailed analysis of these spectra, aided by the 2D NMR experiments COSY, HMQC and HMBC, indicated a structure practically identical to that of plakortide H methyl ester (**1a**). As a consequence, the presence of two more hydrogen atoms in **4a** could be only rationalized by assuming that C-3 and C-6 are not involved in a peroxidic linkage, and they actually carry two hydroxyl groups. This was further proved by the presence of: (i) two D_2O exchangeable signals in the ^1H NMR spectrum of **4a** (measured in DMSO-d_6); (ii) an OH stretching band at ν_{max} 3340 cm^{-1} in the IR (KBr) spectrum. Consequently, structure **4a** was assigned to plakortide I methyl ester.

Using a parallel reasoning, comparison of the spectral data of compound **5a** (amorphous solid, $[\alpha]_D=-4$, molecular formula $C_{22}H_{42}O_4$, determined by HREIMS) with those reported above for plakortide I (**2a**) easily allowed us to assign the reported structure **5a**, corresponding to *seco*-plakortide I. All the ^1H and ^{13}C NMR signals of **5a** were assigned by 2D NMR experiments (COSY, HMQC, and HMBC) and are reported in Table 2.

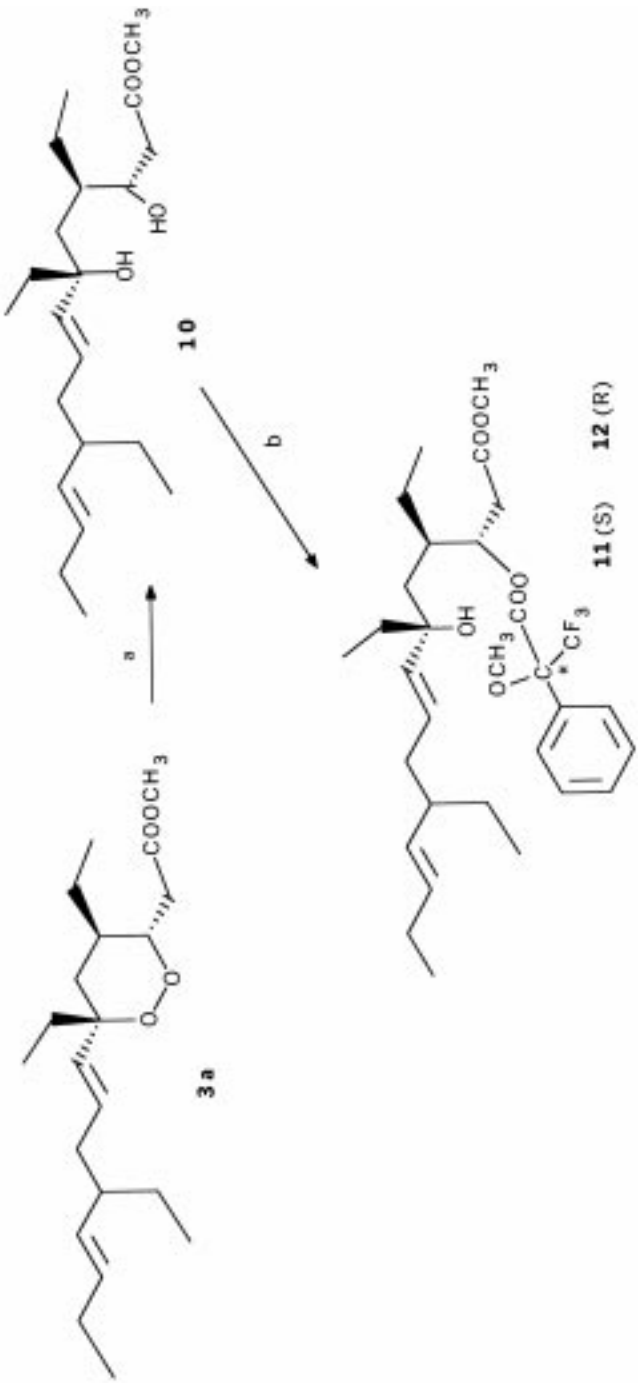
Table 2. ^1H and ^{13}C NMR data of *seco*-Plakortides H (**4a**) and I (**5a**) methyl esters (in $CDCl_3$)

