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The Plakortones, Novel Bicyclic Lactones from the Sponge *Plakortis halichondrioides* : Activators of Cardiac SR-Ca^{2+} -Pumping ATPase

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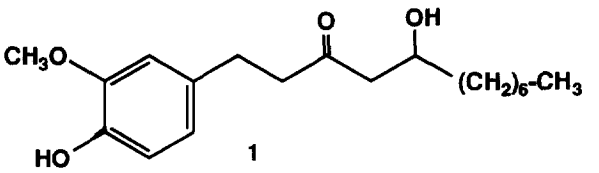
Abstract: Bioassay-guided fractionation of the EtOAc extract of sponge *Plakortis halichondrioides* yielded four novel bicyclic lactones, plakortones A, B, C, D and a novel acid, plakortide E (**3, 4, 5, 6** and **7**). The structures, including stereochemistry, of these compounds were established by interpretation of spectral data. Plakortones A-D (**3-6**) comprise a novel class of activators of cardiac SR-Ca^{2+} -pumping ATPase which were found to be active at micromolar concentrations As part of an SAR study, the α and β diols **9** and **10** of plakortone D were prepared using Sharpless AD procedure.

Introduction: The rapid removal of Ca^{2+} ions from the cytosol, necessary for the efficient relaxation of cardiac muscle cells, is performed mainly by the Ca^{2+}-pumping ATPase of the sarcoplasmic reticulum (SR)[1]. Altered sequestration of Ca^{2+} by the cardiac SR, may contribute to the abnormal relaxation observed in some forms of human heart failure,[2] and possibly to the inverse force-frequency relationship observed in failing myocardium.[3] Therefore, it was proposed to identify compounds capable of increasing Ca^{2+} pumping by the cardiac SR in order to correct relaxation abnormalities.

Although gingerol (**1**), a compound extracted from the rhizome of *Zingiber officinale* Roscoe was reported to produce a cardiotonic action most likely resulting from activation of the Ca^{2+} pumping activity of the SR,[4] no data are available regarding the possible clinical use of this product. Compounds such as heparin[5] and tannic acid[6] were described as activators of the cardiac SR-Ca^{2+}-pump which work by relieving the inhibitory effect of the regulatory protein phospholamban, but they could not be used as therapeutic agents owing to their

limited selectivity. Therefore, no suitable activator of cardiac SR-Ca^{2+} ATPase is available to date for treating cardiac diseases.



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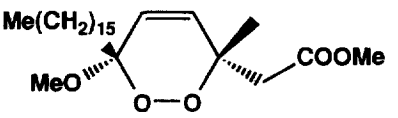

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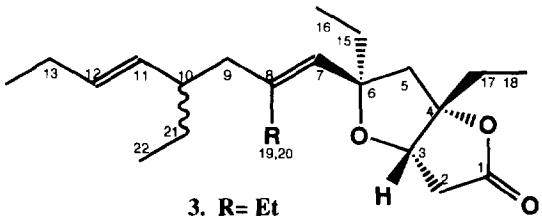
Marine organisms have proven to be a good source of compounds with interesting biological activities,[7] such as, plakorin (**2**),[8] an activator of skeletal muscle SR-Ca^{2+} ATPase which was isolated from a *Plakortis* sponge. Over the years many *Plakortis* sp. sponges, including *Plakortis halichondrioides* have provided chemically and biologically interesting metabolites.[9-29]

Results and Discussion

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 product extracts to stimulate cardiac SR-Ca^{2+} ATPase. Over 2400 plant and marine extracts were screened. An extract from one of the sponges collected in Jamaica showed the ability to stimulate SR-Ca^{2+} ATPase activity and was selected for fractionation. We now report the isolation from *Plakortis halichondrioides* and structure determination of four novel polyketides, plakortones A-D (**3-6**), and a novel acid, plakortide E (**7**), which have useful biological activity.

The freeze-dried sponge was extracted sequentially with ethyl acetate and methanol. The ethyl acetate extract which showed ability to stimulate SR-Ca^{2+} ATPase activity was chromatographed over a column of silica gel to yield several active fractions. Further purification of these active fractions by preparative TLC and HPLC led to the isolation of plakortones A-D (**3-6**) and plakortide E (**7**).



3. R= Et
4. R= Me

Plakortone A (**3**) was isolated as an oil, $[\alpha]_D$= -21.1°. The UV spectrum displayed a strong absorption at 240 nm and the IR spectrum contained a strong band at 1783 cm^{-1} which indicated the presence of a lactone carbonyl. The positive ESIMS of plakortone A (**3**) had a molecular ion at *m/z* 349 and exhibited fragment ions at *m/z* 331 and *m/z* 183 corresponding to the loss of water and loss of the branched side chain, respectively. The molecular formula of plakortone A (**3**) was deduced as $C_{22}H_{36}O_3$ from ^{13}C NMR and high resolution DCIMS [*m/z* 349.2765, $(M+H)^+$] which required five degrees of unsaturation. The ^1H NMR (Table 1) spectrum showed three olefinic multiplets between δ 5.37 and 5.07 and a doublet of doublets at δ 4.26. In addition, there were a number of methylene multiplets between δ 2.72 and 1.15 and five methyl triplets in the upfield region.

