Full Papers

Plakortides, Novel Cyclic Peroxides from the Sponge *Plakortis halichondrioides*: Activators of Cardiac SR-CA^{2+}-Pumping ATPase

Ashok D. Patil,*[,1] Alan J. Freyer,[1] Brad Carte,[1] Randall K. Johnson,[1] and Philippe Lahouratate[2]

Departments of Biomolecular Discovery and Analytical Sciences, SmithKline Beecham Pharmaceuticals, R & D, King of Prussia, Pennsylvania 19406-0939, and Biochemistry & Cellular Biology Group, SmithKline Beecham Pharmaceutiques, Unit'e de Recherche, 4 rue du Chesnay Beauregard, BP 58, 35762 Saint Gregoire, France

As part of a search for novel activators of Ca^{2+} pumping activity of cardiac SR- (sarcoplasmic reticulum), the EtOAc extract of the Jamaican sponge *Plakortis halichondrioides* was shown to be active. Bioassay-guided fractionation of the extract followed by preparative TLC and HPLC yielded several known and novel compounds. Three of the novel cyclic peroxides, plakortides F, G, and H (**3, 4**, and **5**) are the subject of this report. Their structures including relative stereochemistry were established by interpretation of spectral data. Micromolar concentrations of plakortides F−H (**3−5**) were found to significantly enhance Ca^{2+} uptake by SR.

Active Ca^{2+} sequestration by the cardiac sarcoplasmic reticulum (SR), which plays a key role in the removal of Ca^{2+} from the myofilaments, thereby leading to myocardial relaxation, is performed by a Ca^{2+}-dependent ATPase. Although this Ca^{2+} pump constitutes an ideal target to improve cardiac relaxation which is impaired during several forms of heart failure, only a few activators of cardiac SR-Ca^{2+} ATPase have been identified. To date, only gingerol, isolated from the rhizome of ginger,[3] and polyanions such as heparin,[4] tannic acid,[5] and more recently penaresin[6] have been described as activators of cardiac SR-Ca^{2+} uptake. However, none of these compounds could be used as a therapeutic agent due to their poor selectivity. Thus, the pursuit of substances having novel bioactivities from natural sources in order to identify more specific activators of SR-Ca^{2+} uptake is warranted.

Results and Discussion

Marine sponges of the genus *Plakortis* have proven to be a prolific source of compounds with interesting biological activities.[7] As part of our continuing search for biologically active natural products with potential utility in the treatment of cardiac diseases, we initiated a high throughput screen to evaluate the ability of natural products extracts to stimulate cardiac SR-Ca^{2+} ATPase. Over 2400 marine and plant extracts were screened. The EtOAc extract of the Jamaican sponge *Plakortis halichondrioides* Wilson (Plakinidae) exhibited significant enhancement of SR-Ca^{2+} ATPase activity and of SR-Ca^{2+} uptake. As a result, this extract was selected for fractionation.

The freeze-dried sponge was extracted sequentially with EtOAc and MeOH. The active EtOAc extract was chromatographed over a column of Si gel to yield several active fractions. Further purification of these fractions by PTLC and HPLC and in some cases via esterification led to the isolation of a total of 10 metabolites that included two known and eight novel compounds. The known compounds were identified as 3-epiplakortin (**1**)

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* To whom correspondence should be addressed. Tel.: (610) 270-4244.Fax: (610) 270-6996. E-mail: Inet: Ashok_D_Patil@sbphrd.com.
⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1996.

