New cytotoxic cyclic peroxide acids from *Plakortis* sp. marine sponge

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Abstract

Bioassay-guided fractionation of the extract of Jamaican marine sponge *Plakortis* sp. followed by preparative TLC and HPLC yielded several known methyl ester cyclic peroxides (**1a, 2a, 3a, 4, 5**), known plakortides (**6,7**), known bicyclic lactone (**8**) and new cyclic peroxide acids (**1b, 2b, 3b**). The chemical structures were elucidated by extensive interpretation of their spectroscopic data. These natural products showed remarkable in vitro cytotoxicity against several cancer cell lines.

Keywords: Plakortis sponge, polyketides, cyclic peroxides, cytotoxicity

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 or the pyrrolidine containing plakoridines almost all the isolated molecules are assumed to be derived from the polyketide pathway and most of them contain stable cyclic peroxides.[1-4] The first of these compounds to be reported was plakortin, a six membered ring cycloperoxide found in 1978 by Faulkner's group in a *Plakortis halicondrioides*.[5]

Subsequently, a series of related bioactive metabolites have been isolated. They include plakinic acids the strongly antifungal peroxyketals named peroxyplakoric acids and the recently reported activators of cardiac SR-Ca $^{2+}$ ATPase plakortones A-D. In addition,[6-10] an unusual furano α, β–unsaturated ester (**6,7**) has also been described.[4,11,12]

As a part of systematic endeavors to isolate bioactive compounds from marine organisms, we have investigated constituents of the sponge *Plakortis* sp., collected at depths of -10 to -20 m in the Discovery Bay, Jamaica. These efforts resulted in the isolation and characterization of known methyl ester cyclic peroxides (**1a, 2a, 3a, 4, 5**), known plakortides (**6, 7**), known bicyclic lactone (**8**) and new acid peroxides (**1b, 2b, 3b**) (Figure 1).

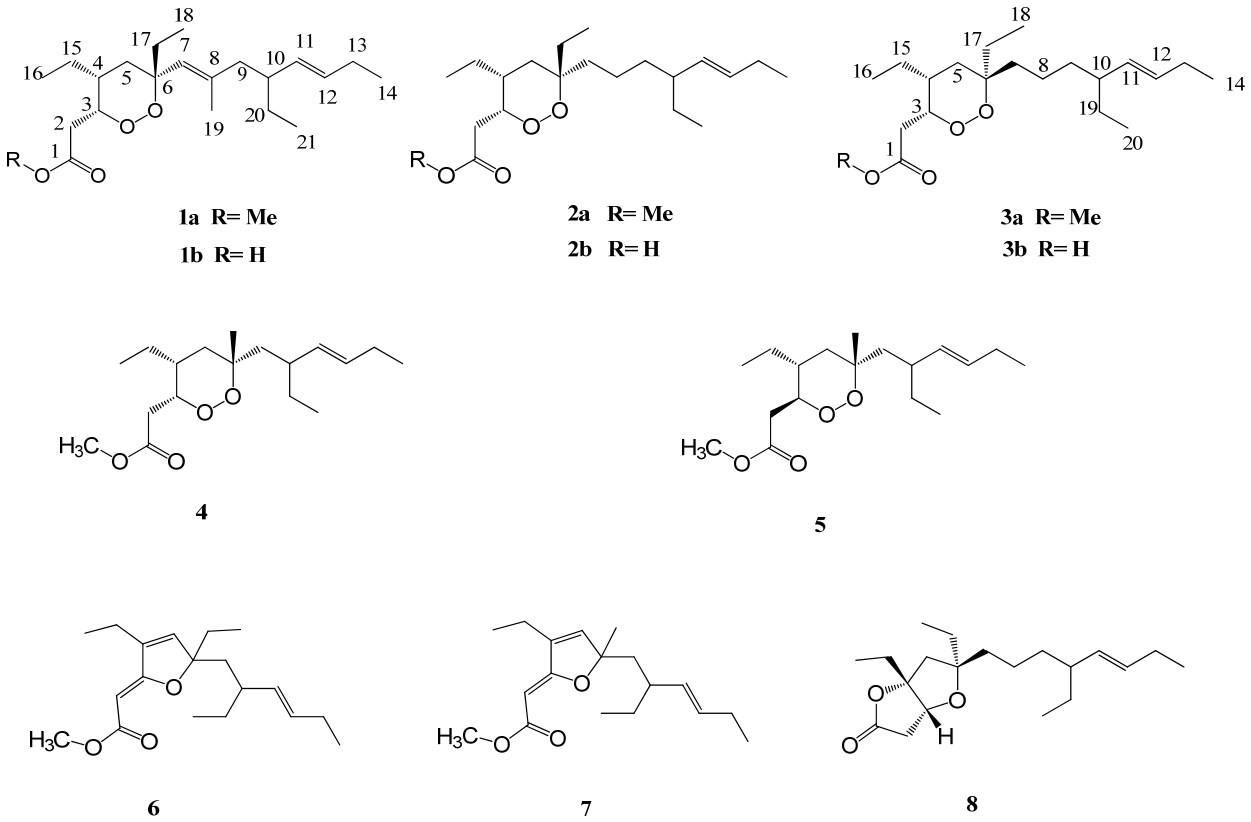


Figure 1. Compounds isolated from *Plakortis* sp.

