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Pachycladins A−E, Prostate Cancer Invasion and Migration Inhibitory Eunicellin-Based Diterpenoids from the Red Sea Soft Coral *Cladiella pachyclados*

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Alcyonaria species are among the important marine invertebrate classes that produce a wealth of chemically diverse bioactive diterpenes. Examples of these are the potent microtubule disruptor sarcodictyins and eleutherobin. The genus *Cladiella* has proven to be a rich source of cytotoxic eunicellin-based diterpenoids. Five new eunicellin diterpenes, pachycladins A−E (**1**−**5**), were isolated from the Red Sea soft coral *Cladiella pachyclados*. The known sclerophytin A Cladiellisin, 3-acetylcladiellisin, 3,6-diacetylcladiellisin, (+)-polyanthelin A, klysimplexin G, klysimplexin E, sclerophytin F methyl ether, (6*Z*)-cladiellin (cladiella-6*Z*,11(17)-dien-3-ol), sclerophytin B, and patagonicol were also identified. The structures of the isolated compounds were elucidated by extensive interpretation of their spectroscopic data. These compounds were evaluated for their ability to inhibit growth, proliferation, invasion, and migration of the prostate cancer cells PC-3. Some of the new metabolites exhibited significant anti-invasive activity.

Natural products have proven to be the most reliable source of new anticancer entities. Nearly 63% of anticancer drugs introduced over the last 25 years are natural products or can be traced back to a natural products origin.[1] A recent review lists 79 natural products or natural product analogues that entered clinical trial as anticancer agents between 2005 and 2007.[2] Soft corals of the class Alcyonaria produce a wealth of bioactive diterpenes. Examples of these are sarcodictyins and eleutherobin, which were isolated in 1995 by the Fenical group and found to be among the most potent cytotoxic natural products.[3] Alcyonaria species also afforded several bioactive diterpenes including cembranes,[4] norcembranes,[5] xeniaenes,[6] briaranes,[7] and eunicellins.[8,9] Previous reports on the chemical constituents of soft corals belonging to the genus *Cladiella* have illustrated the predominance of eunicellin-based diterpenoids.[10−18]

Eunicellin-based diterpenoids were found to display a wide range of bioactivities including anti-inflammatory and antitumor activities.[19−21] So far, there are no reports of anti-invasive or antimigratory activities of this class against different cancer cell lines. The lipophilic extract of the Red Sea soft coral *Cladiella pachyclados* showed antimigratory activity in a wound-healing assay, and therefore the ultimate objective of this study was to isolate and identify the bioactive ingredients of this extract and assess their antimigratory and anti-invasive activities against metastatic prostate cancer.

Results and Discussion

A frozen specimen of *C. pachyclados* was subjected to extraction with organic solvents. Fractionation of the lipophilic extract on Sephadex LH20 and final purification using C-18 reversed-phase chromatography afforded five new (**1**−**5**) and 11 known eunicellin-based diterpenes. The new compounds were given the generic names pachycladins A−E (**1**−**5**). The known sclerophytin A (**6**),[21,22] cladiellisin (**7**),[23] 3-acetylcladiellisin (**8**),[17] 3,6-diacetylcladiellisin (**9**),[17] (+)-polyanthelin A (**10**),[24,25] klysimplexin G (**11**),[26] klysimplexin E (**12**),[26] sclerophytin F methyl ether (**13**),[11] (6*Z*)-cladiellin (cladiella-6*Z*,11(17)-dien-3-ol, **14**),[27] sclerophytin B (**15**),[28] and patagonicol were also identified.[29]