Pos.	4a		5a	
	δC, mult.	δH, mult., *J* in Hz	δC, mult.	δH, mult., *J* in Hz
1	175.2, C		174.5, C	
2a	37.0, CH_2	2.59, dd, 16.9, 10.3	46.3, CH_2	2.57, dd, 16.9, 10.2
b		2.34, dd, 16.9, 1.3		2.36, dd, 16.9, 1.2
3	71.5, CH	4.18, dd, 10.3, 1.3	70.0, CH	4.18, dt, 10.2, 1.2
4	40.5, CH	1.88[a]	40.8 CH	1.91, m
5a	42.3, CH_2	1.57[a]	41.2, CH_2	1.58, dd, 14.7, 8.8
b		1.48[a]		1.49, dd, 14.7, 2.2
6	78.5, C		76.2, C	
7	131.5, CH	4.49, s	131.5, CH	4.97, s
8	132.0, C		136.5, C	
9	42.5, CH_2	2.02[a]	46.4, CH_2	1.92, m
10	48.1, CH	1.91[a]	36.8, CH	1.44, m
11	134.4, CH	5.09, dd, 15.4, 7.3	25.8,CH_2	1.26[a]
12	132.5, CH	5.38, dt, 15.4, 6.6	25.8, CH_2	1.26[a]
13a	25.3, CH_2	2.02[a]	26.2, CH_2	1.17, m
b		2.01[a]		
14	14.4, CH_3	0.97, t, 7.3	12.6, CH_3	0.91, t, 7.3
15a	27.8, CH_2	1.40, m	26.1, CH_2	1.26[a]
b		1.15, m		
16	12.3, CH_3	0.82, t, 7.3	14.8, CH_3	0.91, t, 7.3
17	17.2, CH_3	1.75, s	17.0, CH_2	1.78, s
18a	26.8, CH_2	1.30[a]	28.5, CH_2	1.58[a]
b		1.22[a]		1.28[a]
19	8.2, CH_3	0.89, t, 7.3	8.3, CH_3	0.92, t, 7.3
20a	23.7, CH_2	1.23[a]	29.1, CH_2	1.28[a]
b		1.15, m		1.28[a]
21	12.7, CH_3	0.90, t, 7.3	11.2, CH_3	0.85, t, 7.3
22	52.1, CH_3	3.71, s	52.0, CH_3	3.71, s

[a] overlapped with other signals.

With the gross structure of compounds **1a**, **2a**, **4a** and **5a** (two polyketide cycloperoxides and their diol analogues) in our hands, the following task was to establish the absolute stereochemistry of these molecules, including also that of the known plakortide H (**1**), for which only the relative stereochemistry was reported in the original paper.[8] The reductive cleavage of the cycloperoxide ring represented the key reaction for such a study, since it affords a molecule suitable for the application of the Mosher method for secondary alcohols[10] and moreover, in our case it allowed us to relate the *seco*-compounds to the corresponding cycloperoxides. The key molecule of our reaction scheme (Scheme 1) was the major metabolite **4a**. Unfortunately, the stereochemistry of the chiral center C-10 had to be left unassigned, since any conceivable determination strategy would require much more material than we had in our hands.

Reduction of both compounds **1a** and **4a** with $LiAlH_4$ in dry ether at room temperature afforded the same triol **6** in high yield, $[\alpha]_D=+4$; ^1H NMR data (assignment obtained with 2D methods) are reported in the Experimental. Analogously, treatment of both compounds **2a** and **5a** under the same conditions afforded the triol **7** ($[\alpha]_D=+8$,



Scheme 2. (a) Zn in AcOH; (b) (*R*) and (*S*) MTPA chloride in dry pyridine.

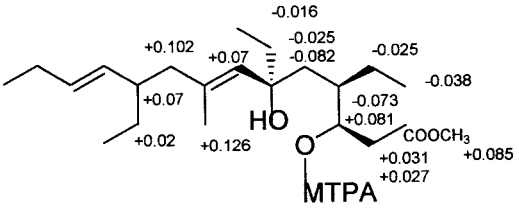


Figure 1. $\Delta\delta$ ($S-R$) values (in ppm) for MTPA ester derivatives of **4a**.