Results and Discussion

The chloroform/methanol extract of the *Plakortis* sponge, was fractionated on silica gel using *n*-hexane-ethyl acetate as gradient solvent. The fractions were monitored by cytotoxicity bioassays using proliferating mouse cell lines: NIH3T3, KAI3T and Vero cells to afford, in order of elution, eleven compounds (**1-8**). The structures of known compounds **4**,[5] **5**, **6**[4], **7**,[12] **8,**[9] and **1a,**

2a, 3a[10] were established by comparing their physical and spectral data with those in the literature.

The new cyclic peroxide acid, plakortide **1b** was isolated as an oil, the molecular formula $C_{21}H_{40}NO_4$ was deduced by HRCIMS of 370.2960 for $[M + NH_4]^+$ and ^{13}C NMR which required four degrees of unsaturation. The ^1H NMR spectrum of **1b** contained signals that were typical of cyclic peroxides having the plakortin ring system and a side chain.[10] It exhibited three olefinic multiplets at δ 5.37, 5.13, 5.09 and a double doublet of doublets at δ 4.46. In addition there were a number of methylene multiplets between δ 3.05 and 1.15, four methyl triplets at δ 0.95, 0.90, 0.84 and 0.83 and methyl singlet at δ 1.70 brs in the upfield region. The proton signals were divided into a number of separate spin systems by interpretation of ^1H ^1H COESY experiments and the resulting assignments were verified by HMQC and HMBC data. In the side chain of the molecule, the two olefinic multiplets at 5.09 (H-11) and 5.37 (H-12) shared a 15.5 Hz trans coupling. The ^1H ^1H COESY NMR spectrum of **1b** showed a correlation between H-12 and the methylene H-13 at (δ 1.99) of an ethyl group. The H-11 was coupled to the H-10 methine multiplet at δ 2.04 which also shared additional correlations with the methylene signals of an ethyl group H-20 at (δ 1.40, 1.17) and a vinylic methylene H-9 at (δ 2.10, 1.93). Decoupling of the brood H-7 olefinic multiplet at δ 5.13 resulted in the sharpening of the signals due to the H-9 methylene group and the H-19 methyl group at δ 1.70. Therefore there must be ethyl group attached to C-10 and methyl group attached to C-8. The remaining two ethyl groups were located in the cyclic peroxide of **1b** which also incorporated a methane multiplet at δ 4.46 (H-3) which further coupled to a non-equivalent methylene pair (H-2). The DEPT NMR spectrum displayed five methyl, seven methylene, six methine and three quaternary carbon signals . The structure of the cyclic peroxide of the plakortide **1b** was established by evidence based on HMBC data. The H-7 multiplet at δ 5.13 showed correlations to C-8, C-9, and Me-19 of the side chain of **1b** and oxygenated quaternary C-6 at δ 84.3 and C-5 methylene at δ 34.4 in the cyclic peroxide moiety.

The H-4 multiplet at δ 2.07 showed correlations to C-5 methylene at δ 34.4, C-6 at δ 84.3, C-15 at δ 25.1, C-16 at δ 11.0 and oxygenated metaine C-3 at δ 78.3 in the cyclic peroxide moiety. The H-3 signals showed a one bond correlation to the oxygenated C-3 at δ 78.3 and long range correlation to the C-2 at δ 31.5, C-4 at δ 35.7, C-15 at δ 25.1, and carboxlic C-1 at δ 177.9

The new cyclic peroxide acid, plakortide **2b** was isolated as an oil, the molecular formula $C_{20}H_{40}NO_4$ was deduced by HRCIMS of 358.2946 for $[M + NH_4]^+$ and ^{13}C NMR which required three degrees of unsaturation. The ^1H NMR spectrum of **2b** contained signals that were typical of a cyclic peroxide having the plakortin ring system[10] and a side chain. It exhibited two olefinic multiplets at δ 5.38 (1H, dt, *J* 15.0, 6.5, H-12), 5.08 (1H, dd, *J* 15.0, 8.5, H-11) with *trans* coupling and a double doublet of doublets at δ 4.47. In addition there were a number of methylene multiplets between δ 3.04 and 1.14 and four methyl triplets at δ 0.96, 0.92, 0.85 and 0.82. The proton signals were divided into a number of separate spin systems by interpretation of ^1H ^1H COESY experiments and the resulting assignments were verified by HMQC and HMBC data. The DEPT ^{13}C NMR spectrum displayed four methyl, nine methylene, five methine and two quaternary carbon signals . The ^1H ^1H COESY NMR spectrum of **2b** showed a correlation

between H-12 and the methylene of an ethyl group H-13/14. The H-11 was coupled to the H-10 methine multiplet at δ 1.78 which also shared additional correlations with the methylene signals of an ethyl H-19/20 group at δ 1.38, 1.18 and methylene at H-9 (δ 1.47, 1.20). The H-9 protons further correlated to the H-8 and H-7 methylene protons. In the HMBC spectrum of **2b** the nonequivalent H-7 protons at δ 1.32 and 1.20 correlated to C-8 and C-9 in the side chaine, to the C-6 quaternary carbon at the junction of the peroxide ring and the side chain, to the C-5 methylene ring carbon, and finally to C-17 of the isolated C-17/18 ethyl group which was attached to C-6. The Me-18 proton at δ 0.85, which are attached to the upfield methyl carbon at δ 7.1, correlated to C-17 and C-6, establishing that this was the ethyl group attached at the fully substituted C-6.