The HRTOFMS analysis of pachycladin A (**1**) suggested the molecular formula $C_{26}H_{44}O_7$. Analysis of ^{1}H and ^{13}C NMR data of **1** (Table 1) and correlations in the ^{1}H−^{1}H COSY and HMBC spectra (Figure 1) led to the establishment of the gross structure **1**. Pachycladin A possesses the known eunicellin skeleton with oxygen functionalities at C-3, C-6, C-7, and C-11.[30−32] Two of these oxygenated carbons bear free hydroxy groups, and the others have acetate and butyrate ester moieties. Initially, the lack of any relevant HMBC correlations linking the ester moieties on their respective carbons made it difficult to assign the exact positions of these groups.[33] The chemical shift of H-6 (δ_H 4.55, d, J = 6.2 Hz) suggested a free secondary alcohol.[32,33] In addition, the downfield shift of the signals of C-3 and C-11 (δ_C 86.1 and 82.3, respectively) suggested the presence of the ester moieties at these positions.[32,33] This was supported by the downfield shift of the methyl singlets H_3-15 and H_3-17 (δ_H 1.37 and 1.47, respectively) compared to H_3-16 (δ_H 1.14, $\Delta\delta$ +0.23 and +0.33, respectively), which unequivocally supported the placement of the esters at C-3 and C-11. The assignment of C-3 acetate and C-11 butyrate was supported by a NOESY experiment. The acetate methyl singlet H_3-2′ showed strong NOESY correlations with the methyl singlet H_3-15, H-1, and H-2, suggesting its placement at C-3. Similarly, the methylene protons H_2-3″ (δ_H 1.66) showed a NOESY correlation with the pseudoequatorial proton H-12a (δ_H 2.26), supporting the placement of the butyrate group at C-11. These NOESY correlations were further justified by a molecular modeling study (Figure 2), which showed a distance range of 2.3−4.4 Å between H_3-2′ and H-12 and nearby functionalities listed above. On the basis of proton coupling constants, NOESY data, and comparison of ^{13}C NMR chemical shifts with published data,[26,30−32] the absolute configuration of **1** was proposed as 1*R,* 2*R,* 6*S,* 7*S,* 9*R,* 10*S,* 11*R,* 14*R*. This was further supported by the fact that the related (−)-sclerophytin A (**6**), with its established absolute configuration by enantioselective synthesis,[22] was co-isolated with **1** and likely shared the same biogenetic pathway.

The molecular formula of pachycladin B (**2**) was $C_{26}H_{42}O_7$ on the basis of the HRTOFMS. Comprehensive NMR data analysis (Table 1) of **2** indicated its close similarity to the known klysimplexin E (**12**),[23] with the replacement of the C-11 acetate with

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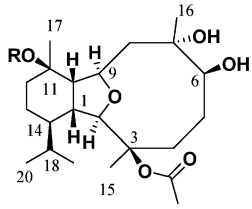
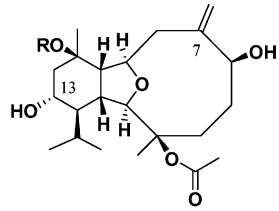
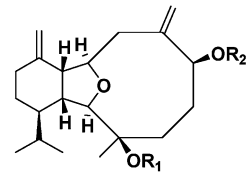
Chart 1



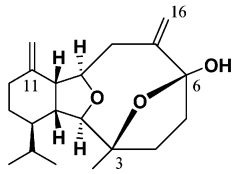
	R
1	COC_3H_7
11	$COCH_3$



	R
2	COC_3H_7
12	$COCH_3$



	R_1	R_2
3	H	$COCH_3$
7	H	H
8	$COCH_3$	H
9	$COCH_3$	$COCH_3$



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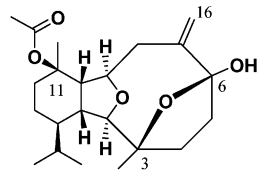

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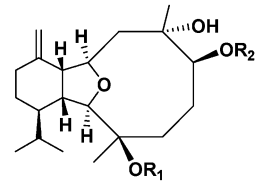
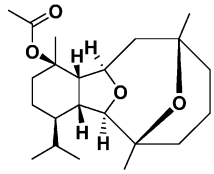


	R_1	R_2
6	H	H
13	H	CH_3
15	$COCH_3$	H



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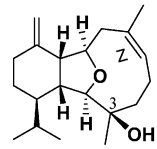

14

Table 1. ^1H and ^{13}C NMR Data of Compounds **1**−**3**[a]