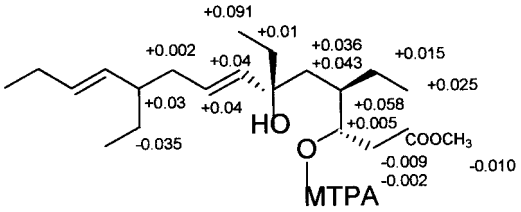


Figure 2. $\Delta\delta$ ($S-R$) values (in ppm) for MTPA ester derivatives of **10**.

^{1}H NMR assignment reported in the Experimental). Then, in order to connect the stereochemistry of **1a** and **4a** with that of the corresponding 11,12 dihydro analogues **2a** and **5a**, compound **6** was reduced with H_2/10% Pd, on charcoal catalyst yielding a molecule identical to the above reported triol **7**, by selective hydrogenation of the $\Delta^{11,12}$ double bond. The results of these reactions unambiguously demonstrate that compounds **1a**, **2a**, **4a**, and **5a** actually possess the same stereochemistry at the stereogenic centers C-3, C-4, C-6, and C-10. Their absolute configuration, except that of C-10, was then established by application of the modified Mosher method for secondary alcohols on the major compound **4a**. Treatment of two aliquots of **4a** with (−)- and (+)-MTPA chloride in dry pyridine provided mono ester derivatives **8** (*S*) and **9** (*R*), respectively, whose $\Delta\delta$ (*S-R*) values (Fig. 1), in accordance with the Mosher model, indicated the *R* configuration at the C-3. When coupled with the previously reported relative geometry of plakortide H, this result assigned the absolute configurations of C-4 (*R*) and C-6 (*R*) of **4a**, as well as those of the structurally related compounds **1a**, **2a**, and **5a**.

Plakortide J methyl ester (**3a**, $[\alpha]_D=-47$), a colorless oil, has the molecular formula $C_{21}H_{36}O_4$, determined by HR-EIMS. The IR absorption band at ν_{max} 1744 cm^{-1} was assigned to an ester carbonyl (confirmed by a signal at δ 175.1 in the ^{13}C NMR spectrum), and the small band at ν_{max} 1189 cm^{-1} was suggestive of a peroxidic linkage. The ^{13}C NMR spectrum (Table 1) of **3a** contained 21 carbon resonances including signals for three oxygen-bearing sp^3 carbons: those at δ 88.3 (C) and 82.5 (CH) appeared quite similar to the reported values of carbon atoms flanking the peroxide bond,[9] while that at δ 52.5 could be easily attributed to the ester methoxy group. In addition, resonances of two disubstituted double bonds (doublets at δ 130.4, 132.4, 133.4, and 133.5) were also present, and accounted for the two remaining formal unsaturations implied by the molecular formula. Finally, the four methyl signals at δ 12.5, 12.7, 13.6, and 16.1, in the ^{13}C NMR spectrum, corresponded to four methyl triplets at δ 0.90, 0.82, 0.82, and 0.97 respectively, in the ^{1}H NMR spectrum (Table 1), as associated by the HMQC NMR spectrum. The COSY spectrum of plakortide J methyl ester (**3a**) indicated the presence of an isolated ethyl group (H_2-17/H_3-18) and of two larger spin systems. The first one extends from H_2-2 (δ 2.62 and 2.28) to the multiplet at δ 4.21 (H-3), which correlates with a methine signal at δ 1.59 (H-4). This latter signal showed in turn cross-peaks with the mutually coupled signals of H_2-5 (δ 1.21 and 2.03), and with the methylene protons at δ 1.43 and 1.10 (H_2-19), both coupled to the methyl group at δ 0.90 (H_3-20). The second spin system starts with the doublet of H-7 and extending until H_3-14, it includes the above determined two double bonds and also the ethyl group (H_2-15/H_3-16) linked at the allylic C-10. The *trans* geometry of both double bonds was deduced by the large coupling constants $J_{H\text{-}7/H\text{-}8}$ (14.7 Hz) and $J_{H\text{-}11/H\text{-}12}$ (15.4 Hz). These fragments were interconnected based on some key HMBC correlation peaks: e.g. H-7 with C-17, C-6, and C-5; H-3 with C-1, C-5, and C-19 to give the plain structure of **3a**, which is a novel cyclic peroxide having a plakortide-type skeleton.