The proton signals were divided into a number of separate spin systems by interpretation of ^1H decoupling and COSY experiments and the resulting assignments were verified by HMQC and HMBC NMR data. In the side chain of the molecule, the two olefinic multiplets at δ 5.07 (H-11) and 5.37 (H-12) shared a 15.3 Hz trans coupling. The ^1H-^1H COSY NMR spectrum of **3** showed a correlation between H-12 and the methylene of an ethyl group. H-11 was coupled to the H-10 methine multiplet at δ 1.96 which also shared additional correlations with the methylene signals of an ethyl group at H-21 (δ 1.37, 1.15) and a vinylic methylene at H-9 (δ 2.08, 1.89). Decoupling of the narrow H-7 olefinic multiplet at δ 5.06 resulted in the sharpening of the signals due to the H-9 methylene group and the H-19 methylene, which was part of another ethyl group. Therefore, there must be ethyl groups attached to C-10 and C-8. The remaining two ethyl groups were located in the bicyclic lactone portion of **3** which also incorporated a methine multiplet at δ 4.26 (H-3) which further coupled to a non-equivalent methylene pair (H-2). There was an isolated methylene group (H-5) also present.

The ^{13}C GASPE spectrum (Table 2) displayed five methyls, eight methylenes, five methines and four quaternary carbon signals which are assigned in Table 2. The structure of the bicyclic portion of the plakortone A (**3**) was established by evidence based on HMBC

TABLE 1. ^{1}H NMR Data ($CDCl_3$) for Plakortones A-D (3-6) and Plakortide E Methyl ester (8)

H no.	3	4	5	6	8
2	2.72 β (1H,dd,4.9,18.4) 2.66 α (1H,dd,1.5,18.4)	2.71 β (1H,dd,5.1,18.4) 2.64 α (1H,dd,1.3,18.4)	2.69 β (1H,dd,4.8,18.2) 2.61 α (1H,dd,1.0,18.2)	2.70 β (1H,dd,4.4,18.3) 2.63 α (1H,dd,1.3,18.3)	6.07 (1H,d,15.8)
3	4.26 (1H,dd,1.5,4.9)	4.21 (1H,dd,1.3,5.1)	4.34 (1H,dd,1.0,4.8)	4.33 (1H,dd,1.3,4.4)	6.85 (1H,d,15.8)
5	2.30 α (1H,d,13.8) 2.14 β (1H,d,13.8)	2.24 α (1H,d,13.7) 2.13 β (1H,d,13.7)	2.28 α (1H,d,14.5) 1.83 β (1H,d,14.5)	2.26 α (1H,d,14.4) 1.89 β (1H,d,14.4)	2.54 β (1H,d,12.0) 2.44 α (1H,d,12.0)
7	5.06 (1H,m)	5.03 (1H,q,1.3)	1.38 (2H,d,5.7)	1.53 (1H,m) 1.35 (1H,m)	5.11 (1H,q,1.3)
8			1.57 (1H,m)	1.21 (1H,m) 1.16 (1H,m)	
9	2.08 (1H,m) 1.89 (1H,m)	2.00 (1H,m) 1.85 (1H,m)	1.20 (1H,m) 1.10 (1H,m)	1.32 (1H,m) 1.18 (1H,m)	2.00 (1H,m) 1.85 (1H,m)
10	1.96 (1H,m)	1.98 (1H,m)	1.85 (1H,m)	1.76 (1H,m)	2.00 (1H,m)
11	5.07 (1H,ddt,8.4,15.3,1.5)	5.06 (1H,ddt,8.4,15.3,1.5)	4.99 (1H,ddt,9.0,15.3,1.5)	5.07 (1H,ddt,8.9,15.3,1.5)	5.05 (1H,ddt,8.4,15.3,1.5)
12	5.37 (1H,dt,15.3,6.4)	5.36 (1H,dt,15.3,6.3)	5.39 (1H,dt,15.3,6.4)	5.38 (1H,dt,15.3,6.4)	5.34 (1H,dt,15.3,6.4)
13	1.96 (2H,m)	1.96 (2H,m)	1.99 (2H,m)	2.00 (2H,m)	1.97 (2H,m)
14	0.96 (3H,t,7.4)	0.95 (3H,t,7.4)	0.95 (3H,t,7.4)	0.97 (3H,t,7.4)	0.93 (3H,t,7.4)
15	1.70 (2H,m)	1.73 (2H,m)	1.60 (2H,m)	1.55 (2H,m)	1.86 (1H,m) 1.64 (1H,m)
16	0.88 (3H,t,7.4)	0.86 (3H,t,7.4)	0.82 (3H,t,7.4)	0.84 (3H,t,7.4)	0.88 (3H,t,7.4)
17	1.76 (2H,m)	1.73 (2H,m)	1.74 (2H,m)	1.74 (2H,m)	1.78 (2H,m)
18	0.98 (3H,t,7.4)	0.96 (3H,t,7.4)	1.00 (3H,t,7.4)	1.02 (3H,t,7.4)	0.90 (3H,t,7.4)
19	2.18 (2H,m)	1.69 (3H,d,1.3)	0.89 (3H,d,6.5)		1.61 (3H,d,1.3)
20	0.97 (3H,t,7.4)				1.35 (1H,m) 1.10 (1H,m)
21	1.37 (1H,m) 1.15 (1H,m)	1.35 (1H,m) 1.15 (1H,m)	1.30 (1H,m) 1.17 (1H,m)	1.35 (1H,m) 1.18 (1H,m)	0.80 (3H,t,7.4)
22	0.84 (3H,t,7.4)	0.83 (3H,t,7.4)	0.81 (3H,t,7.4)	0.82 (3H,t,7.4)	
					3.73 (3H,s,OCH_3)

data. The H-7 multiplet at δ 5.06 showed correlations to C-8, C-9 and C-19 of the side chain of **3** and to the oxygenated quaternary C-6 at δ 87.0 and C-5 methylene at δ 49.2 in the bicyclic lactone moiety. Furthermore, correlations showed that the C-15/16 ethyl group was attached at C-6. The inequivalent H-5 doublets at δ 2.30 and 2.14 correlated to C-6, C-7 and C-15 as well as to the oxygenated quaternary C-4 at δ 97.3 and C-17 which was the methylene of the sole remaining ethyl group. The H-3 signal showed a one bond correlation to the oxygenated C-3 which resonated at δ 79.7 and long range correlation to the lactone carbonyl C-1 at δ 175.7. It was noteworthy that the methyl carbons of the two ethyl groups, C-16 and C-18, which were pendant from the bicyclic lactone moiety, resonated noticeably upfield near δ 8. This observation was of diagnostic value.