Table 1. ^{1}H- and ^{13}C-NMR Assignments for Plakortides F−H (**3**−**5**) in CDCl$_3$

no.	plakortide F ^{1}H	^{13}C	plakortide G ^{1}H	^{13}C	plakortide H ^{1}H	^{13}C
1		172.2		172.2		172.3
2	3.01 (1H, dd, 9.6, 15.6)	31.3	3.02 (1H, dd, 9.6, 15.6)	31.3	3.02 (1H, dd, 9.6, 15.6)	31.3
	2.37 (1H, dd, 3.6, 15.6)		2.37 (1H, dd, 3.6, 15.6)		2.36 (1H, dd, 3.6, 15.6)	
3	4.49 (1H, ddd, 3.6, 5.2, 9.6)	78.6	4.48 (1H, ddd, 3.6, 5.2, 9.6)	78.6	4.49 (1H, ddd, 3.6, 5.2, 9.6)	78.7
4	2.14 (1H, m)	34.5	2.16 (1H, m)	34.4	2.07 (1H, m)	35.3
5	1.54 (1H, m, eq)	32.7	1.56 (1H, m, eq)	33.1	1.58 (1H, m, eq)	32.4
	1.21 (1H, m, ax)		1.26 (1H, dd, ax, 12.3, 14.0)		1.25 (1H, m, ax)	
6		82.4		82.5		84.1
7	1.94 (1H, m)	31.9	1.31 (1H, m)	35.5	5.13 (1H, q, 1.3)	127.2
	1.49 (1H, m)		1.20 (1H, m)			
8	1.27 (1H, m)	20.9	1.30 (1H, m)	20.1		137.1
	1.21 (1H, m)		1.21 (1H, m)			
9	1.38 (1H, m)	35.7	1.47 (1H, m)	36.3	2.10 (1H, m)	47.4
	1.19 (1H, m)		1.20 (1H, m)		1.94 (1H, m)	
10	1.79 (1H, m)	44.5	1.78 (1H, m)	44.2	2.05 (1H, m)	42.4
11	5.09 (1H, ddt, 8.8, 15.3, 1.5)	133.5	5.06 (1H, ddt, 8.4, 15.3, 1.5)	133.2	5.05 (1H, ddt, 8.4, 15.3, 1.5)	133.0
12	5.39 (1H, dt, 15.3, 6.4)	132.1	5.38 (1H, dt, 15.3, 6.3)	132.3	5.38 (1H, dt, 15.3, 6.3)	131.7
13	2.00 (2H, m)	25.7	2.01 (2H, m)	25.7	2.00 (2H, m)	25.6
14	0.96 (3H, t, 7.5)	14.2	0.97 (3H, t, 7.4)	14.3	0.97 (3H, t, 7.4)	13.9
15	1.48 (1H, m)	29.7	2.05 (1H, m)	24.7	2.02 (1H, m)	25.0
	13.6 (1H, m)		1.53 (1H, m)		1.50 (1H, m)	
16	0.85 (3H, t, 7.6)	7.1	0.85 (3H, t, 7.6)	7.5	0.85 (3H, t, 7.6)	7.6
17	1.24 (1H, m)	25.2	1.22 (1H, m)	25.2	1.20 (1H, m)	25.2
	1.14 (1H, m)		1.14 (1H, m)		1.12 (1H, m)	
18	0.92 (3H, t, 7.4)	11.0	0.92 (3H, t, 7.4)	11.0	0.91 (3H, t, 7.4)	11.0
19	1.38 (1H, m)	28.2	1.37 (1H, m)	28.3	1.70 (3H, d, 1.3)	16.9
	1.19 (1H, m)		1.19 (1H, m)			
20	0.82 (3H, t, 7.4)	11.7	0.82 (3H, t, 7.4)	11.7	1.37 (1H, m)	27.9
					1.17 (1H, m)	
21					0.82 (3H, t, 7.4)	11.6
OCH3	3.71 (3H, s)	51.8	3.71 (3H, s)	51.8	3.70 (3H, s)	51.8