The relative stereochemistry around the peroxide ring of **3a** was established by some spatial interactions, evidenced through a ROESY experiment. The cross-peak of H-2a (δ 2.28) with the axial H-4 indicated the 3,4-*trans* geometry, whereas the spatial proximity between H-7 (δ 5.48) and H-4 suggested the *cis* relative orientation of the ethyl groups linked at C-4 and C-6, respectively. The absolute stereochemistry of the chiral centers belonging to the cycloperoxide ring of **3a** has been established using the reactions reported in Scheme 2. In particular, treatment of **3a** with an excess of Zn in acetic acid furnished sufficient yields of diol **10**, which was then esterified at C-3 by (−)- and (+)-MTPA chloride in dry pyridine. Upon analysis of the obtained MTPA derivatives, **11** (*S*) and **12** (*R*), respectively, according to the modified Mosher method (Fig. 2), the stereochemistry of C-3 was established as *S*. Consequently, absolute configurations of C-4 (*R*) and C-6 (*S*) were assigned on the basis of the above deduced relative geometry, and this allowed us to establish the absolute stereostructure of plakortide J as shown in **3a**.

In conclusion, the marine sponge *Plakortis simplex* elaborates a peculiar series of secondary metabolites composed of cycloperoxides from both the plakortin and plakortide structural series, which differ not only in the length of the alkyl chains linked at position 6, but also in the stereochemistry of cycloperoxide ring chiral centers. In addition, two unique diols of the plakortide series have been found in the same sponge. The contemporary presence of cycloperoxides and of diols could be related to the biological role of cycloperoxide-containing polyketides in *Plakortis* sponges.

The methyl esters of plakortides H (**1a**), I (**2a**), and J (**3a**) and the diols *seco*-plakortides H methyl ester (**4a**) and *seco*-plakortides I methyl ester (**5a**) have been evaluated for cytotoxic activity against WEHI 164, a murine fibrosarcoma cell line. In addition, the inseparable mixtures of the corresponding carboxylic acids (**1**–**2**–**3** and **4**–**5**) have also been subjected to the same test. Results, expressed as IC_{50}, are listed in the Experimental section. Plakortides H–J methyl esters inhibited the cell growth at 72 h with IC_{50} ranging from 7 to 9 μg/mL, the most active of the series being compound **1a**. It should be noted that plakortide H had

been reported in 1996 by Patil et al. as activator of cardiac SR–Ca^{2+} ATPase, while no data were known about its cytotoxic activity.[8] The diols were generally much less active than the corresponding cycloperoxides and, as already evidenced for related compounds,[11] the carboxylic acids showed a cytotoxic activity higher (IC_{50} ranging from 2 to 4 μg/mL) than that of their methyl esters.

Experimental

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 spectra were recorded on a Bruker model IFS-48 spectrophotometer. Low and high resolution EI (70 or 45 eV) and FAB (glycerol matrix) mass spectra were performed on a VG Prospec-Autospec (FISONS) mass spectrometer. ^1H (500.13 MHz) and ^{13}C (125.77 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; DMSO-d_6: δ_H=2.50). For a more accurate measurement of the coupling constants, the one-dimensional ^1H NMR spectra were transformed at 64 K points achieving a digital resolution of 0.09 Hz. 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 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 8.0 Hz. Medium-pressure liquid chromatographies (MPLC) were performed using a Büchi 861 apparatus with RP18 and SI60 (230–400 mesh) stationary phases. High performance liquid chromatography (HPLC) separations were achieved on a Beckman apparatus equipped with RI detector and LUNA (250×4 mm) columns.

Collection, extraction and isolation

A specimen of *Plakortis simplex* was collected in Summer 1998 along the coasts of Berry Island (Bahamas), 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. no. 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, in sequence, with methanol (4×500 mL) and with chloroform (4×500 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 EtOAc/*n*-hexane 6:4 were methylated by a saturated solution of CH_2N_2 in Et_2O. After removal of the organic solvent the residue was purified by HPLC (eluant *n*-hexane/EtOAc 98:2, flow 0.8 mL/min) affording, in order of elution, the methyl esters plakortide I (**2a**, 1.0 mg), plakortide H (1, 2.1 mg), and plakortide J (**3a**, 1.1 mg), in a pure state. Fractions eluted with EtOAc/MeOH 7:3 were methylated as above and then purified by HPLC (eluant *n*-hexane/EtOAc 85:15, flow 0.6 mL/min) affording, in order of elution, the methyl esters *seco*-plakortide I (**5a**, 0.8 mg) and *seco*-plakortide H (**4a**, 5.1 mg), in a pure state.