The relative stereochemistry of the bicyclic lactone in plakortone A was determined by nOe difference data which are summarized in Figure 1. Saturation of H-7 caused an enhancement of the H-9 methylene protons establishing that the C-7/8 double bond possessed the E-configuration. Irradiation of H-7 also enhanced H-5β and H-3 suggesting that they were on the same face of bicyclic lactone. The H-3 signal shared nOes with the H-17 methylene protons and H-2β. Thus the H-17/18 ethyl group was also situated on the same face of the bicyclic plane as H-3 and H-7. The relative stereochemistry at C-10 was not be determined nor was the absolute stereochemistry of **3**.

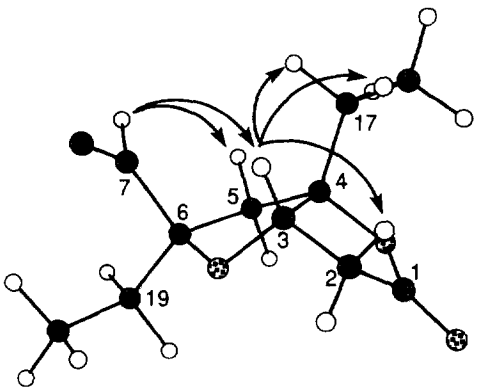


Figure 1. Molecular model of the bicyclic lactone portion of plakortone D with arrows representing nOe enhancements.

With the structure of plakortone A (**3**) in hand, we readily assigned structures to the closely related plakortones B-D (**4-6**). Plakortone B (**4**), which was isolated as a colorless oil, displayed a molecular ion at *m/z* 335.2541 $(M+H)^+$. The molecular formula was determined to be $C_{21}H_{34}O_3$ by HRDCIMS, CH_2 less than for **3**. The UV and IR spectra were similar to those of **3**, and the ^1H NMR spectrum ($CDCl_3$) was almost identical with that of **3**, except that in place of the five methyl triplets near δ 1.0, plakortone B (**4**) had four methyl triplets near δ 1.0 and a methyl doublet at δ 1.69 which shared a 1.3 Hz coupling

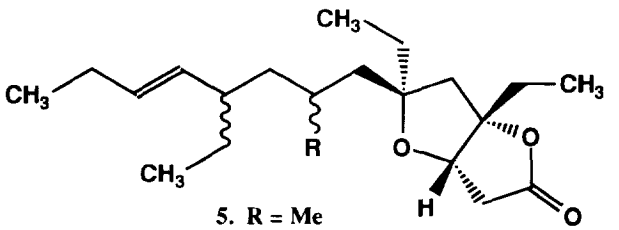
TABLE 2. ^{13}C NMR Data ($CDCl_3$) for Plakortones A-D (3-6) and Plakortide E Methyl Ester (8)

C no.	3	4	5	6	8
1	175.7	175.6	175.5	175.5	166.9
2	36.8	36.7	37.4	37.4	119.9
3	79.7	79.5	80.2	80.5	149.6
4	97.3	97.2	97.6	97.8	87.1
5	49.2	49.0	45.9	45.0	55.9
6	87.0	86.9	88.0	87.5	89.1
7	129.8	129.5	45.9	38.4	126.7
8	142.8	137.1	26.3	21.5	136.4
9	42.2	46.9	44.7	35.4	46.5
10	42.7	42.6	42.1	44.3	42.5
11	132.9	132.7	133.2	133.2	132.7
12	132.0	131.9	132.3	132.3	131.9
13	25.6	25.5	25.6	25.6	25.5
14	14.0	14.0	14.1	14.2	14.0
15	34.3	33.7	31.6	31.4	32.1
16	8.8	8.7	8.6	8.4	8.8
17	30.3	30.3	30.3	30.3	30.8
18	8.4	8.3	8.5	8.6	8.8
19	22.7	16.7	20.7	28.3	17.7
20	12.4				27.6
21	27.9	27.8	28.9		11.5
22	11.6	11.5	11.7	11.7	
					51.5 (OCH_3)

with H-7 reflecting the replacement of the ethyl group at olefinic C-8 by a methyl group. In addition, ^{13}C NMR signals for the 21 carbons appeared at positions identical (bicyclic lactone ring) or very similar (side chain) to those in **3**, except for the C-19 vinylic methyl group which resonated at δ 16.7.

The nOe data suggested that the relative stereochemistry of plakortone B (**4**) was identical to that of plakortone A (**3**). An additional nOe was observed between the H-19 methyl doublet and H-3 which is reasonable for the proposed stereochemistry in which the side chain of **4** is on the same face of the bicyclic lactone moiety as is H-3.

Plakortone C (**5**), also obtained as an oil, showed a molecular ion at *m/z* 337.2717 by HRDCIMS ($C_{21}H_{37}O_3$ $[M+H]^+$), 2 mass units higher than that of **4**. The UV and IR absorptions were identical to those of **3** and **4** and the ^1H NMR spectrum was similar to that of **4** except that it lacked the H-7 multiplet at δ 5.03 and H-19 methyl at δ 1.69. The occurrence of a methyl doublet at δ 0.89, which shared a 6.5 Hz coupling with a methine multiplet at δ 1.57 assigned to H-8, suggested that the C-7/8 double bond had been reduced in **5**. The ^{13}C GASPE NMR spectrum displayed 21 carbons and confirmed the presence of an additional methine (δ 26.3, C-8) and methylene (δ 45.9, C-7). These assignments were supported by the COSY and HMQC NMR spectra. The relative stereochemistry of **5** was identical to that of **3** and **4**. Irradiation of H-3 enhanced H-2β, the H-17 methylene protons, the H-7 methylene doublet at δ 1.38 and the H-19 methyl doublet at δ 0.89. These enhancements established that the side chain of plakortone C (5) was once again on the same face of the bicyclic lactone moiety as H-3 and the ethyl group at C-4.