In the other extended spin system the H-3 methine multiplet at δ 4.47 correlated to the nonequivalent H-2 methylene doublet of doublets δ 3.04 (dd, *J* 15.5, 9.5), 2.38 (dd, *J* 15.5, 3.0) and C-1 at δ 177.3 of the carboxylic group on one side and the C-4 and C-5 in the ring. The Me-16 triplet at δ 0.92 correlated to C-15 (δ 24.2) and C-4 (δ 34.4), confirming that it was part of the remaining ethyl substituent attached at C-4.

The relative stereochemistry about the peroxide ring was established by NOESY correlations, the results of which are summarized with arrows in Figure 2. On saturation of the H-2 methylene protons, correlations with H-3 signal and H-5ax were viewed, establishing that H-2 and H-5ax were on the same face of the peroxide ring. Both the H-3 and Me-16 hydrogens showed a correlation with the axial H-4 signal. The H-4 multiplet shared a 4.5 Hz coupling constant with H-3 and a 12.5 Hz axial/axial coupling constant with H-5ax. The H-4 showed a correlation with the H-5eq. The H-5eq also showed correlation with H-17 methylene protons. Thus, H-4, H-3 and H-17 must be in the opposite face of the peroxide ring.

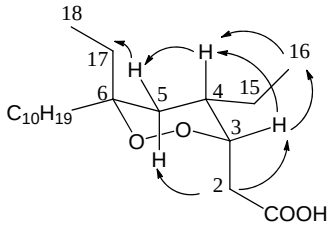


Figure 2. Relative stereochemistry of plakortide (**2b**) with arrows representing nosey H NMR.

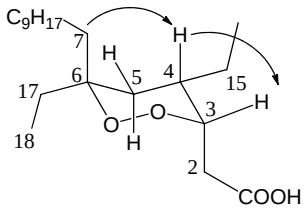


Figure 3. Relative stereochemistry of plakortide (**3b**) with arrows representing nosey H NMR.

The new cyclic peroxide acid, plakortide **3b** was isolated as an oil, the molecular formula $C_{20}H_{40}NO_4$ was deduced by HRCIMS of 358.2935 for $[M + NH_4]^+$. The NMR spectrum was similar to that of **2b**. The COESY spectrum of plakortide **3b** indicated the presence of nearly identical spin systems in **2b** and **3b**. The DEPT ^{13}C NMR and HMQC spectrum of **3b** exhibited 20 carbon signals as was the case for **2b**, and most of the resonances had virtually the same chemical shifts except for two carbon signals. The C-7 resonance in **3b** was observed at δ 32.1, 3.0 ppm further upfield than its δ 35.1 counterpart in **2b**, and the C-17 resonance in **3b** was observed at δ 29.5, 4.4 ppm further downfield than its δ 25.1 value in plakortide **2b**. Since the NMR data indicated that **2b** and **3b** were very similar in structure, these differences suggested that plakortide **3b** is a diastereomer of **2b** with inverted stereochemistry at C-6. This conclusion was further supported by measuring coupling constants and NOESY experiments which are summarized in Figure 3. On saturation of the H-7` at δ 1.49, the methylene protons in the side chain correlated with the axial H-4 signals at δ 2.15, and H-4 showed also correlation with H-3 signal. Thus, H-4 must be on the same face of the peroxide ring as is H-3 and the side chain containing H-7`. Therefore, the C-6 sterochemistry of plakortide **2b** is opposite of that of plakortide **3b**. This conclusion was consistent with the downfield shift of C-7, which is closer to the peroxide ringe oxygens in plakortide **2b** than in **3b**, and the up field shift of C-17 which is further away from the ring oxygen in plakortide **2b** than **3b**.

Finally, cytotoxic assays of the individual compounds after purification (Table 1) were performed using proliferating mouse cell lines, NIH3T3, KA3IT and one Verocells.[13, 14] Compounds **1a** and **2a, 3a** exhibited very low cytotoxicity and **6-8** exhibited moderate cytotoxicity on all cell lines, but compounds **1b** and **2b, 3b** exhibited high cytotoxicity on all cell lines.