and the α,β-unsaturated ester (**2**) by comparison of spectral data with literature data.[8] The novel plakortones A−D and plakortide E will be described elsewhere. We now report the structure determination of three novel cyclic peroxides, plakortides F−H (**3**−**5**), which have interesting biological activity.
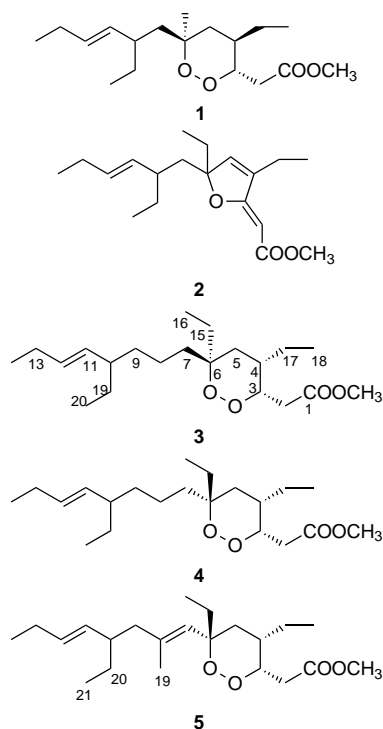
Plakortide F (**3**) was isolated as an oil, [α]$^{25}_D$ = −159.5°, and its molecular formula was determined as C$_{21}$H$_{38}$O$_4$ by HRDCIMS (*m/z* 354.2763, M$^+$). The IR

spectrum showed a band at 1743 cm^{-1} that was assigned to an ester carbonyl. The ^{1}H NMR spectrum of **3** (Table 1) contained signals that were typical of a cyclic peroxide having the plakortin ring system and a side chain. It exhibited two olefinic multiplets at δ 5.39 and 5.09 which shared a 15.3 Hz trans coupling and a methine multiplet at δ 4.49. The ^{13}C GASPE NMR spectrum (Table 1) of plakortide F (**3**) indicated the presence of 21 carbon signals. It contained signals at δ 172.2 and 51.8 for the carbomethoxy group, at δ 82.4 and 78.6 for the carbon atoms flanking the peroxide bond, and at δ 133.5 and 132.1 which were assigned to the disubstituted double bond. The four methyl signals at δ 7.1, 11.0, 11.7, and 14.2 in the GASPE NMR spectrum corresponded to four methyl triplets at δ 0.96, 0.92, 0.85, and 0.82 in the ^{1}H NMR spectrum as determined by the HMQC NMR data. Two of the three requisite degrees of unsaturation are thus accounted for in the ^{13}C NMR data, and plakortide F (**3**) must therefore contain one ring.

The COSY spectrum of plakortide F (**3**) indicated the presence of two extended spin systems and an isolated ethyl substituent. COSY data also revealed that all four methyl triplets were part of four separate ethyl groups. The olefinic proton at δ 5.39 (H-12) correlated to the H-13/14 ethyl group, and the δ 5.09 signal (H-11) correlated to the H-10 methine which further coupled to the H-19/20 ethyl group and the H-9 methylene protons. The H-9 protons further correlated to the H-8 and H-7 methylene protons. In the other extended spin system the δ 4.49 multiplet (H-3) correlated to the nonequivalent H-2 methylene doublet of doublets on one side and to the H-4 methine on the other. H-4 further correlated to the inequivalent H-5 methylene protons and to the H-17/18 ethyl group. The remaining isolated
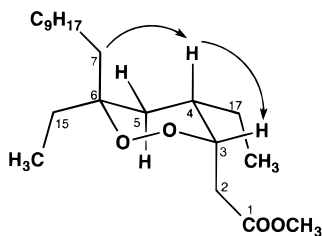
Figure 1. Relative stereochemistry of plakortide F (**3**) with arrows representing NOE enhancements.

Figure 2. Relative stereochemistry of plakortide G (**4**) with arrows representing NOE enhancements.

ethyl group consisted of H-15 at δ 1.48 and 1.36 and H-16 at 0.85.

In the HMBC spectrum of **3**, the H-12 olefin multiplet at δ 5.39 correlated to the C-13 and C-14 ethyl carbons and the H-11 olefin multiplet at δ 5.09 correlated to the adjacent C-10 methine and C-9 in the side chain and to C-19 of the ethyl substituent attached to C-10. CH_3-20 at δ 0.82 correlated to C-19 and C-10, establishing that this was the ethyl group attached at C-10. The nonequivalent H-7 protons at δ 1.94 and 1.49 correlated to C-8 and C-9 in the side chain, 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-15 of the isolated ethyl group which was attached to C-6. The CH_3-16 protons at δ 0.85, which are attached to the upfield methyl carbon at δ 7.1, correlated to C-15 and C-6, establishing that this was the ethyl group attached at the fully substituted C-6. The H-3 methine multiplet at δ 4.49 correlated to C-2 and C-1 of the methyl ester as well as to C-4 and C-5 in the ring. The CH_3-18 triplet at δ 0.92 correlated to C-17 and C-4, confirming that it was part of the remaining ethyl substituent attached at C-4. The δ 3.71 methoxyl group correlated to ester carbonyl C-1.