	1		**2**		**3**	
position	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	42.4, CH	2.21, m	43.0, CH	2.22, dd (11.7, 7.3)	44.3, CH	2.17, m
2	92.3, CH	3.47, brs	91.7, CH	3.57, brs	91.7, CH	3.64, brs
3	86.1, C		84.6, C		74.0, C	
4	36.4, CH_2	1.84 m, 2.58, dd (14.6, 8.8)	30.1, CH_2	1.82, 2H, m	34.5, CH_2	1.60, m, 1.81, m
5	30.6, CH_2	1.42, 2H, m	35.5, CH_2	1.72, m, 2.06, m	32.3, CH_2	1.85, m, 2.03, m
6	80.7, CH	4.55, d (6.2)	73.2, CH	4.28, dd (11.0, 3.9)	75.4, CH	5.26, dd (11.4, 4.4)
7	77.3, C		150.0, C		148.1, C	
8	47.8, CH_2	2.15, m, 1.82, m	41.3, CH_2	2.45, d (13.2), 2.81, dd (13.7, 4.6)	39.0, CH_2	2.20, d (13.2), 3.00, dd (13.9, 5.5)
9	75.7, CH	4.04, ddd (11.2, 7.7, 3.7)	79.2, CH	4.11 dd (10.4, 3.8)	79.8, CH	4.10, dd (10.1, 5.7)
10	53.2, CH	3.05, dd (7.1, 7.1)	45.6, CH	3.05 dd (10.3, 7.3)	48.0, CH	2.90, dd (10.2, 8.0)
11	82.3, C		82.3, C		146.4, C	
12	32.1, CH_2	2.26, m, 1.57, m	42.2, CH_2	1.49, m, 2.40, dd (13.9, 3.7)	31.9, CH_2	2.03, m, 2.21, m
13	17.7, CH_2	1.34, 2H, m	66.8, CH	3.87, ddd (11.0, 11.0, 4.0)	25.4, CH_2	1.00, m, 1.74, m
14	42.7, CH	1.23, m	50.3, CH	1.22, dd (11.0, 11.0)	44.3, CH	1.28, m
15	23.3, CH_3	1.37, 3H, s	22.7, CH_3	1.54, 3H, s	26.9, CH_3	1.24, 3H, s
16	22.9, CH_3	1.14, 3H, s	117.1, CH_2	5.20, s, 5.45, s	118.3, CH_2	5.10, s, 5.42, s
17	22.6, CH_3	1.47, 3H, s	25.2, CH_3	1.55, 3H, s	111.2, CH_2	4.63, s, 4.78, dd (2.4, 2.4)
18	29.1, CH	1.72, m	28.5, CH	1.87, m	27.9, CH	1.81, m
19	15.4, CH_3	0.80, 3H, d (7.0)	15.9, CH_3	0.93, 3H, d (7.0)	15.2, CH_3	0.71, 3H, d (7.0)
20	21.9, CH_3	0.93, 3H, d (7.0)	24.8, CH_3	1.16, 3H, d (7.0)	22.1, CH_3	0.95, 3H, d (7.0)
1′	170.2, C		170.1, C		170.7, C	
2′	24.8, CH_3	1.97, 3H, s	22.5, CH_3	1.99, 3H, s	21.5, CH_3	1.97, 3H, s
1″	172.8, C		172.7, C			
2″	37.4, CH_2	2.34, 2H, m	37.4, CH_2	2.13, 2H, t (7.5)		
3″	18.7, CH_2	1.66, 2H, m	18.7, CH_2	1.58, 2H, q (15)		
4″	13.7, CH_3	0.98, 3H, t (14.6)	13.7, CH_3	0.92, 3H, t (7.3)		

[a] In $CDCl_3$, *J* in Hz; 400 MHz for ^1H and 100 MHz for ^{13}C NMR. Carbon multiplicities were determined by APT experiments, C = quaternary, CH = methine, CH_2 = methylene, CH_3 = methyl carbons.

a butyrate ester. The assignments of the C-3 acetate and C-11 butyrate were supported by a NOESY experiment, as discussed previously with **1**. On the basis of comparison of the ^{13}C and ^1H NMR chemical shifts as well as the proposed absolute stereochemistry with **1** and **6**, the configuration of pachycladin B (**2**) was established to be 1*R,* 2*R,* 3*R,* 6*S,* 9*R,* 10*S,* 11*R,* 13*R,* 14*R*.

Analysis of the HRTOFMS and NMR data of pachycladin C (**3**, Table 1) indicated its 6-acetylcladiellisin identity. This was evident

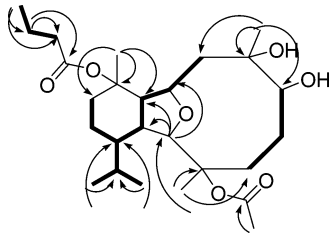

Figure 1. Selected ^{1}H−^{1}H COSY (bold lines) and HMBC correlations (arrows) of compound **1**.

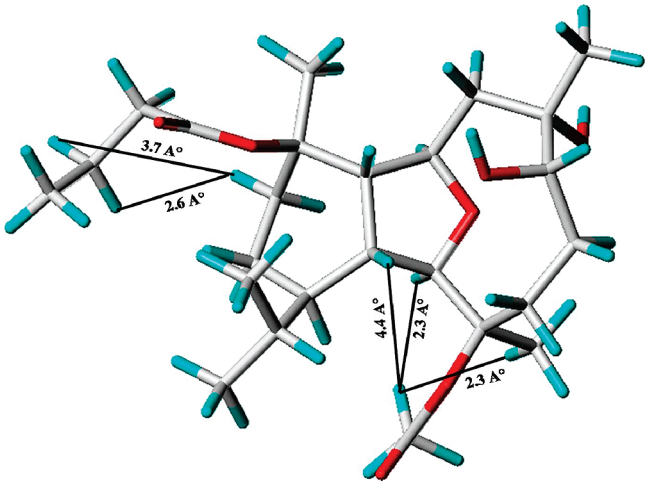

Figure 2. Pachycladin A (**1**) local minimum energy generated by SYBYL 8.1. Of interest are the 2.3−4.4 Å distance range between H_3-2′ and H-1, H-2, and H_3-15 and the 2.6−3.7 Å distance between H_2-3″ and H-12a, which support the NOESY correlations.