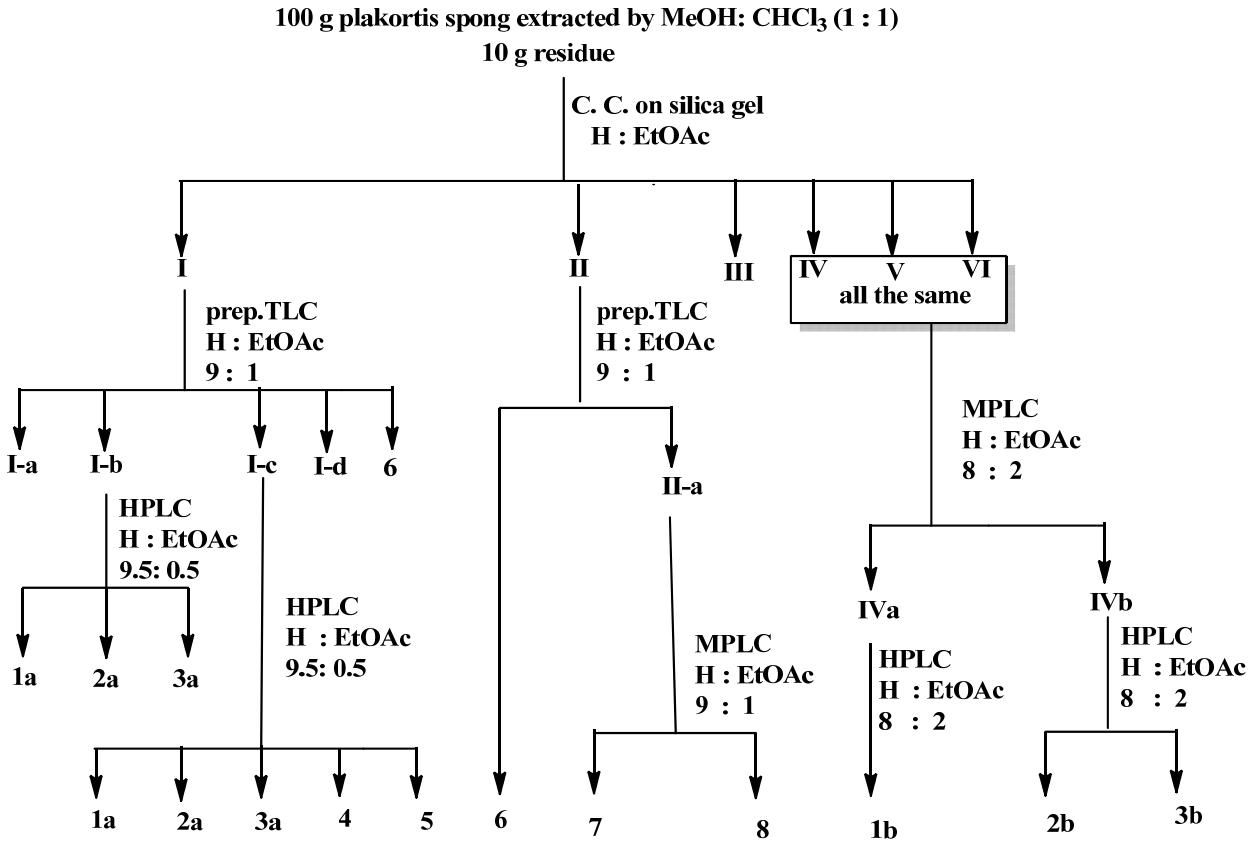
Table 1. Cytotoxicity [IC_{50} (μg/mL)] of the alcohol extract and the purified compounds from marine sponge *Plakortis* sp. in *vitro* proliferating mouse cell lines

Sample	Cell Lines		
	NIH3T3	SSVNIH3T3	KA3IT
Alcohol extract of *plakotis* sponge	1	1	1
(1a)	>100	>100	>100
(3a)	>100	>100	>100
(6)	21	14	14
(7)	28	14	14
(8)	14	10.5	10.5
(1b)	<0.7	<0.7	<0.7
(2b)	<0.7	<0.7	<0.7
(3b)	<0.7	<0.7	<0.7

Experimental Section

General. ^{1}H NMR spectra were recorded at 300 or 500 MHz and ^{13}C NMR at 75 MHz. Chemical shifts are given in δ (ppm) relative to TMS as internal standard. Electron impact mass spectra were determined at 70 ev on a Kratos MS-25 instrument. Thin layer chromatography was performed on silica gel (kieselgel 60, F254) of 0.25 mm layer thickness. Preparative thin layer chromatography (PTLC) was performed on silica gel plates (20 cm x 20 cm) of 500 μm thicknesses. The spray reagent used is ammonium molybdate, which was prepared from, a solution of molybdenum trioxide (8.64 g in 7 mL NH_4OH, stirred for 30 min) was added to a solution of amm. Cr IV sulphate (4 g in 50 mL 50% H_2SO_4, stirred for 30 min) and completed to 250 mL by dist. water. The obtained solution was stirred overnight. The chromatoplate, after spraying, was heated at 60-70^{o}C until the spots attained maximum color intensity.

Extraction and isolation. The sponge specimens were collected at depths of -10 to -20 m in the Discovery Bay, Jamaica. Each specimen was freeze-dried (100 g) and then ground and extracted with methanol/chloroform (1:1). The extract was evaporated under vacuum to afford ~10 g of a brown oily residue. This oil was chromatographed on silica gel (200 g, 50 x 3 cm) eluting first with hexane followed by a gradient of hexane-ethyl acetate. Fractions of ~100 mL were collected. The fractions containing material of the same R_f were combined and further purified by sequential preparative TLC, MPLC, HPLC to give pure compounds in the order described below.



Scheme 1. Isolation of the metabolite from *Plakortis* sp.

Fraction **I** eluted by *n*-hexane/ethyl acetate (19:1), was purified by prep. TLC using hexane/ethyl acetate (9:1) to provide five bands. The second band (R_f. 0.7) was purified by normal phase HPLC using hexane/ethyl acetate (19:1) to give three pure substances (**1a, 2a, 3a**) in order of increasing elution time / polarity.