The relative stereochemistry about the peroxide ring was established by NOE difference experiments, the results of which are summarized with arrows in Figure 1. Saturation of the δ 1.49 H-7 methylene protons in the side chain enhanced the axial H-4 signal, and irradiation of H-4 caused an increase in the 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. The stereochemistry at C-10 in the side chain could not be determined. Consistent with the β axial assignment of H-4 is the 5.2 Hz coupling constant (axial/equatorial) it shared with H-3. In addition, decoupling H-5ax at δ 1.21 caused the H-4 multiplet to collapse losing a large axial/axial coupling of approximately 12 Hz. Irradiation of H-5eq at δ 1.54 changed the H-4 multiplet by eliminating an axial/equatorial coupling of approximately 5 Hz.

Plakortide G (**4**) was also isolated as a colorless oil, $[\alpha]^{25}_D$ = +67.2° and displayed a molecular ion at m/z 354.2759 (M$^+$) in its HRDCIMS. The UV and IR spectra were similar to those of plakortide F (**3**), and the ^{1}H NMR spectrum was similar to that of **3**, suggesting that it might be a diastereomer of **3**. The COSY spectrum of plakortide G (**4**) indicated the presence of nearly identical spin systems in **3** and **4**. The ^{13}C GASPE spectrum of plakortide G (**4**) exhibited 21 carbon signals as was the case for **3**, and most of the resonances had virtually the same chemical shifts except for two carbon signals. The C-7 resonance in **4** was observed at δ 35.5, 3.6 ppm further downfield than its δ 31.9 counterpart in **3**, and the C-15 resonance in **4** was observed at δ 24.7,
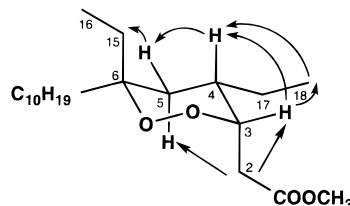
5.0 ppm further upfield than its δ 29.7 value in plakortide F (**3**). Since the NMR data indicated that **3** and **4** were very similar in structure, these differences suggested that plakortide G (**4**) is a diastereomer of F (**3**) with inverted stereochemistry at C-6. This conclusion was further supported by measuring coupling constants and NOE difference experiments which are summarized in Figure 2.

Saturation of the H-2 methylene protons enhanced the H-3 signal and H-5ax, establishing that H-2 and H-5ax were on the same face of the peroxide ring. Irradiation of both the H-3 and CH_3-18 hydrogens caused an increase in the axial H-4 signal. The H-4 multiplet shared a 5.2 Hz coupling constant with H-3 and a 12.3 Hz axial/axial coupling constant with H-5ax. NOE saturation of H-4 enhanced H-5eq, and upon irradiation of H-5eq the H-15 methylene protons were affected. Therefore, the C-6 stereochemistry of plakortide G (**4**) must be the opposite of that of plakortide F (**3**). This conclusion is consistent with the downfield shift of C-7, which is closer to the peroxide ring oxygens in plakortide **4** than in **3**, and the upfield shift of C-15, which is further away from the ring oxygens in plakortide **4** than in **3**. The change in the sign of the $[\alpha]_D$ values between **3** and **4** is consistent with this change in C-6 stereochemistry.