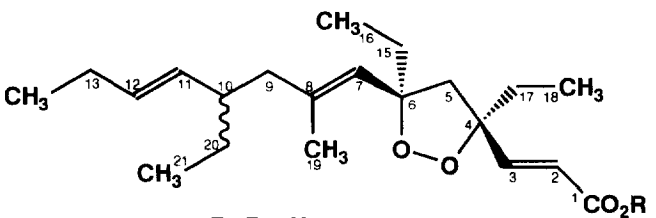


5. R = Me
6. R = H

Plakortone D (**6**) was isolated as an oil with an $[\alpha]_D$= -26.3° and had a molecular weight of 322 Daltons by ESIMS, 14 Daltons less than that of plakortone C (**5**). The molecular formula of $C_{20}H_{35}O_3$ $(M+H)^+$ was deduced from the HRDCIMS. The UV and IR spectra of **6** were identical to those of **3**-**5** and the ^1H NMR spectrum of **6** closely resembled that of plakortone C (**5**) except that it had only four methyl triplets. The ^{13}C NMR spectrum showed the presence of four methyl signals (two of which resonated near δ

8.5), one additional methylene carbon (δ 38.4) and one less methine signal than in **5** suggesting that the C-19 methyl group was absent in **6**. The relative stereochemistry of plakortone D (**6**) was identical to that of plakortones A-C (**3-5**). Irradiation of the H-17 methylene protons enhanced H-2β and H-3 indicating that they were cis-oriented. Saturation of H-5α enhanced the H-16 methyl protons, both of which occupied a position on the opposite face of the bicyclic lactone moiety away from H-17, H-2β and H-3.

Plakortide E (**7**), $[\alpha]_D$= +63.9°, was isolated as a low melting solid with a $C_{22}H_{36}O_4$ molecular formula requiring five degrees of unsaturation. Many similarities were observed between the ^1H NMR spectrum of **7** and those of plakortones A-D, especially between the signals corresponding to the two double bonds and the vinyl methyl group attached at C-8 in the side chain. The H-2 methylene proton signal, however, was not present. Additionally two sharp olefinic doublets sharing a 15.8 Hz trans coupling were observed at δ6.07 and δ6.85. In the IR spectrum a sharp, intense absorption at 1690 cm^{-1} suggested that the carbonyl was an α,β-unsaturated acid. Treatment of **7** with diazomethane yielded methyl ester **8** (^1H methyl singlet at δ 3.73 and ^{13}C carbonyl at 166.9), confirming the presence of an acid group in **7**. The carbonyl double bond and the three olefinic double bonds accounted for four of the five degrees of unsaturation suggesting that plakortide E (**7**) had a single ring.



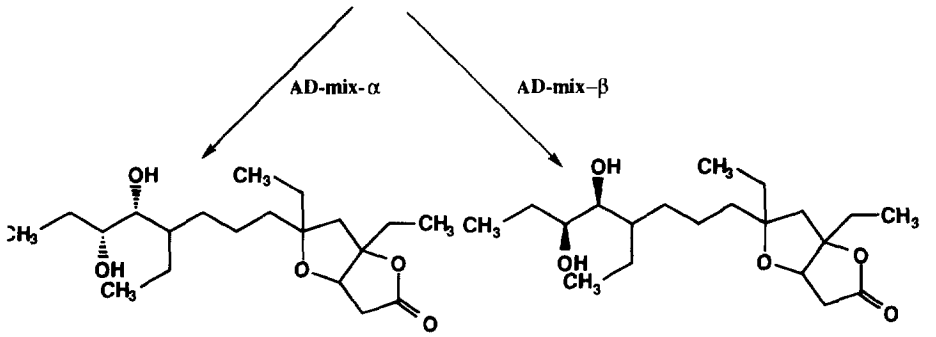
7. R = H
8. R= Me

From the NMR data, it was evident that the remaining oxygens in **7** must be linked via a peroxide functionality forming a substituted 1,2-dioxalane moiety. Examination of the COSY and the HMBC experiments indicated the presence of a five-membered ring in **7** rather than a six-membered peroxide ring. Five member rings are common in *Plakortis* sp. sponges.[15-20] Specifically, the H-7 multiplet (δ 5.11) showed correlation to C-8, C-9 and C-19 in the side chain portion of **7** and to the oxygenated quaternary C-6 at δ 89.1 and methylene C-5 at δ 55.9 in dioxalane ring. Other correlations showed that an ethyl group was attached at the quaternary C-6. The H-5 doublets (δ 2.54, 2.44) correlated to C-6, C-7

and C-15 as well as to the oxygenated quaternary C-4 at δ 87.1. The olefinic H-2 and H-3 doublets correlated with C-4 and the C-1 acid carbonyl at δ 166.9.

Analysis of the nOe difference data led to the determination of the relative stereochemistry of the dioxalane ring in plakortide E (**7**). Saturation of H-7 caused enhancement of the H-9 methylene protons establishing that the C-7/8 double bond possessed the E configuration. Irradiation of H-7 also enhanced H-5β suggesting that both had a cis orientation. The H-5β signal shared nOes with H-3 and H-2 while the H-5α shared nOes with H-15 and H-17 of the two ethyl groups. This meant that the two ethyl groups attached at C-4 and C-6 are cis oriented as is H-5α.