based on the downfield shifts of H-6/C-6 as compared to the known cladiellisin (**7**)[23] ($\Delta\delta$ +0.88 and +2.5, respectively) and the presence of new signals for an acetate moiety at δ 170.7 (C-1′) and 1.97/21.5 (H_3-2′/C-2′). The isolation and identification of 3-acetylcladiellisin (**8**) and 3,6-diacetylcladiellisin (**9**) from the same extract further supported the assignment of **3** as 6-acetylcladiellisin. The absolute configuration of 1*R,* 2*R,* 3*R,* 6*S,* 9*R,* 10*R,* 14*R* was proposed for pachycladin C (**3**) on the basis of the absolute configuration of the parent co-isolated **7** that was previously established.[23]

The HRTOFMS data of pachycladin D (**4**) established the molecular formula of $C_{20}H_{30}O_3$. Analysis of ^{1}H and ^{13}C NMR data of **4** (Table 2) showed close similarity to vigulariol with an exocyclic methylene at C-7.[34] The exomethylene olefinic protons H_2-16 (δ_H 5.14 and 6.11) showed a ^2J-HMBC correlation with the quaternary olefinic carbon C-7 (δ_C 150.7) and ^3J-HMBC correlations with the downfield dioxygenated quaternary carbon C-6 (δ_C 107.2) and the methylene carbon C-8 (δ_C 40.0), confirming the $\Delta^{7,16}$ system. The configuration of **4** was determined to be 1*R,* 2*R,* 3*R,* 6*S,* 9*R,* 10*R,* 14*R* on the basis of coupling constant values, observed NOESY data, and comparison with literature[34] and the previously proposed absolute configuration of **1**.

The HRTOFMS of pachycladin E (**5**) established the molecular formula $C_{22}H_{34}O_5$, consistent with six degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **5** (Table 2) indicated its close similarity to those of **4** with the replacement of the $\Delta^{11,17}$ exomethylene with C-11 β-acetoxy and C-17 α-methyl functionalities. The methyl singlet H_3-17 (δ_H 1.42) showed a ^2J-HMBC correlation with the quaternary oxygenated carbon C-11 (δ_C 78.5) and ^3J-HMBC correlations with the methine carbon C-10 (δ_C 50.0) and the methylene carbon C-12 (δ_C 29.5), supporting its placement at C-11. The acetoxy methyl H_3-2′ showed a ^2J-HMBC correlation with the carbonyl carbon C-1′ (δ_C 170.5). Furthermore, the protons H-1 and H-9 showed ^3J-HMBC correlations with the quaternary oxygenated carbon C-11. The configuration of **5** was determined as 1*R,* 2*R,* 3*R,* 6*S,* 9*R,* 10*S,* 11*R,* 14*R*, on the same basis previously used for **2**−**4**.

It is worth noting that the compound formulated as **10** in this investigation appears to be spectroscopically identical to a natural product reported by Bowden et al.[24] from an Australian soft coral *Briareum* species but with a tetracyclic skeleton as previously revised and named by Ospina et al.[25] as (−)-polyanthelin A. Compound **10** was revealed to be the (+)-enantiomer of polyanthelin A, on the basis of comparison of its specific rotation value ($[\alpha]_D^{25}$ +8.0, *c* 0.73, $CHCl_3$) with those reported by Bowden et al.[24] ($[\alpha]_D$ +8.9, *c* 0.22, $CHCl_3$) and Ospina et al.[25] ($[\alpha]_D^{20}$ −9.9, *c* 1.0, $CHCl_3$).

All isolated compounds were evaluated for their ability to inhibit the proliferation, migration, and invasion of the human prostate cancer PC-3 cell line using MTT, wound-healing, and Cultrex Basement Membrane Extract cell invasion assays, respectively.[35−37] None of the compounds showed any effect on the proliferation of PC-3 cells up to a 50 μM dose, indicating the lack of cytotoxicity toward this cell line. Compounds were then tested in the wound-healing assay at a 50 μM dose for the ability to inhibit the migration of PC-3 cells (Figures 3 and 4). Their activity was compared with a 200 μM dose of the antimetastatic marine natural product lead 4-hydroxyphenylmethylene hydantoin.[38,39] Compounds **1**, **4**, **6**, **8**, **9**, **10**, and **13** showed potent antimigratory activity, comparable to the positive control,[38,39] while **2**, **3**, **14**, and **15** showed moderate activities. A dose of 50 μM of **11** showed similar antimigratory activity to the positive control and compound **1** but with a notable cell shape change. To avoid correlation of the antimigratory activity with possible cytotoxic activity, klysimplexin G (**11**) was retested at a 10 μM dose. This dose induced a comparable antimigratory effect comparable to that induced by a 200 μM dose of 4-hydroxyphenylmethylene hydantoin without affecting cell shape or viability.