Plakortide H (1a). Colorless oil (20 mg, ~0.002% dry wt.). HRCIMS calcd. for $C_{22}H_{42}NO_4$ $[M + NH_4]^+$ 384.3114 found 384.3094. ^{1}H NMR (500 MHz,$CDCl_3$) δ 5.37 (1H, dt, *J* 15.5, 6, H-12), 5.13 (1H, br s, H-7), 5.09 (1H, dd, *J* 15.5, 8.5, H-11), 4.46 (1H,ddd, *J* 9, 5, 3.5, H-3), 3.71 (3H, s, OCH_3), 3.05 (1H, dd, *J* 15.5, 9, H-2), 2.38 (1H, dd, *J* 15.5, 3.5, H-2`), 2.10 (1H, m, H-9), 2.07 (1H, m, H-4), 2.04 (1H, m, H-10), 2.02 (1H, m, H-17), 1.99 (2H, m, H-13), 1.93 (1H, m, H-9`), 1.70 (3H, br s, H-19), 1.57 (2H, m, H-5), 1.49 (1H, m, H-17`), 1.40 (1H, m, H-20), 1.24 (1H, m, H-5`), 1.20 (1H, m, H-15), 1.17 (1H, m, H-20`), 1.12 (1H, m, H-15`), 0.95 (3H, t, *J* 7.5, Me-14), 0.90 (3H, t, *J* 7.5, Me-16), 0.84 (3H, t, *J* 7, Me-18), 0.83 (3H, t, *J* 7, Me-21). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.9 (C-1), 137.4 (C-8), 133.4(C-11), 132.1 (C-12), 127.0 (C-7), 84.3 (C-6), 78.3 (C-3), 51.8 (OCH_3), 47.5 (C-9), 44.2 (C-10), 35.7 (C-4), 34.4 (C-5), 31.5 (C-2), 28.2 (C-20), 25.7 (C-13), 25.1 (C-15), 25.0 (C-17), 16.9 (C-19), 14.3 (C-14), 11.7 (C-21), 11.0 (C-16), 7.1 (C-18).

Plakortide G (2a). Colorless oil (20 mg, ~0.002% dry wt.). HRCIMS calcd. for $C_{21}H_{42}NO_4$ [M + NH_4]$^+$ 372.3113 found 372.3124. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38 (1H, dt, *J* 15.0, 6.5, H-12), 5.08 (1H, dd, *J* 15.0, 8.5, H-11), 4.47 (1H,ddd, *J* 9.0, 4.5, 3.0, H-3), 3.71 (3H, s, OCH_3), 3.04 (1H, dd, *J* 15.5, 10.0, H-2), 2.38 (1H, dd, *J* 15.5, 3.0, H-2`), 2.18 (1H, m, H-4), 2.03 (1H, m, H-17), 2.00 (2H, m, H-13), 1.78 (1H, m, H-10), 1.52 (1H, m, H-5), 1.50 (1H, m, H-17`), 1.47 (1H, m, H-9), 1.38 (1H, m, H-19), 1.32 (1H, m, H-7), 1.30(1H, m, H-8), 1.26(1H, m, H-5`), 1.21(1H, m, H-15), 1.20 (1H, m, H-8`), 1.20 (2H, m, H-7`and H-9`), 1.18 (1H, m, H-19`), 1.14 (1H, m, H-15`), 0.96 (3H, t, *J* 7, Me-14), 0.92 (3H, t, *J* 7.5, Me-16), 0.85 (3H, t, *J* 7.5, Me-18) and 0.82 (3H, t, *J* 7, Me-20). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.3 (C-1), 133.5 (C-11), 132.1 (C-12), 82.6 (C-6), 78.3 (C-3), 51.8 (OCH_3), 44.5 (C-10), 35.7 (C-9), 35.1 (C-7) 34.4 (C-4), 32.7 (C-5), 31.9 (C-2), 28.2 (C-19), 25.7 (C-13), 25.1 (C-17), 24.2 (C-15), 20.9 (C-8), 14.3 (C-14), 11.7 (C-20), 11.1 (C-16), 7.1 (C-18).

Plakortide F (3a). Colorless oil (10 mg, ~0.001% dry wt.). HRCIMS calcd. for $C_{21}H_{42}NO_4$ [M + NH_4]$^+$ 372.3113 found 372.3124. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38 (1H, dt, *J* 15.0, 6.5, H-12), 5.04 (1H, dd, *J* 15.0, 8.5, H-11), 4.47 (1H,ddd, *J* 9.0, 4.5, 3.0, H-3), 3.71 (3H, s, OCH_3), 3.04 (1H, dd, *J* 15.5, 10.0, H-2), 2.38 (1H, dd, *J* 15.5, 3.0, H-2`), 2.18 (1H, m, H-4), 2.00 (2H, m, H-13), 1.93 (1H, m, H-7), 1.78 (1H, m, H-10), 1.52 (1H, m, H-5), 1.49 (1H, m, H-7`), 1.47 (1H, m, H-17), 1.38 (1H, m, H-9), 1.36 (1H, m, H-19) 1.32 (1H, m, H-17`), 1.27 (1H, m, H-8), 1.24 (1H, m, H-15), 1.22 (1H, m, H-5`), 1.20 (1H, m, H-8`), 1.19 (1H, m, H-9`), 1.18 (1H, m, H-19`), 0.96 (3H, t, *J* 7, Me-14), 0.92 (3H, t, *J* 7.5, Me-16), 0.84 (3H, t, *J* 7.5, Me-18) and 0.82 (3H, t, *J* 7, Me-20). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.3 (C-1), 133.5 (C-11), 132.1 (C-12), 82.6 (C-6), 78.3 (C-3), 51.8 (OCH_3), 44.5 (C-10), 35.7 (C-9), 34.4 (C-4), 32.7 (C-5), 32.1 (C-7), 31.9 (C-2), 29.5 (C-17), 28.2 (C-19), 25.7 (C-13), 25.1 (C-15), 20.9 (C-8), 14.3 (C-14), 11.7 (C-20), 11.0(C-16), 7.1 (C-18).