Plakortone D (6)



9 **10**

As part of an SAR, we could find very few compounds from our compound bank that were structurally related to the plakortones. In the preliminary assays plakortones A-D (**3-6**) failed to show any activity in cardiac skinned fibers (pluricellular preparation). In order to reduce the molecular anisotropy of these compounds, which was proposed as a possible reason for their lack of activity, and at the same time attempt to increase their hydrophilicity, the Sharpless asymmetric dihydroxylation (AD) procedure[30] was performed on the double bond present in the side chain of plakortone D (**6**) in order to produce α- and β- diols. This was achieved by using commercially available AD-mix-α and AD-mix-β. Virtually quantitative transformations yielded plakortone D-11,12-α-diol (**9**) and plakortone D-11,12-β-diol (**10**) which were characterized by NMR. The ^{1}H NMR spectra of **9** and **10** were identical to that of plakortone D (**6**) except that the vinylic protons at C-11 (δ 5.07) and C-12 (δ 5. 38) were replaced by two oxygenated methine protons which appeared at δ 3.36 and

3.52 respectively. In the ^{13}C NMR spectra of **9** and **10**, two olefinic carbons at δ 133.2 and 132.3 were replaced by two oxygenated aliphatic carbons at δ 74.9 and 73.3 respectively.

Biological Activity: The four novel plakortones were evaluated for their ability to enhance oxalate-supported Ca^{2+} uptake using isolated cardiac SR-vesicles. In this assay based on the on-line monitoring of $[Ca^{2+}]_{free}$ decline via the fluorescence of fluo-3 (to assess the amount of Ca^{2+} stored by isolated cardiac SR-vesicles), the plakortones did not alter basal fluorescence of the assay medium. Therefore, this assay was suitable for assessing the effect of these natural products on SR-Ca^{2+} -pumping ATPase. As shown in Table 3, plakortones A-D significantly enhanced initial rates of SR-Ca^{2+} uptake. Although this stimulation was concentration-dependent for plakortones B-D (**4-6**), the maximum stimulating effect was obtained near 3 μg/ml and the activating effect reached a plateau between 3 and 30 μg/ml. In contrast, the stimulating effect of plakortone A (**3**) was still increasing at 30 μg/ml, but higher concentration could not be assayed due to limited solubility of this compound.

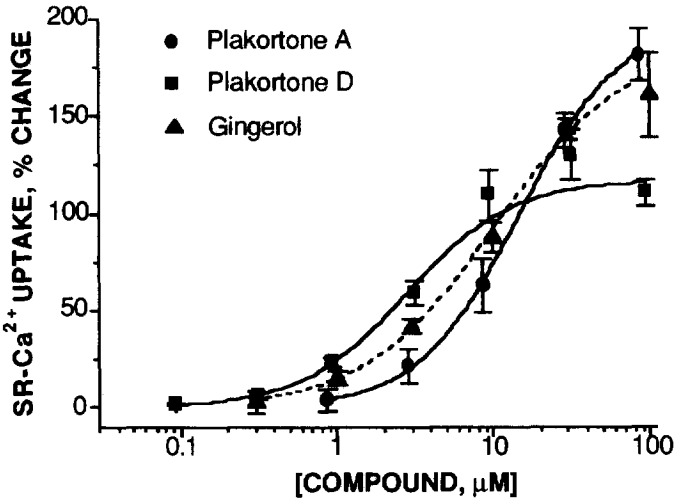


Figure 2. Concentration-response curve obtained on SR-Ca^{2+} uptake by isolated cardiac SR-vesicles, with plakortone A and plakortone D in comparison with gingerol. Values are mean±SEM n=4-6) and curves were fitted to Hill equation using the software Origin 3.5.

In order to further investigate the potential interest of these novel activators of SR-Ca^{2+} uptake, their activities have been compared to that of gingerol which is one of the few

available activators of cardiac SR-Ca^{2+} -pumping ATPase.[4] Fig. 2 shows that both the level of activation and the potency displayed by plakortone A (**3**) and plakortone D (**6**) are similar to that observed with gingerol. After fitting the experimental data to the Hill equation, the best potency was observed for plakortone D (**6**) with EC_{50} (μM) of 2.8±0.4 as compared to 10.1±3.7 and 14.5±4.3 for gingerol and plakortone A (**3**), respectively.

Table 3. Stimulation of cardiac SR-Ca^{2+} uptake by plakortones.

Compound	Concentration 3 μg /ml	Concentration 30 μg / ml
Plakortone A	62.7±13.8	181.1±13.8
Plakortone B	90.1±23.8	93.3±13.1
Plakortone C	119.5±18.8	105.0±5.0
Plakortone D	109.4±13.1	110.5±6.7

Values shown are mean ± SEM (n=4-6) of percent stimulation of the initial rate of oxalate-supported Ca^{2+} uptake in the presence of compound relatively to the activity in the presence of the solvent.

As was previously mentioned about the crude extract, the pure plakortones also stimulated the cardiac SR-Ca^{2+} ATPase, assessed by measuring the amount of Pi liberated during incubation. For example, the enhancement of this enzymatic activity reached 32.3±6.0% with 30 μM plakortone D. This suggests that the mechanism of stimulatory action of these compounds on the Ca^{2+} of SR is related to the direct activation of SR-Ca^{2+} ATPase. The extent of stimulation of Ca^{2+} ATPase by plakortones is less than that of the Ca^{2+} pumping activity reported for gingerol[4]. This difference may be due to the experimental conditions used to assess Ca^{2+} ATPase and Ca^{2+} uptake activities.