Plakortide H (**5**) was isolated as a oil, $[\alpha]^{25}_D$ = +5.5°, and its molecular formula was determined as $C_{22}H_{38}O_4$ by HRDCIMS (m/z 366.2744, M$^+$), one carbon more than in **3** or **4**. The UV, IR, and ^{1}H NMR spectra of **5** closely resembled that of plakortide G (**4**) except that the ^{1}H-NMR spectrum of plakortide H (**5**) contained an additional unsaturation as evidenced by the emergence of a narrow olefinic quartet at δ 5.13 (δC 127.2) and a methyl doublet at δ 1.70 (δC 16.9). In the COSY spectrum of **5**, the methyl doublet at δ 1.70 correlated to the H-7 olefinic quartet and to the H-9 methylene protons at δ 2.10 and 1.94 which in turn correlated to the H-10 proton at δ 2.05, indicating that the second double bond in plakortide H (**5**) was located between C-7 and C-8. This conclusion was also supported by HMBC data, revealing cross peaks between signals observed at δ 1.70 (19-CH_3) and 127.2 (7-CH), 137.1 (C-8), and 47.4 (9-CH_2) and δ 5.13 (7-CH) and 137.1, 47.4, 11.6 (21-CH_3), and 84.1(C-6).

The H-7 quartet showed an NOE to the H-9 methylene protons, establishing the *E* geometry for the C-7/8 double bond. Similar coupling constants, chemical shifts, and NOES for plakortide G (**4**) established that **5** had the same relative stereochemistry about the peroxide ring.

Biological Activity

As shown in Figure 3, we found that the three novel plakortides and 3-epiplakortin were able to significantly
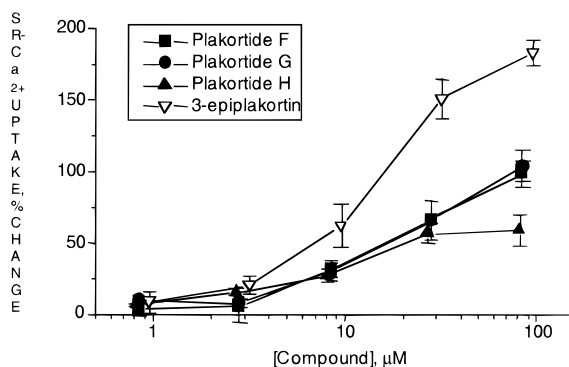
Figure 3. Stimulation of SR-Ca2+ uptake by cyclic peroxides isolated from *P. halichondrioides*. Values are mean ± SEM of the effect on four different SR-vesicle preparations.

enhance Ca^{2+} uptake by the SR. Although all four compounds were active in the same range of concentration (10−100 μM), the level of stimulation reached with 3-epiplakortin was significantly larger than for the plakortides F, G, and H (**3**, **4**, and **5**). The finding that these novel cyclic peroxides isolated from the marine sponge *P. halichondrioides* can enhance SR-Ca^{2+} uptake is in agreement with the previous report that plakorin, another cyclic peroxide isolated from the Okinawan marine sponge *Plakortis* sp. activated SR-Ca^{2+} ATPase activity from rabbit fast skeletal muscle.[9]

It is of interest to note that the activity of plakortide F (**3**) and its diastereomer plakortide G (**4**) is exactly the same, which suggests that a specific orientation of the hydrophobic side chain with respect to the peroxide ring is not essential for the activity. The larger activity observed with 3-epiplakortin (**1**) may result from the shorter side chain (C_8H_{15}). In contrast, the weaker activity observed for plakortide H (**5**), especially at higher concentration, may result from the longer hydrophobic side chain ($C_{11}H_{19}$). Indeed, within a series of activators of cardiac SR Ca^{2+} ATPase which also consisted essentially of a polar nucleus attached to a hydrophobic side chain, it was found that the ability of the compounds to stimulate SR Ca^{2+} ATPase activity was dependent on the size of the lateral chain.

In conclusion, we have identified three novel plakortides which stimulate cardiac SR Ca^{2+} -pumping ATPase, and we have discovered that 3-epiplakortin is a strong activator of SR Ca^{2+} -pumping ATPase. Although the relatively high concentrations needed to observe activation of SR Ca^{2+} uptake with these compounds limits their interest as potential therapeutic agents, their identification as activators of cardiac SR-Ca2+ uptake provides novel tools for studies aimed at investigating the catalytic and transport mechanism of SR Ca^{2+} pumps. Furthermore, they could be used to elucidate the causal relationship between the Ca^{2+} pumping activity of SR and cardiac muscle contractility.