Plakortide I methyl ester (2a). Colorless oil. $[\alpha]_D$=−42 (c=0.01, $CHCl_3$). IR (film): ν_{max} 2960, 2927, 1745, 1455, and 1190 cm^{-1}. ^1H and ^{13}C NMR: see Table 1. EIMS (45 eV): *m/z* 368 (M^+, 15), 337 (75), 215 (100). HREIMS: *m/z* 368.2941 (calcd for $C_{22}H_{40}O_4$, 368.2927). HMBC ($CDCl_3$, H/C): 2/1; 2/3; 2/4; 3/1; 3/20; 3/5; 4/21; 4/6; 5/18; 5/6; 5/7; 7/6; 7/18; 7/5; 9/7; 9/17; 9/15; 10/16; 10/12; 10/8; 15/9; 17/8; 17/7; 17/9; 22/1.

***seco*-Plakortide H methyl ester (4a).** Amorphous colorless solid. $[\alpha]_D$=+13 (c=0.01, $CHCl_3$). IR (KBr): ν_{max} 3340, 2969, 1735, 1450 cm^{-1}. ^1H and ^{13}C NMR: see Table 2. EIMS (45 eV): *m/z* 368 (M^+, 55), 350 (75), 339 (100). HREIMS: *m/z* 368.2920 (calcd for $C_{22}H_{40}O_4$, 368.2927). HMBC ($CDCl_3$, H/C): 2/1; 2/3; 2/4; 3/1; 3/20; 3/5; 4/21; 4/6; 5/18; 5/6; 5/7; 7/6; 7/18; 7/5; 9/7; 9/17; 9/15; 10/16; 10/12; 10/8; 15/9; 17/8; 17/7; 17/9; 22/1.

***seco*-Plakortide I methyl ester (5a).** Amorphous colorless solid. $[\alpha]_D$=−4 (c=0.008, $CHCl_3$). IR (KBr): ν_{max} 3342, 2967, 1735, 1450 cm^{-1}. ^1H and ^{13}C NMR: see Table 2. EIMS (45 eV): *m/z* 370 (M^+, 55), 352 (75), 341 (100). HREIMS: *m/z* 370.3091 (calcd for $C_{22}H_{42}O_4$, 370.3083). HMBC ($CDCl_3$, H/C): 2/1; 2/3; 2/4; 3/1; 3/20; 3/5; 4/21; 4/6; 5/18; 5/6; 5/7; 7/6; 7/18; 7/5; 9/7; 9/17; 9/15; 10/16; 10/12; 10/8; 15/9; 17/8; 17/7; 17/9; 22/1.

Reduction of plakortide H methyl ester (1a) and of *seco*-plakortide H methyl ester (4a). Plakortide H methyl ester (1.5 mg) was dissolved in anhydrous diethyl ether and then $LiAlH_4$ (1 mg) was added. The mixture was left at room temperature under stirring, and the progress of the reduction was monitored by TLC. After completion of the reaction (20 h), H_2O (1 mL) was added and the solution extracted with chloroform. Evaporation in vacuo of the organic phase yielded a crude fraction, that was purified by HPLC using a Si gel column (*n*-hexane–EtOAc 8:2 as eluent) affording triol **6** (1 mg), in a pure state. Reduction of compound **4a** (1.5 mg) using the same procedure furnished 1.1 mg of an alcohol identical to **6**, by comparison of $[\alpha]_D$ value and ^1H NMR data.

Compound 6. Amorphous solid. $[\alpha]_D$=+4 (c=0.01, $CHCl_3$). EIMS: *m/z* 340. ^1H NMR ($CDCl_3$, 500 MHz): δ 5.38 (1H, dt, J=15.4, 5.9 Hz, H-12); 5.09 (1H, dd, J=15.4, 8.1 Hz, H-11); 4.94 (1H, s, H-7); 3.93 (1H, m, H-1a); 3.89 (1H, m, H-3); 3.83 (1H, m, H-1b); 2.02 (1H, overl., H-10); 2.00 (2H, m, H_2-13); 1.90 (2H, m, H_2-9); 1.78