The third band (R_f. 0.5) was also purified by normal phase HPLC using *n* –hexane/ethyl acetate (19:1) to give the three pure substances (**1a, 2a, 3a**) reported above in addition two pure substances (**4, 5**).

Plakortine (4). Colorless oil (10 mg, ~0.001% dry wt.). HREIMS calcd. for $C_{18}H_{32}O_4$ 312.2301 found 312.2302. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 5.38 (1H, dt, *J* 15, 6.5, H-10), 5.10 (1H, dd, *J* 15, 8.5, H-9), 4.49 (1H, ddd, *J* 9.5, 5, 3.5, H-3), 3.71 (3H, s, OCH_3), 3.04 (1H, dd, *J* 15.5, 9.5, H-2), 2.37 (1H, dd, *J* 15.5, 3.5, H-2`), 2.14 (1H, m, H-4), 2.00 (2H, m), 1.41 (1H, m), 1.37 (3H, s, H-15), 1.20 (2H, m), 0.97 (3H, t, *J* 7, Me-14), 0.90 (3H, t, *J* 7, Me-17), 0.81 (3H, t, *J* 7, Me-12). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.9 (C_{quat}), 134.4(CH), 131.5 (CH), 81.0 (C_{quat}), 78.8 (CH), 51.5 (CH_3), 46.5(CH_2), 40.2 (CH), 36.0 (CH_2), 34.9 (CH), 31.4 (CH_2), 29.9 (CH_2), 29.5 (CH_2), 25.2 (CH_2), 21.3 (CH_3), 13.9 (CH_3), 11.5 (CH_3), 11.0 (CH_3).

3-Epiplakortine (5). Colorless oil (10 mg, ~0.001% dry wt.). HREIMS calcd. for $C_{18}H_{32}O_4$ 312.228 found 312.230. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38 (1H, dt, *J* 15, 6.5, H-10), 5.10 (1H, dd, *J* 15, 8.5, H-9), 4.15 (1H, ddd, *J* 9.0, 9.0, 3.0, H-3), 3.69 (3H, s, OCH_3), 2.65 (1H, dd, *J* 15.5, 3.0, H-2), 2.38 (1H, m, H-2`), 2.01 (3H, m), 1.81 (1H, dd, *J* 13.5, 4.5, H-7), 1.61 (1H, m),

1.43(3H, m), 1.37 (3H, s, H-15), 1.21 (2H, m), 0.97 (3H, t, *J* 7, Me-14), 0.90 (3H, t, *J* 7, Me-17), 0.81 (3H, t, *J* 7, Me-12). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.9 (C_{quat}), 134.4(CH), 131.5 (CH), 81.0 (C_{quat}), 78.8 (CH), 51.5 (CH_3), 46.5(CH_2), 40.2 (CH), 36.0 (CH_2), 34.9 (CH), 31.4 (CH_2), 29.9 (CH_2), 29.5 (CH_2), 25.2 (CH_2), 21.3 (CH_3), 13.9 (CH_3), 11.5 (CH_3), 11.0 (CH_3).

The fifth band (R_f. 0.34) was found a pure substance (**6**)

Methyl (2Z, 6R, 8R, 9E)-3,6- epoxy-4,6,8-triethyl-2,4,9-dodecatrienoate (6). Colorless oil (50 mg, ~0.05% dry wt.). GC/MS [70 eV, m/e (rel. int.)] 306 (18)[M^+,$C_{19}H_{30}O_3$], 275 (10), 195 (100), 181 (100), 163 (10), 149 (22), 55 (60). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.19 (1H, s, H–5), 5.23 (1H, dt, *J* 15, 6.5, H-10), 5.03 (1H, dd, *J* 15,8.5, H-9), 4.80 (1H, s, H-2), 3.69 (3H, s, $COOCH_3$), 2.13 (2H, m), 1.97 (2H, m), 1.92 (1H), 1.84 (1H, m), 1.77 (1H, m), 1.75 (1H, m), 1.72 (1H, m), 1.38 (1H, m), 1.15 (3H, t, *J* 7, Me-18), 1.14 (1H, m), 0.95 (3H, t, *J* 7, Me-12), 0.78 (3H, t, *J* 7, Me-16) and 0.77 (3H, t, *J* 7, Me-14). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 172.1 (C_{quat}), 166.9(C_{quat}), 140.2(CH), 139.2(C_{quat}), 133.9(CH), 132.2 (CH), 97.7 (C_{quat}), 83.2 (CH), 50.4 (OCH_3), 43.5 (CH_2), 39.6 (CH), 32.3 (CH_2), 29.4 (CH_2), 25.6 (CH_2), 18.5 (CH_2), 13.9(CH_3), 11.7 (CH_3), 11.3 (CH_3), 7.9 (CH_3).