The antimetastatic lead 4-mercaptoethylphenylmethylene hydantoin (50 μM) was used as a positive control in the Cultrex cell invasion assay (Figure 5).[38−41] Out of all tested compounds, pachycladin A (**1**) was the most active followed by sclerophytin F methyl ether (**13**), polyanthelin A (**10**), and sclerophytin A (**6**). The activities of these compounds were several-fold that of the positive drug control, indicating the potential of eunicellins as a new anti-invasive class. On the basis of the results of the antimigratory and the anti-invasive assays, it can be concluded that the C-11 butyrate and/or $\Delta^{11,17}$ functionalities are essential for optimal activity. A 10 μM dose of **11** showed better anti-invasive activity than the 50 μM dose of the positive control without any apparent cytotoxicity or cell shape change. The introduction of *Z*-$\Delta^{6,7}$ maintained the activity, as illustrated by the activity of **14**. However, additional studies are needed to identify essential pharmacophores required for anti-invasive and antimigratory activities of this unique natural products class.

In conclusion, compounds with exomethylene functionalities at C-7 and C-11 demonstrate the lowest antimigratory activities. Upon the replacement of the exomethylene moiety at C-7 with a quaternary oxygenated carbon, the activities dramatically increase, as observed for compounds **6**, **10**, and **13**. The activity was maximal with the replacement of both exomethylene groups with oxygenated quaternary carbons, and the increment in activity was dependent on the side chain at C-11.

Experimental Section

General Experimental Procedures. A Rudolph Research Analytical Autopol III polarimeter was used to measure optical rotation. The IR spectra were recorded on a Varian 800 FT-IR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or C_5D_5N using TMS as an internal standard, on a JEOL Eclipse NMR spectrometer operating

Table 2. ^{1}H and ^{13}C NMR Data of Compounds **4** and **5**[a]

	4[b]		**5**	
position	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	46.4, CH	2.15, m	42.9, CH	2.30, m
2	91.4, CH	3.59, br s	91.4, CH	3.49, br s
3	78.1, C		87.9, C	
4	38.7,CH_2	2.05, m, 3.25, m	37.6, CH_2	1.86, m, 2.96, m
5	37.0, CH_2	1.47, m, 2.23, dd (11.7, 8.4)	36.6, CH_2	2.00, m, 2.16, m
6	107.2, C		107.1, C	
7	150.7, C		146.2, C	
8	40.0, CH_2	2.59, dd (16.0, 3.1), 3.23, m	42.2, CH_2	2.46, dd, (16.1, 3.2), 3.15, m
9	81.3, CH	4.14, ddd (7.7, 3.7, 3.7)	78.5, CH	3.96, ddd (9.0, 3.1, 3.1)
10	47.6, CH	3.85, dd (8.1, 8.1)	50.0, CH	3.08, ddd (9.0, 7.2, 1.5)
11	149.5, C		78.5, C	
12	31.6, CH_2	2.35, m, 2.43, m	29.5, CH_2	1.31, m, 2.57, m
13	24.9, CH_2	0.90, m, 1.66, m	17.9, CH_2	1.16, m, 1.40, m
14	43.4, CH	1.30, m	42.5, CH	1.30, m
15	24.4, CH_3	1.23, 3H, s	24.4, CH_3	1.19, 3H, s
16	115.3, CH_2	5.14, dd (2.6, 2.6), 6.11, d (0.7)	115.3, CH_2	4.97, s, 5.50, s
17	110.0, CH_2	4.75, s, 4.78, dd (3.1, 3.1)	24.1, CH_3	1.42, 3H, s
18	24.4, CH	1.56, dd (3.3, 12.8)	29.2, CH	1.64, m
19	15.3, CH_3	0.63, 3H, d (7.0)	15.3, CH_3	0.77, 3H, d (7.0)
20	21.9, CH_3	0.86, 3H, d (7.0)	21.8, CH_3	0.92, 3H, d (7.0)
1′			170.5, C	
2′			22.6, CH_3	1.97, 3H, s

[a] In $CDCl_3$, J in Hz; 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. Carbon multiplicities were determined by APT experiments, C = quaternary, CH = methine, CH_2 = methylene, CH_3 = methyl carbons. [b] In C_5D_5N.