(2H, overl., H_2-2); 1.76 (3H, s, H_3-17); 1.68 (1H, dd, J=14.7, 8.8 Hz, H-5a); 1.48, (1H, dd, J=14.7, 1.5 Hz, H-5b); 1.42 (1H, overl., H-4); 1.40 (1H, overl., H-15a); 1.28 (2H, overl., H_2-18); 1.28 (2H, overl., H_2-20); 1.15 (1H, m, H-15b); 0.96 (3H, t, J=7.3 Hz, H_3-14); 0.90 (3H, t, J=7.3 Hz, H_3-19); 0.90 (3H, t, J=7.3 Hz, H_3-21), 0.84 (3H, t, J=7.3 Hz, H_3-16).

Reduction of plakortide I methyl ester (2a) and of *seco*-plakortide I methyl ester (5a). Plakortide I (0.8 mg) and *seco*-plakortide I methyl ester (0.6 mg) were reduced with $LiAlH_4$ using the same procedure described above. After the usual work up, both of them afforded the triol **7** (in total, 1 mg).

Compound 7. Amorphous solid. $[\alpha]_D$=+8 (c=0.01, $CHCl_3$). EIMS: m/z 342. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 4.98 (1H, s, H-7); 3.93 (1H, m, H-1a); 3.89 (1H, m, H-3); 3.83 (1H, m, H-1b); 1.91 (2H, m, H_2-9); 1.78 (2H, overl., H_2-2); 1.77 (1H, s, H-17); 1.66 (1H, dd, J=14.7, 8.8 Hz, H-5a); 1.45 (1H, overl., H-5b); 1.45 (1H, overl., H-10); 1.42 (1H, overl., H-4); 1.40 (1H, m, H-15a); 1.28 (2H, overl., H_2-18); 1.27 (2H, overl., H_2-20); 1.26 (2H, overl., H_2-11); 1.26 (2H, overl., H_2-12); 1.15 (1H, m, H-15b); 1.13 (2H, overl., H_2-13); 0.90 (3H, t, J=7.3 Hz, H_3-14); 0.90 (3H, t, J=7.3 Hz, H-19); 0.90 (3H, t, J=7.3 Hz, H-21); 0.82 (3H, t, J=7.3 Hz, H_3-16).

Catalytic hydrogenation of triol 6. Palladium on charcoal catalyst (10%) was added to 1.8 mg of triol **6** in dry EtOH. The solution was stirred at rt under an atmosphere of hydrogen for 20 min. 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 compound **7** (0.8 mg) in a pure state.

Preparation of MTPA esters of compound 4a. Diol **4a** (1.5 mg) was dissolved in 60 μL of dry pyridine, treated with (−) MTPA chloride (30 μL) and then maintained at rt, 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 **8** in a pure state (1.3 mg). Using (+) MTPA chloride, the same procedure afforded the (*R*)-MTPA ester **9** in the same yield.

Compound 8 [(*S*) MTPA ester]. Amorphous solid. IR (KBr): ν_{max} 3350, 2866, 1685, 1570, 1540, 1468 cm^{-1}. FABMS (glycerol matrix, positive ions) m/z 585 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.35 and 7.45 (5H, m, MTPA phenyl protons); 5.83 (1H, m, H-3); 5.31 (1H, dt, J=15.4, 5.9 Hz, H-12); 5.01 (1H, dd, J=15.4, 8.8 Hz, H-11); 4.89 (1H, s, H-7); 3.70 (3H, s, H_3-22); 3.59 (3H, s, MTPA OCH_3), 2.65 (1H, dd, J=16.1, 8.1 Hz, H-2a); 2.51 (1H, dd, J=16.1, 5.1 Hz, H-2b); 1.98 (2H, overl., H_2-13); 1.95 (1H, overl., H-10); 1.95 (2H, overl., H_2-9); 1.74 (3H, s, H_3-17); 1.63 (1H, dd, J=9.6, 3.7 Hz, H-5a); 1.47 (1H, m, H-4); 1.44 (1H, overl., H-5b); 1.31 (1H, m, H-18a); 1.25 (1H, m, H-18b); 1.15 (2H, overl., H_2-20); 1.07 (2H, m, H_2-15); 0.90 (t, J=7.3 Hz, H_3-14); 0.83 (t, J=7.3 Hz, H_3-16); 0.81 (t, J=7.3 Hz, H_3-19); 0.76 (t, J=7.3 Hz, H_3-21).