Subsequent results obtained after examination of the effect of plakortone D (**6**) on SR-Ca^{2+} uptake by isolated myocytes (described elsewhere) showed that the sequestration of Ca^{2+} by the SR in this preparation is also enhanced by plakortone D.

Unfortunately, both the α- and β-diols **9** and **10** of plakortone D showed no activity on SR-Ca^{2+} uptake. This is in agreement with previous findings. For a series of activators of cardiac SR-Ca^{2+} ATPase activity which consisted essentially of a polar nucleus attached to a hydrophobic side chain, introduction of a polar group into the side chain at a distance farther than one methylene group from the nucleus leads to a decrease in activating effect, or

even to the disappearance of the activating effect when the distance between the polar nucleus and the polar group reached five methylene groups.

In conclusion, a novel class of activators of cardiac SR-Ca^{2+} -pumping ATPase, active at micromolar concentrations, has been identified. After the previous report[8] of the isolation of cyclic peroxides from the *Plakortis* sp. sponge capable of stimulating SR-Ca^{2+} ATPase, this study confirms interest in *Plakortis* sp. sponges as potential sources of bioactive compounds. Although, the effect of these novel compounds on *in-vitro* cardiac relaxation could not be examined owing to their limited solubility in aqueous media, they constitute valuable tools for study of the molecular mechanism of Ca2+ transport by SR-Ca2+ pumps. Furthermore, such compounds provide the basis for chemical synthesis of analogs possessing similar biochemical activity and improved solubility, which may represent improved pharmacology in order to aid the impaired relaxation of the diseased heart.

Experimental Section

General. 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 quadrupole mass spectrometer. LRDCI and HRDCIMS spectra were acquired on a VG-70SE instrument using methane and ammonia 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, was used for HPLC separations employing a Lichrosorb SI 60 (7 μm) column. UV spectra were recorded on a Beckman DV-7 spectrophotometer. Reagent grade chemicals (Fisher and Baker) were used throughout.

Biological Assay.

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

Cardiac SR-Ca^{2+} uptake was determined as previously reported[31] on isolated SR-vesicles prepared from dog left ventricle according to Jones et al.,[32] and using a fluorimetric method based on that described by Kargacin et al.[33] 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), 0.045 K_2H_2EGTA, and 0.105 $K_2CaEGTA$ to give a pCa of 6.0 which was calculated

using Fabiato's computer program.[34] Fluorescence was measured in a 3-ml cuvette using a Fluoromax (Jobin-Yvon) fluorimeter using the DM3000F (Spex) software. The excitation wavelength was set at 506 nm, and emission was monitored at 526 nm with band passes 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.[35] 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- Ca^{2+} complex. After the vesicles have been equilibrated for 1 min in the assay medium containing the natural product (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 precipitated 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 initial rates of 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.

Ca^{2+} ATPase activity

The rate of ATP hydrolysis was determined at 30°C by colorimetrically[36] measuring the amount of P_i liberated during a 15 min incubation of 40 µg/ml SR-vesicles prepared according to Jones et al.[32] in a medium containing (in mM) 100 KCl, 2 $MgCl_2$, 50 MOPS-KOH (pH 6.8), 2 Na_2ATP, 1 EGTA and 0.8 $CaCl_2$. The calcium ionophore A-23187 (2 µM) was included in the assay system so that Ca^{2+} ions accumulated by SR-vesicles during the assay would not inhibit the Ca^{2+} pumps from inner surfaces of the vesicles. The Ca^{2+} ATPase activity was taken as the difference between the quantity of P_i liberated in the presence and absence of added $CaCl_2$. The assay ran with a robotic sample processor (Tecan RSP 5052) which distributed the buffers with and without $CaCl_2$, then SR-vesicles were preincubated for 20 min in the assay medium (final volume, 1 ml) containing the compound (or MeOH 1% as solvent) before initiating the ATPase reaction by the addition of Na_2ATP. After exactly 15 min, the ATPase reaction was stopped by the addition of the colorimetric reagent before reading the absorbance in each test tube using a Beckman DU-60 spectrophotometer connected to a Gilson (model 222) sample processor.

Collection, Extraction, and Isolation. The sponge, voucher number JAM90-003, was collected by hand using closed-circuit mixed gas rebreathers on November 26, 1990 at a

depth of 100 feet on the west fore 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 as *Plakortis halichondrioides* (Wilson), (Order Homosclerophoride, Family Plakinidae). A voucher sample has been deposited in the Scripps Institution of Oceanography Benthic Invertebrate Collection, registry number # P1145. The freeze-dried sponge (321 g) was extracted with ethyl acetate and methanol to give 23.1 and 61 g extracts respectively. The pale yellow ethyl acetate extract (20 g), which showed ability to stimulate the SR-Ca^{2+} ATPase activity, was applied to a silica gel column 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 silica gel column using ethyl acetate:hexane mixture (15:85) to give several fractions. Initial fractions yielded an inactive oil (2.64 g). The next several fractions charred on silica gel plates upon treatment with vanillin /H_2SO_4 giving characteristic blue spots that intensified in color over 12 h to a deep ink-blue appearance and had almost identical R_f values. These fractions were combined to give an oily residue (0.81 g). After Si gel preparative TLC (acetone:hexane:20:80) this residue gave five fractions. The first four fractions were further subjected to Si gel HPLC (monitored by refractive index) using ethyl acetate:hexane (12:88) as the solvent system to obtain plakortones A (**3**, 21 mg), B (**4**, 44 mg), C (**5**, 59 mg), D (**6**, 57 mg). The fifth fraction was purified by reversed phase C-18 HPLC using H_2O:CH_3CN (8:92) to yield plakortide E (**7**, 110 mg) in pure form.