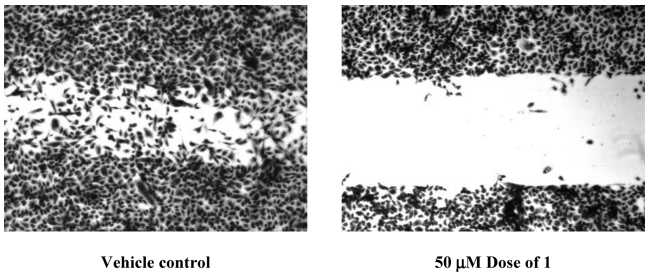

Figure 3. Relative number of migrated PC-3 cells from one side to the other side of the vehicle control and 50 μM dose of **1** after 24 h of wounding.

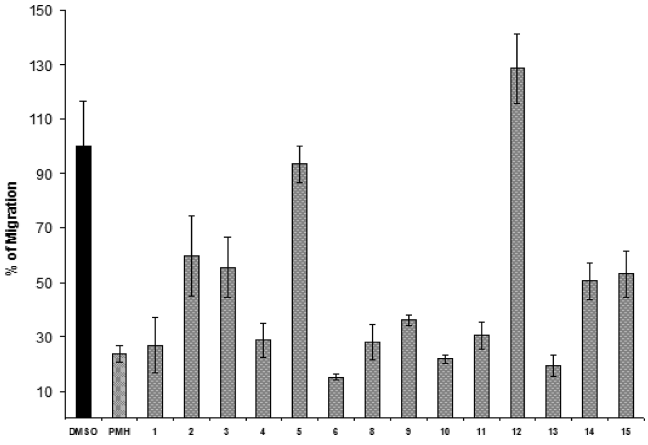

Figure 4. Effect of 50 μM dose of each of **1**−**10** and **12**−**15** and 10 μM dose of **11** on the percent of migration on the highly metastatic PC-3 in a wound-healing assay. A 200 μM dose of 4-hydroxyphenylmethylene hydantoin (PMH) was used as a positive drug control.[38,39]

at 400 MHz for ^{1}H and 100 MHz for ^{13}C. The HRTOFMS experiments were conducted at Louisiana State University on an Agilent 6200-TOFLCMS and the University of Mississippi on a Burker Bioapex FT mass spectrometer. For CC, Sephadex LH20 and C-18 Si gel (Bakerbond, Octadecyl 40 μm) were used. The TLC analyses were carried out on precoated Si gel 60 F_{254} 500 μm TLC plates (EMD Chemicals),

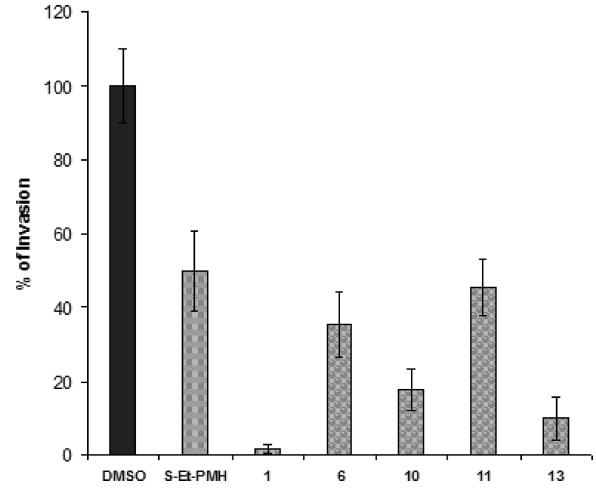

Figure 5. Anti-invasive activities of a 50 μM dose of each of **1**, **6**, **10**, and **13** and a 10 μM dose of **11** against PC-3 cells using a Cultrex assay kit. A 50 μM dose of 4-mercaptoethylphenylmethylene hydantoin (S-Et-PMH) was used a positive drug control.[38−41]

using variable proportions of *n*-hexane−ethyl acetate as developing system. As visualizing reagent, 1% vanillin in concentrated H_2SO_4 was used.

Biological Materials. The soft coral *Cladiella pachyclados* (Klunzinger, 1877) was collected from Hurghada at the Egyptian Red Sea coast by scuba at depths of −5 m in July 2007. *C. pachyclados* has a wide distribution throughout the entire Indo-Pacific region. It inhabits shallow reef areas and usually grows in small patches intermingled with other species of the family Alcyoniidae. It grows in areas exposed to strong surge but needs ample light. In the northern Red Sea it is the most abundant species of that genus and was collected from numerous reefs in the Gulf of Aqaba, Gulf of Suez, and other northern Red Sea reefs. The colonies are firm to fleshy and typically lobate. The brown polyps are retractile and possess numerous symbiotic algae (zooxanthellae). The colonies are white-gray. The sclerites are small dumbbells, and in the polyps there are figure-eights. A voucher specimen was deposited in the Red Sea Invertebrates Collection of the Department of Pharmacognosy at Suez Canal University under the registration number 2007DY68.