Compound 9 [(*R*) MTPA ester]. Amorphous solid. IR (KBr): ν_{max} 3350, 2869, 1740, 1687, 1573, 1541, 1468. FABMS (glycerol matrix, positive ions) m/z 585 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.32 and 7.55 (5H, m, MTPA phenyl protons), 5.75 (1H, m, H-3); 5.23 (1H, dt, J=15.4, 6.1 Hz, H-12); 4.93 (1H, dd, J=15.4, 8.8 Hz, H-11); 4.88 (1H, s, H-7); 3.64 (3H, s, MTPA OCH_3); 3.61 (3H, s, H_3-22); 2.62 (1H, dd, J=15.4, 8.1 Hz, H-2a); 2.48 (1H, dd, J=15.4, 4.4 Hz, H-2b); 1.91 (2H, overl., H_2-13); 1.88 (1H, overl., H-10); 1.85 (2H, overl., H_2-9); 1.71 (1H, dd, J=9.2, 4.8 Hz, H-5a); 1.61 (3H, s, H_3-17); 1.55 (1H, m, H-4); 1.46 (1H, dd, J=9.2, 4.4 Hz, H-5b); 1.32 (1H, m, H-18a); 1.27 (1H, m, H-18b); 1.17 (2H, overl., H_2-20); 1.05 (2H, m, H_2-15); 0.80 (3H, t, J=7.3 Hz, H_3-19); 0.80 (3H, t, J=7.3 Hz, H_3-21); 0.75 (3H, t, J=7.3 Hz, H_3-14); 0.75 (3H, t, J=7.3 Hz, H_3-16).

Plakortide J methyl ester (3a). Colorless oil. $[\alpha]_D$=−47 (c=0.01, $CHCl_3$). IR (film): ν_{max} 1744, 1455, and 1189 cm^{-1}. ^{1}H and ^{13}C NMR: see Table 1. EIMS (45 eV): m/z 352 (M^+, 15), 337 (75), 321 (100). HREIMS: m/z 352.2622 (calcd for $C_{21}H_{36}O_4$, 352.2614). HMBC ($CDCl_3$, H/C): 2/1; 2/4; 3/1; 3/19; 3/5; 4/20; 4/6; 5/17; 5/7; 7/6; 7/17; 7/5; 9/7; 9/15; 10/16; 10/8; 15/9; 17/7; 21/1.

Reduction of plakortide J methyl ester. Plakortide J (1.0 mg, 0.003 mmol) in 50 μL of dry ether was treated with 100 μL of acetic acid and an excess (20 mg, 0.3 mmol) of Zn and then stirred vigorously for 24 hr. at room temperature. After confirmation of disappearance of the starting material by Si gel TLC, the solution was neutralized with Na_2CO_3 and the solid removed by filtration. The solvent was then evaporated under reduced pressure and the obtained product was partitioned between H_2O and $CHCl_3$. The organic phase contained compound **10** (0.8 mg), in a pure state.

Compound 10. Amorphous solid. $[\alpha]_D$=−8 (c=0.008, $CHCl_3$). EIMS: m/z 354. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.40 (1H, overl., H-12); 5.38 (1H, overl., H-8), 5.29 (1H, d, J=14.7 Hz, H-7); 5.14 (1H, dd, J=15.4, 8.1 Hz, H-11); 4.08 (1H, m, H-3); 3.70 (3H, s, H_3-21); 2.53 (1H, dd, J=12.5, 8.4 Hz, H-2a); 2.32 (1H, dd, J=12.5, 5.9, H-2b); 2.08 (1H, overl., H-10); 2.07 (2H, overl., H_2-9); 2.04 (1H, overl., H-5a); 2.03 (2H, overl., H_2-13); 1.62 (1H, m, H-4); 1.45 (1H, m, H-15a); 1.40 (1H, m, H-19a); 1.25 (2H, overl., H_2-17); 1.25 (1H, overl., H-5b); 1.20 (1H, m, H-15b); 1.10 (1H, m, H-19b); 0.97 (3H, t, J=7.3 Hz, H_3-14); 0.90 (3H, t, J=7.3 Hz, H_3-20); 0.87 (3H, t, J=7.3 Hz, H_3-16); 0.87 (3H, t, J=7.3 Hz, H_3-18).