Plakortone A (3): Colorless oil, $[\alpha]^{25}_D$ -21.1° (*c* = 0.038, $CHCl_3$); UV ($CHCl_3$) λ_{max} 240, 228, 217 nm; IR (neat) ν max 3100-2800 , 1783 (C=O, lactone), 1674, 1631, 1463, 1220, 1155, 1076, 1567, 975 cm^{-1}; ^1H NMR , see Table 1; ^{13}C NMR, see Table 2; LRESIMS 349 $(M+H)^+$; positive HRDCIMS (methane) calcd M*r* for $C_{22}H_{37}O_3$ 349.2742 $(M+H)^+$, found M*r* 349.2765, 331.269, 183.095.

Plakortone B (4): Colorless oil, $[\alpha]^{25}_D$ - 9.2° (*c* = 0.72, $CHCl_3$); UV ($CHCl_3$) λ_{max} 241, 229 nm; IR (neat) νmax 3100-2800, 1781 (C=O, lactone), 1670, 1625, 1463, 1220,1155, 1079, 1057, 970 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; LRESIMS 335 $(M+H)^+$; positive HRDCIMS (methane) calcd M*r* for $C_{21}H_{35}O_3$ 335.2586 $(M+H)^+$, found M*r* 335.2451, 317.243, 183.102.

Plakortone C (5): Colorless oil, $[\alpha]^{25}D$ - 24.9° (*c* = 1.23, $CHCl_3$); UV ($CHCl_3$) λ_{max} 241, 227, 223 nm; IR (neat) vmax 3000-2800, 1781 (C=O, lactone), 1675, 1462, 1175, 1153, 1076, 1062 and 970 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; LRESIMS 337 $(M+H)^+$; positive HRDCIMS (methane) calcd M*r* for $C_{21}H_{37}O_3$ 337.2743 $(M+H)^+$, found M*r* 337.2717, 319.257, 183.101.

Plakortone D (6): Colorless oil, $[\alpha]^{25}D$ -26.3° (*c* = 1.27 $CHCl_3$); UV ($CHCl_3$) λ_{max} 241, 227, 219 nm; IR (neat) vmax 3000-2800, 1781 (C=O, lactone), 1674, 1626, 1462, 1167, 1153, 1079, 1067 and 970 cm-1; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; LRESIMS 323 $(M+H)^+$; positive HRDCIMS (methane) calcd M*r* for $C_{20}H_{35}O_3$ 323.2586 $(M+H)^+$, found M*r* 323.2574, 305.248, 183.102.

Plakortide E (7): Colorless oil, $[\alpha]^{25}D$ + 63.9° (*c* = 2.0 $CHCl_3$); UV ($CHCl_3$) λ_{max} 244 and 219 nm; IR (neat) vmax 3400-2300, 3100-2800, 1690 (C=O, acid), 1659, 1591, 1461, 1399, 1313 and 967 cm^{-1}; LRESIMS 351 $(M+H)^+$; ^1H NMR ($CDCl_3$): δ 6.69 (1H, d, *J* = 15.8 Hz, H-3), 5.98 (1H, d, *J* = 15.8 Hz, H-2), 5.34 (1H, dt, *J* = 15.2, 6.3 Hz, H-12), 5.12 (1H, m, H-7), 5.05 (1H, ddt, *J* = 15.2, 8.3, 1.4 Hz, H-11), 4.94 (1H, bs, COOH), 2.53 (1H, d, *J* = 12.0 Hz, H-5β), 2.42 (1H, d, *J* = 12.0 Hz, H-5α), 2.00 (2H, m, H-9, H-10), 1.98 (2H, m, H-13), 1.85 (2H, m, H-9, H-15), 1.77 (2H, m, H-17, H-17), 1.63 (1H, m, H-15), 1.61 (3H, d, *J* = 1.0 Hz, H-19), 1.35 (1H, m, H-20), 1.11 (1H, m, H-20), 0.93 (3H, t, *J* = 7.4 Hz, H-14), 0.87 (6H, t, *J* = 7.4 Hz, H-16, H-18), 0.80 (3H, t, *J* = 7.4 Hz, H-21); ^{13}C NMR ($CDCl_3$): δ 173.0 (COOH), 146.9 (C-3), 136.5 (C-8), 132.8 (C-11), 131.9 (C-12), 126.9 (C-7), 123.9 (C-2), 89.1 (C-6), 87.2 (C-4), 55.8 (C-5), 46.6 (C-9), 42.6 (C-10), 32.1 (C-15), 31.0 (C-17), 27.6 (C-20), 25.6 (C-13), 17.7 (C-19), 14.0 (C-14), 11.6 (C-21), 8.9 (C-18), 8.8 (C-16).

Preparation of plakortide E methyl ester (8): A solution of diazomethane in ether (3 ml) was added to compound **7** (20 mg) in ether (10 ml). After 2 h, the solvent was evaporated and residue was purified by Si gel PTLC (EtOAc:Hexane:8:92) to afford methyl ester **8** as an oil. Colorless oil, $[\alpha]^{25}D$ + 75.1° (*c*= 2.23 $CHCl_3$); UV ($CHCl_3$) λ_{max} 246 and 218 nm; IR (neat) vmax 3100-2800, 1727 (C=O, ester), 1661, 1461, 1436, 1308, 1272, 1195, 1171 and 980 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR see Table 2; LRESIMS 365 $(M+H)^+$; positive HRDCIMS (methane) calcd M*r* for $C_{22}H_{37}O_4$ 365.2692 $(M+H)^+$, found M*r* 365.2681.