Fraction **II** eluted by *n*-hexane/ethyl acetate (9:1), was purified by prep. TLC using hexane/ethyl acetate (9:1) to provide two bands. The first band (R_f 0.4) was found to be a pure substance (**6**) which was reported above.

The second band (R_f. 0.37) was purified by normal phase HPLC using *n*-hexane/ethyl acetate (9 : 1) to give two pure substances (**7, 8**) in order of increasing elution time / polarity.

Methyl-3,6-epoxy-4,8-diethyl-6-methyl-2,4,9-dodecatrienoate (7). Colorless oil (30 mg, ~0.03% dry wt.). GC/MS [70 eV, m/e (rel. int.)] 292 (10) [M^+,$C_{18}H_{28}O_3$], 277 (4), 261 (6), 181 (100), 149 (20), 55 (40). HRCIMS calcd. for $C_{18}H_{28}O_3$ $[M]^+$ 292.2038 found 292.2035. ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.25 (1H, s, H–5), 5.25 (1H, dt, *J* 15, 6.5, H-10), 5.03 (1H, dd, *J* 15, 8, H-9), 4.82 (1H, s, H-2), 3.69 (3H, s, OCH_3), 2.11 (2H, qt, *J* 7.5, 2.5, H-16), 1.97 (2H, m, H-11), 1.94 (1H, m, H-7), 1.77 (1H, m, H-8), 1.74 (1H, m, H-7), 1.42 (3H, s, Me-15) 1.36 (1H, m, H-13), 1.15 (1H, m, H-13`), 1.14 (3H, t, *J* 7.5, Me-17), 0.95 (3H, t, *J* 7.5, Me-12) and 0.77 (3H, t, *J* 7.5, Me-14). ^{13}C -NMR($CDCl_3$, 125 MHz) δ 171.6 (C_{quat}), 166.9 (C_{quat}), 141.7 (CH), 138.1 (C_{quat}), 133.8 (CH), 132.2 (CH), 95.1 (C_{quat}), 83.8 (CH), 50.6 (OCH_3), 45.0 (CH_2), 40.1 (CH), 29.4 (CH_2), 26.4 (CH_3), 25.6(CH_2), 18.5 (CH_2), 13.9 (CH_3), 11.6 (CH_3), 11.4 (CH_3).

Plakortone D (8). Colorless oil (40 mg, ~0.04% dry wt.). GC/MS [70 eV, m/e (rel. int.)] 322 (2) [M^+,$C_{20}H_{34}O_3$], 293 (15), 183 (100), 57 (40). HREIMS calcd. for $C_{20}H_{34}O_3$ $[M]^+$ 322.2508 found 322.2503. ^{1}H NMR (500 MHz,$CDCl_3$) δ 5.38 (1H, dt, *J* 15, 6.5, H-12), 5.06 (1H, dd, *J* 15, 8.5, H-11), 4.34 (1H, d, *J* 5, H-3), 2.87 (2H, m, H-2), 2.26 (1H, d, *J* 14, H-5α), 1.99 (2H, p, *J* 6, H-13), 1.90 (1H, d, *J* 14, H-5β), 1.77 (1H, m, H-10), 1.74 (2H, m, H-17), 1.55 (2H, m, H-15), 1.53 (1H, m, H-7), 1.40- 1.15 (6H, m, H-19, 9, 8) 1.02 (3H, t, *J* 7, Me-18), 0.97 (3H, t, *J* 7.5, Me-14), 0.85 (3H, t, *J* 7.5, Me-16) and 0.83 (3H, t, *J* 7, Me-20). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 175.6 (C_{quat}), 133.3(CH), 132.4 (CH), 97.9 (C_{quat}), 87.5 (C_{quat}), 80.6 (CH), 45.0 (CH_2), 44.3

(CH), 39.4 (CH_2), 37.5 (CH_2), 35.4 (CH_2), 31.5 (CH_2), 30.4 (CH_2), 28.3 (CH_2), 25.7 (CH_2), 21.5 (CH_2), 14.3 (CH_3), 11.8 (CH_3), 8.6 (CH_3), 8.5 (CH_3).

Fraction (**IV**) eluted by *n*-hexane/ethyl acetate (4:1) ~ 2g were further purified by MPLC using hexane/ – ethyl acetate (4:1) to provide two fractions. The first fraction (**IV-1**) was again purified by HPLC normal phase using Hexane/ethyl acetate (4:1) to provide pure substance (**1b**). The second fraction (**IV-2**) was also purified by HPLC normal phase using *n*-hexane/ethyl acetate (4:1) to provide two pure substances (**2b, 3b**)