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Nicolet Model 20 DXB FTIR spectrometer. All homonuclear and heteronuclear one- and two-dimensional NMR data were recorded on a Bruker AMX-400 spectrometer in $CDCl_3$. Low-resolution electrospray ionization (ESI) and deuterium exchange mass spectra were obtained in the positive mode on a Perkin-Elmer Sciex API-III triple quadruple mass spectrom-

eter. The LRDCI and HRDCIMS were acquired on a VG-70SE using CH_4 and NH_3 gases. Analytical and preparative TLC were carried out on precoated Si gel G (Kiesel gel G254) and reversed-phase (Whatman KC18F) plates. A Rainin HPXL solvent delivery system equipped with a refractive index detector, Model 156, and a Beckman 114M solvent delivery system equipped with UV detector were used for HPLC separations employing a Lichrosorb SI 60 (7 μm) and a Whatman Magnum-9 ODS-3 column, respectively. The UV spectra were recorded on a Beckman DU-7 spectrophotometer. Optical rotations were recorded on a Perkin-Elmer 241 MC polarimeter. Reagent grade chemicals (Fisher and Baker) were used.

Biological Assays. Oxalate-supported SR-Ca^{2+} uptake by isolated cardiac SR-vesicles.

Cardiac SR-Ca^{2+} uptake was determined as previously reported[10] on isolated SR-vesicles prepared from dog left ventricle according to Jones et al.[11] and using a fluorimetric method based on that described by Kargacin et al.[12] The Ca^{2+}-selective fluorescent dye, fluo-3 (pentaamonium salt, Molecular Probes Inc.), was added outside the membrane vesicles to continuously monitor the decline in free Ca^{2+} concentration ($[Ca^{2+}]_{free}$) produced as a result of ATP-dependent Ca^{2+} uptake by the SR. Ca^{2+} uptake was determined at 30 °C in a buffer containing (in mM) 119.7 KCl, 5 NaN_3, 6 $MgCl_2$, 1.5 Na_2ATP, 20 K-HEPES (pH 7.02), K_2H_2EGTA 0.045, and $K_2CaEGTA$ 0.105 to give a pCa of 6.0 which was calculated using Fabiato's computer program.[13] Fluorescence was measured in a 3-mL cuvette with a Fluoromax (Jobin-Yvon) fluorimeter using the DM3000F (Spex) software. Excitation wavelength was set at 506 nm, and emission was monitored at 526 nm with bandpasses of 0.85 nm. The spectrofluorimeter was operated in the ratio mode (emission signal/reference signal) to compensate for any fluctuation in the excitation source intensity. The emission signal was integrated for 0.5 s, and samples were mixed with a small magnetic stirrer in the cuvette thermostated at 30 °C. The relationship between $[Ca^{2+}]_{free}$ and the fluorescence of fluo-3 was $[Ca^{2+}]_{free} = K_d \times [F - F_{min}/(F_{max} - F)]$, according to Grynkiewickz et al.[14] where F is the fluorescence at any unknown $[Ca^{2+}]_{free}$, F_{min} is the fluorescence in the absence of Ca^{2+}, F_{max} is the fluorescence at saturating Ca^{2+}, and K_d (422 nM in our conditions) is the dissociation constant for fluo-3 and Ca^{2+}. After the vesicles have been equilibrated for 1 min in the assay medium containing the natural product to be assayed (or 1% methanol as solvent), the oxalate-supported uptake of Ca^{2+} by isolated SR vesicles was initiated by the addition of 2.5 mM K-oxalate which precipitates intra-SR Ca^{2+}, thus preventing an increase in $[Ca^{2+}]_{free}$ in the SR, which would otherwise slow the rate of net Ca^{2+} uptake. The effect of the natural products on SR-Ca^{2+} uptake was expressed as the percent change of the rate of $[Ca^{2+}]_{free}$ decline, measured for 50 s after the addition of K-oxalate, in the presence of the compound and compared to that obtained with the solvent alone.