Extraction and Isolation. The frozen soft coral (2 kg) was extracted with a mixture of MeOH−CH_2Cl_2 (4 × 4 L) at room temperature. The combined extracts were concentrated under vacuum, and the dried extract (140.3 g) was subjected to CC on Sephadex LH20 using $CHCl_3$−MeOH gradient elution to afford five major fractions, which were subjected to repeated RP-C18 Si gel chromatography, affording the new pachycladin A (**1**, 18 mg), pachycladin B (**2**, 22 mg), pachycladin C (**3**, 29 mg), pachycladin D (**4**, 56 mg), and pachycladin E (**5**, 12 mg) along with the known sclerophytin A (**6**, 330 mg),[21,22] cladiellisin (**7**, 420 mg),[23] 3-acetylcladiellisin (**8**, 14 mg),[17] 3,6-diacetylcladiellisin (**9**, 9.7 mg),[17] polyanthelin A (**10**, 30 mg),[24,25] klysimplexin G (**11**, 6 mg),[26] klysimplexin E (**12**, 10 mg),[26] sclerophytin F methyl ether (**13**, 10 mg),[11] (6*Z*)-cladiellin (cladiella-6*Z*,11(17)-dien-3-ol) (**14**, 19 mg),[27] sclerophytin B (**15**, 10 mg),[28] and patagonicol (75 mg).[29]

Pachycladin A (1): colorless oil; $[\alpha]_D^{25}$ −16.0 (*c* 0.48, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3689, 3031, 2957, 2931, 2874, 1725, 1602, 1463, 1369, 1266, 1157 cm^{-1}; NMR data, see Table 1; HRTOFMS *m*/*z* 451.3065, $[(M + H) - H_2O]^+$ (calcd for $C_{26}H_{43}O_6$, 451.3059).

Pachycladin B (2): colorless oil; $[\alpha]_D^{25}$ −12.7 (*c* 0.23, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3688, 3600, 3446, 2958, 2933, 2874, 1729, 1602, 1464, 1370, 1262, 1177, 994 cm^{-1}; NMR data, see Table 1; HRTOFMS *m*/*z* 449.2896, $[(M + H) - H_2O]^+$ (calcd for $C_{26}H_{41}O_6$, 449.2903).

Pachycladin C (3, 6-acetylcladiellisin): colorless oil; $[\alpha]_D^{25}$ −20.1 (*c* 0.31, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3688, 3587, 3526, 3024, 2957, 2937, 2873, 1729, 1602, 1464, 1371, 1261 cm^{-1}; NMR data, see Table 1; HRTOFMS *m*/*z* 363.2535, $[M + H]^+$ (calcd for $C_{22}H_{35}O_4$, 363.2535).

Pachycladin D (4). yellowish-white, gummy residue; $[\alpha]_D^{25}$ −24.2 (*c* 0.12, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3689, 3609, 3024, 2960, 2929, 1724, 1602, 1456, 1370, 1263, 1076, 928 cm^{-1}; NMR data, see Table 2; HRTOFMS *m*/*z* 357.1830, $[M + K]^+$ (calcd for $C_{20}H_{30}O_3K$, 357.1832).

Pachycladin E (5): viscous, yellow oil; $[\alpha]_D^{25}$ +19.9 (*c* 0.22, $CHCl_3$); IR ν_{max} ($CHCl_3$) 3649, 3608, 2960, 2934, 2874, 1723, 1602, 1456, 1370, 1264, 1146, 1075 cm^{-1}; NMR data, see Table 2; HRTOFMS *m*/*z* 401.2303, $[M + Na]^+$ (calcd for $C_{22}H_{34}O_5Na$, 401.2304).

Sclerophytin A (6): $[\alpha]_D^{25}$ −7.8 (*c* 1.34, $CHCl_3$) [lit. $[\alpha]_D^{20}$ −6.9 (*c* 0.087, $CHCl_3$)].[22]

Cladiellisin (7): $[\alpha]_D^{25}$ −9.3 (*c* 0.48, $CHCl_3$) [lit. $[\alpha]_D^{23}$ −21.3 (*c* 0.51, $CHCl_3$)].[23]

Polyanthelin A (10): $[\alpha]_D^{25}$ +8.0 (*c* 0.73, $CHCl_3$) [lit. $[\alpha]_D$ +8.9 (*c* 0.22, $CHCl_3$),[24] $[\alpha]_D^{20}$ −9.9 (*c* 1.0, $CHCl_3$)].[25]

Klysimplexin G (11): $[\alpha]_D^{25}$ −2.5 (*c* 0.21, $CHCl_3$) [lit. $[\alpha]_D^{22}$ −54 (*c* 0.23, $CHCl_3$)].[26]

Biological Assays. Cell Culture. Prostate cancer cell line PC-3 was purchased from ATCC. The cell line was grown in 10% fetal bovine serum (FBS) and RPMI 1640 (GIBCO-Invitrogen) supplemented with 2 mmol/L glutamine, 100 μg/mL penicillin G, and 100 μg/mL streptomycin at 37 °C under 5% CO_2.