Preparation of plakortone D-11, 12-α-diol (9) and plakortone D-11,12-β-diol (10): A 25 ml round bottom flask, equipped with a magnetic stirrer, was charged with t-butyl alcohol (2.5 ml), H_2O (2.5 ml) and AD-mix-α (0.7 g). Stirring at room temperature produced a

bright yellow solution. Methane sulfonamide (0.15 g) was added at this point, and the mixture was stirred for an additional five minutes. Plakortone D (**6**, 0.025 g) was dissolved in CH_2Cl_2 (0.25 ml) and added to the reaction mixture which was stirred at rt. Progress of the reaction was monitored by TLC. After 16 h, all the starting material (**6**) had disappeared and a single product was formed. The reaction mixture was evaporated to dryness and the yellow residue extracted with CH_2Cl_2:EtOAc (1:1, 3 x 25 ml) . Evaporation of the solvent gave a colorless residue which was first purified by using RP-18 PTLC (H_2O:MeOH:15:85) and later Si gel PTLC (MeOH:CH_2Cl_2:7:93) to yield plakortone D-11,12-α-diol (**9**, 24 mg) as a colorless, viscous oil, $[\alpha]^{25}D$ - 27.3° (*c*= 1.33 $CHCl_3$); UV ($CHCl_3$) λ_{max} 242, and 219 nm; IR (neat) vmax 3450 (OH), 3000-2800, 1779 (C=O, lactone), 1463, 1229, 1201, 1170, 1153, 1030, 1069 and 972 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.34 (1H, dd, *J* = 1.1, 4.5 Hz, H-3), 3.54 (1H, dt, *J* = 4.2, 6.8 Hz, H-12), 3.37 (1H, dd, *J* = 4.2, 5.2 Hz, H-11), 2.71 (1H, dd, *J* = 4.5, 8.3 Hz, H-2β), 2.64 (1H, ddm, *J* = 1.0, 18.3 Hz, H-2α), 2.29 (1H, d, *J* = 14.4 Hz, H-5α), 2.20 (2H, bs, 2OH's), 1.89 (1H, d, *J* = 14.4 Hz, H-5β), 1.74 (2H, m, H-17, H-17), 1.56 (3H, m, H-13, H-15, H-15), 1.48 (2H, m, H-7, H-7), 1.42 (5H, m, H-8, H-9, H-13, H-19, H-19), 1.40 (1H, m, H-10), 1.28 (2H, m, H-8, H-9), 1.01 (3H, t, *J* = 7.4 Hz, H-18), 0.98 (3H, t, *J* = 7.4 Hz, H-14), 0.90 (3H, t, *J* = 7.4 Hz, H-20), 0.85 (3H, t, *J* = 7.4 Hz, H-16); ^{13}C NMR ($CDCl_3$): δ 175.6 (C-1), 97.8 (C-4), 87.4 (C-6), 80.6 (C-3), 75.3 (C-11), 73.4 (C-12), 45.2 (C-5), 41.3 (C-10), 38.9 (C-7), 37.4 (C-2), 31.3 (C-15), 30.4 (C-17), 30.2 (C-9), 26.8 (C-13), 21.4 (C-8), 21.1 (C-19), 11.6 (C-20), 9.9 (C-14), 8.6 (C-18), 8.5 (C-16); LRESIMS 357 $(M+H)^+$; positive HRDCIMS (methane) calcd *Mr* for $C_{20}H_{36}O_5$ 357.2641, found *Mr* 357.2677.

Plakortone D-11,12-β-diol (**10**) was prepared under identical conditions described above using AD-mix-β and plakortone D (**6**, 0.025 g) to afford **10** as a colorless, viscous oil, $[\alpha]^{25}D$ -9.8° (*c*= 2.34, $CHCl_3$); UV ($CHCl_3$) λ_{max} 241 and 219 nm; IR (neat) vmax 3456 (OH), 3000-2800, 1779 (C=O, lactone), 1463, 1230, 1170, 1153, 1030, 1071, 1060 and 971 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.33 (1H, dd, *J* = 1.0, 4.6 Hz, H-3), 3.52 (1H, dt, *J* = 4.6, 6.5 Hz, H-12), 3.36 (1H, dd, *J* = 4.2, 4.6 Hz, H-11), 2.69 (1H, dd, *J* = 4.6, 18.3 Hz, H-2β), 2.62 (1H, ddm, *J* = 1.0, 18.3 Hz, H-2α), 2.34 (2H, bs, 2 OH's), 2.26 (1H, d, *J* = 14.4 Hz, H-5α), 1.87 (1H, d, *J* = 14.4 Hz, H-5β), 1.74 (2H, m, H-17, H-17), 1.56 (3H, m, H-13, H-15, H-15), 1.48 (2H, m, H-7, H-7), 1.42 (5H, m, H-8, H-9, H-13, H-19, H-19), 1.40 (1H, m, H-10), 1.27 (1H, m, H-9), 1.15 (1H, m, H-8), 1.00 (3H, t, *J* = 7.4 Hz, H-18), 0.96 (3H, t, *J* = 7.4 Hz, H-14), 0.88 (3H, t, *J* = 7.4 Hz, H-20), 0.83 (3H, t, *J* = 7.4 Hz, H-16); ^{13}C NMR ($CDCl_3$): δ 175.6 (C-1), 97.8 (C-4), 87.4 (C-6), 80.5 (C-3), 74.9 (C-11), 73.3 (C-12), 45.1 (C-5), 41.4 (C-10), 38.9 (C-7), 37.4 (C-2), 31.3 (C-15), 30.3 (C-17), 28.8 (C-9), 26.7 (C-13), 22.9 (C-19), 21.5 (C-8), 11.3 (C-20), 9.9 (C-14), 8.6 (C-18), 8.5 (C-16); LRESIMS 357 $(M+H)^+$.

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