Collection, Extraction, and Isolation. The sponge, voucher no. JAM90-003, was collected by hand using closed-circuit mixed gas rebreathers on Nov 26, 1990, at a depth of 100 feet on the west fore reef at Discovery Bay, Jamaica, and specimens were frozen immediately

and kept at −20 °C until extraction. The sample has been identified by Mary K. Harper (SIO) as *P. halichondrioides* Wilson (Order Homosclerophorid, Family Plakinidae). A voucher sample has been deposited in the Scripps Institution of Oceanography Benthic Invertebrate Collection, registry no. P1145. The freeze-dried sponge (321 g) was extracted with EtOAc and MeOH to give 23.1 and 61 g extracts, respectively. The pale yellow EtOAc extract (20 g) which showed ability to stimulate the SR-Ca^{2+} ATPase activity was applied to a column of Si gel and eluted with acetone:hexane (20:80). Several fractions (15 mL each) were collected and monitored by Si gel TLC. Like fractions were combined to give four (A−D) individual fractions. The active fraction D (3.51 g) was further chromatographed on a Si gel column using EtOAc:hexane mixture (15:85) to give several fractions. Initial fractions yielded inactive oil (2.64 g). The next fractions, which on Si gel plates charred upon treatment with vanillin /H$_2$SO$_4$ to give characteristic blue spots that intensified in color over 12 h to deep ink-blue appearance and had almost identical R_f values, were combined to give an oily residue (0.81 g). This residue after Si gel PTLC (acetone:hexane:20:80) produced six fractions. The first four fractions were further subjected to Si gel HPLC (monitored by refractive index detector) using EtOAc:hexane as solvent system (12:88) to yield plakortones A−D and the α,β-unsaturated ester **2**. The fifth fraction was purified by reversed-phase C-18 HPLC using H$_2$O:CH$_3$-CN (8:92) to yield plakortide E. The last fraction (0.271 g), which appeared to be more polar and unstable by TLC, was dissolved in ether and treated with CH$_2$N$_2$ to yield a pale yellow oil. RP-18 PTLC of the methyl ester using H$_2$O−CH$_3$CN (8:92) gave two fractions. Si gel HPLC of the first fraction furnished 3-epiplakortin, **1** (113 mg), and plakortide H, **5** (21 mg). The second fraction, which appeared to be homogeneous by both Si gel and RP-18 TLC, was resolved by repeated RP-18 HPLC (gradient, CH$_3$CN:H$_2$O, 70:30−99:5, UV detection) into plakortide F, **3** (73 mg), and plakortide G, **4** (33 mg).

Plakortide F (3): colorless oil; $[\alpha]^{25}_D$ −159.5° (*c* = 0.9); UV λ_{max} (MeOH) 209 nm; IR ν_{max} (neat) 2961, 2934, 2875, 1743, 1461, 968 cm^{-1}; HRDCIMS *m/z* 354.2753 (calcd for C$_{21}$H$_{38}$O$_4$, 354.2770); ^{1}H and ^{13}C NMR see Table 1.

Plakortide G (4): colorless oil; $[\alpha]^{25}_D$ +67.2° (*c* = 0.83); UV λ_{max} (MeOH) 213 nm; IR ν_{max} (neat) 2962, 2935, 2875, 1743, 1461, 968 cm^{-1}; HRDCIMS *m/z* 354.2759 (calcd for C$_{21}$H$_{38}$O$_4$, 354.2770); ^{1}H and ^{13}C NMR see Table 1.

Plakortide H (5): colorless oil; $[\alpha]^{25}_D$ +5.5° (*c* = 2.9); UV λ_{max} (MeOH) 221 nm; IR ν_{max} (neat) 2962, 2934, 2875,1743, 1666, 1461, 968 cm^{-1}; HRDCIMS *m/z* 366.2744 (calcd for C$_{22}$H$_{38}$O$_4$, 366.2770); ^{1}H and ^{13}C NMR see Table 1.

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