Plakortide I (1b). Colorless oil (10 mg, ~0.01% dry wt.). HRCIMS calcd. for $C_{21}H_{40}NO_4$ [M + NH_4]$^+$ 370.2957 found 370.2960. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.37 (1H, dt, *J* 15.5, 6, H-12), 5.13 (1H, br s, H-7), 5.09 (1H, dd, *J* 15.5, 8.5, H-11), 4.46 (1H,ddd, *J* 9, 5, 3.5, H-3), 3.05 (1H, dd, *J* 15.5, 9, H-2), 2.38 (1H, dd, *J* 15.5, 3.5, H-2`), 2.10 (1H, m, H-9), 2.07 (1H, m, H-4), 2.04 (1H, m, H-10), 2.02 (1H, m, H-17), 1.99 (2H, m, H-13), 1.93 (1H, m, H-9`), 1.70 (3H, br s, H-19), 1.57 (2H, m, H-5), 1.49 (1H, m, H-17`), 1.40 (1H, m, H-20), 1.24 (1H, m, H-5`), 1.20 (1H, m, H-15), 1.17 (1H, m, H-20`), 1.12 (1H, m, H-15`), 0.95 (3H, t, *J* 7.5, Me-14), 0.90 (3H, t, *J* 7.5, Me-16), 0.84 (3H, t, *J* 7, Me-18), 0.83 (3H, t, *J* 7, Me-21). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.9 (C-1), 137.4 (C-8), 133.4(C-11), 132.1 (C-12), 127.0 (C-7), 84.3 (C-6), 78.3 (C-3), 47.5 (C-9), 44.2 (C-10), 35.7 (C-4), 34.4 (C-5), 31.5 (C-2), 28.2 (C-20), 25.7 (C-13), 25.1 (C-15), 25.0 (C-17), 16.9 (C-19), 14.3 (C-14), 11.7 (C-21), 11.0 (C-16), 7.1 (C-18).

Plakortide J (2b). Colorless oil (10 mg, ~0.01% dry wt.). HRCIMS calcd. for $C_{20}H_{40}NO_4$ [M + NH_4]$^+$ 358.2957 found 358.2946 . ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38 (1H, dt, *J* 15.0, 6.5, H-12), 5.08 (1H, dd, *J* 15.0, 8.5, H-11), 4.47 (1H,ddd, *J* 9.0, 4.5, 3.0, H-3), 3.04 (1H, dd, *J* 15.5, 10.0, H-2), 2.38 (1H, dd, *J* 15.5, 3.0, H-2`), 2.18 (1H, m, H-4), 2.03 (1H, m, H-17), 2.00 (2H, m, H-13), 1.78 (1H, m, H-10), 1.52 (1H, m, H-5), 1.50 (1H, m, H-17`), 1.47(1H, m, H-9), 1.38 (1H, m, H-19), 1.32 (1H, m, H-7), 1.30 (1H, m, H-8), 1.26 (1H, m, H-5`), 1.21 (1H, m, H-15), 1.20 (1H, m, H-8`), 1.20 (2H, m, H-7`and H-9`), 1.18 (1H, m, H-19`), 1.14 (1H, m, H-15`), 0.96 (3H, t, *J* 7, Me-14), 0.92 (3H, t, *J* 7.5, Me-16), 0.85 (3H, t, *J* 7.5, Me-18) and 0.82 (3H, t, *J* 7, Me-20). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.7 (C-1), 133.5 (C-11), 132.1 (C-12), 82.6 (C-6), 78.3 (C-3), 44.5 (C-10), 35.7 (C-9), 35.1 (C-7) 34.4 (C-4), 32.7 (C-5), 31.9 (C-2), 28.2 (C-19), 25.7 (C-13), 25.1 (C-17), 24.2 (C-15), 20.9 (C-8), 14.3 (C-14), 11.7 (C-20), 11.1 (C-16), 7.1 (C-18).

Plakortide K (3b). Colorless oil (10 mg, ~0.01% dry wt.). HRCIMS calcd. for $C_{20}H_{40}NO_4$ [M + NH_4]$^+$ 358.2957 found 358.2935. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.38 (1H, dt, *J* 15.0, 6.5, H-12), 5.06 (1H, dd, *J* 15.0, 8.5, H-11), 4.49 (1H,ddd, *J* 9.0, 4.5, 3.0, H-3), 3.01 (1H, dd, *J* 15.5, 10.0, H-2), 2.36 (1H, dd, *J* 15.5, 3.0, H-2`), 2.15 (1H, m, H-4), 2.00 (2H, m, H-13), 1.93 (1H, m, H-7), 1.78 (1H, m, H-10), 1.52 (1H, m, H-5), 1.49 (1H, m, H-7`), 1.47 (1H, m, H-17), 1.38 (1H, m, H-9), 1.36 (1H, m, H-19) 1.32 (1H, m, H-17`), 1.27 (1H, m, H-8), 1.24 (1H, m, H-15), 1.22 (1H, m, H-5`), 1.20 (1H, m, H-8`), 1.19 (1H, m, H-9`), 1.18 (1H, m, H-19`), 0.96 (3H, t, *J* 7, Me-14), 0.92 (3H, t, *J* 7.5, Me-16), 0.84 (3H, t, *J* 7.5, Me-18) and 0.82 (3H, t, *J* 7, Me-20). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.7 (C-1), 133.5 (C-11), 132.1 (C-12), 82.6 (C-6), 78.3 (C-3), 44.5 (C-

10), 35.7(C-9), 34.4 (C-4), 32.7 (C-5), 32.1(C-7), 31.9(C-2), 29.5(C-17), 28.2(C-19), 25.7(C-13), 25.1 (C-15), 20.9 (C-8), 14.3 (C-14), 11.7 (C-20), 11.0 (C-16), 7.1 (C-18).

Cytotoxicity. The cytotoxic activities of the tested compounds against a normal fibroblast line (NIH3T3 and virally transformed form KA3IT) were assayed by a modification method as previously described.[13-15]

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