Preparation of MTPA esters of compound 10. Compound **10** (0.4 mg) was dissolved in 20 μL of dry pyridine, treated with (−) MTPA chloride (10 μL) and then maintained at rt, with stirring, overnight. After removal of the solvent, (*S*)-MTPA ester **11** was obtained (0.6 mg). Using (+) MTPA chloride, the same procedure afforded the (*R*)-MTPA ester **12** (0.5 mg).

Compound 11 [(*S*) MTPA ester]. Amorphous solid. FABMS (glycerol matrix, positive ions) m/z 571 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.35 and 7.45 (5H, m, MTPA phenyl protons); 5.50 (1H, m, H-3); 5.41 (1H,

overl., H-12); 5.38 (1H, overl., H-8); 5.35 (1H, overl., H-7); 5.14 (1H, dd, J=15.4, 8.1 Hz, H-11); 3.60 (3H, s, H_3-21); 3.59 (3H, s, MTPA OCH_3); 2.53 (1H, dd, J=15.2, 8.7 Hz, H-2a); 2.31 (1H, dd, J=15.2, 6.7 Hz, H-2b); 2.13 (2H, overl., H_2-9); 2.07 (1H, overl., H-10); 2.06 (1H, overl.; H-5a); 2.04 (2H, overl., H_2-13); 1.64 (1H, m, H-4); 1.44 (1H, m, H-15a); 1.42 (1H, m, H-19a); 1.29 (2H, overl., H_2-17); 1.25 (1H, overl., H-5b); 1.20 (1H, m, H-15b); 1.20 (1H, m, H-19b); 0.98 (3H, t, J=7.3 Hz, H_3-14); 0.90 (3H, t, J=7.3 Hz, H_3-16); 0.90 (3H, t, J=7.3 Hz, H_3-18); 0.90 (3H, t, J=7.3 Hz, H_3-20).

Compound 12 [(*R*) MTPA ester]. Amorphous solid. FABMS (glycerol matrix, positive ions) *m/z* 571 $[M+H]^+$. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.55 and 7.32 (5H, m, MTPA phenyl protons); 5.50 (1H, m, H-3); 5.38 (1H, overl., H-12); 5.34 (1H, overl., H-8); 5.31 (1H, overl., H-7); 5.08 (1H, dd, J=15.4, 8.1 Hz, H-11); 3.71 (3H, s, H_3-21); 3.64 (3H, s, MTPA OCH_3); 2.54 (1H, dd, J=14.1, 8.7 Hz, H-2a); 2.31 (1H, dd, J=14.1, 6.7 Hz, H-2b); 2.13 (2H, overl., H_2-9); 2.04 (1H, overl., H-10); 2.02 (1H, overl.; H-5a); 2.01 (2H, overl., H_2-13); 1.58 (1H, m, H-4); 1.47 (1H, m, H-15a); 1.41 (1H, m, H-19a); 1.28 (2H, overl., H_2-17); 1.20 (1H, overl., H-5b); 1.20 (1H, m, H-15b); 1.20 (1H, m, H-19b); 0.87 (3H, t, J=7.3 Hz, H_3-20); 0.85 (3H, t, J=7.3 Hz, H_3-16); 0.82 (3H, t, J=7.3 Hz, H_3-14); 0.81 (3H, t, J=7.3 Hz, H_3-18).

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 Ingelheim). Thereafter, the medium was replaced with 50 μL of fresh medium, and 50 μL of 1:4 v/v serial dilutions of test compounds **1a**, **2a**, **3a**, **4a**, **5a**, and the mixtures **1**–**2**–**3** and **4**–**5** were added and the cells were incubated for 72 h. The cell viability was assessed through the MTT conversion assay.[12] After incubation, 25 μL of MTT (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 dissolved with 100 μL of a solution containing 50% (v/v) SDS with an adjusted pH of 4.5.[13] 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 compound was calculated as % dead cells=100-(OD treated/OD control)×100. The results are expressed as IC_{50} (μg/mL, the concentration that inhibited the cell growth by 50%): 7.1 for **1a**; 9.5 for **2a**; 8.2 for **3a**; 14.1 for **4a**; 19.2 for **5a**; 2.5 for the mixture **1**–**2**–**3**; 4.0 for the mixture **4**–**5**.

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