Proliferation Assay. The antiproliferative effects of the isolated compounds were tested in culture on malignant PC-3 epithelial cell line using the MTT kit (TACS, Trevigen, Inc.). After passing the cells 3 or 4 times, growing cells were incubated in a 96-well plate at a density of 8×10^3 cells per well and allowed to attach for 24 h. Complete growth medium was then replaced with 100 μL of RPMI serum-free medium (GIBCO-Invitrogen) containing various doses (50, 20, 10, and 5 μM) of each compound, and culture was continued at 37 °C under 5% CO_2. After 96 h, the incubated cells were treated with MTT solution (10 μL/well) at 37 °C for 4 h. The color reaction stopped by the addition of solubilization/stop solution (100 μL/well), and incubation at 37 °C continued to dissolve the formazan product completely. Absorbance of the samples was measured at 550 nm with an ELISA plate reader (BioTek). The number of cells per well was calculated against a standard curve prepared by plating various concentrations of cells, as determined by hemocytometer, at the start of each experiment. IC_{50} values for the compounds were calculated using nonlinear regression (curve fit) of log concentration versus number of cells/well implemented in GraphPad Prism 5.0.

Growth curves were determined to ensure that cells used in experiments were within the exponential growth phase. Cell proliferation was assessed by monitoring the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The reduction of MTT is catalyzed by mitochondrial dehydrogenase enzymes and is therefore a measure of cell viability.

Briefly, cells (100 μL/well) were seeded at seeding densities of 1×10^5, 1×10^4, or 1×10^3 cells/mL into 96-well microtiter plates and allowed to adhere for 24 h. Cell viability was assessed on a daily basis by adding 10 μL of filter-sterilized MTT (5 mg/mL in PBS) to each well. Following a 4 h incubation period with MTT, 100 μL of solublizing agent was added to each well, and the blue formazan crystals trapped in cells dissolved for 2−4 h at 37 °C or overnight at room temperature in a dark place. The absorbance was measured with a plate reader at 550 nm.

Wound-Healing Assay. The human metastatic prostate cancer PC-3 cells were cultured in RPMI 1640 medium containing 10 mM HEPES, 4 mM L-glutamine, 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (50 μg/mL), and grown in a 5% CO_2 atmosphere at 37 °C. Cells were plated onto sterile 24-well plates and allowed to recover for a confluent cell monolayer formed in each well (>90% confluence). Wounds were then inflicted to each cell monolayer using a sterile 200 μL pipet tip. Media were removed, cell monolayers were washed once with PBS, and then fresh media containing test compounds were added to each well. Test compounds were prepared in DMSO at different concentrations and added to the plates, each in triplicate using 4-hydroxyphenylmethylene hydantoin as positive control and DMSO as a vehicle control.[38,39] The incubation was carried out for 24 h under serum-starved conditions, after which media was removed and cells were fixed and stained using Diff Quick staining (Dade Behring Diagnostics). The number of cells migrated on the scratched wound were counted under the microscope in three or more randomly selected fields (magnification: 400×). Final results are expressed as mean ± SEM per 400× field. All treatments, including the controls, were documented photographically.

Cultrex Cell Invasion Assay. Anti-invasive activities were measured using Trevigen's Cultrex Cell Invasion Assay as previously described.[40,41] About 50 μL of basement membrane extract (BME) coat was added per well. After incubation for 4 h at 37 °C in 5% CO_2, 50 000/50 μL of PC-3 cells in serum-free RPMI medium was added per well to the top chamber, containing the tested compound at the desired concentration (10 μM for **11**, 50 μM for all other compounds). About 150 μL of RPMI medium was added to the lower chamber, containing 10% FBS and penicillin/streptomycin, and using fibronectin (1 μL/mL) and *N*-formyl-met-leu-phe (10 nM) as chemoattractants. Cells were allowed to migrate to the lower chamber at 37 °C in a CO_2 incubator. After 24 h, the top and bottom chambers were aspirated and washed with washing buffer provided with the kit. About 100 μL of Cell Dissociation Solution/Calcein-AM solution was added to the bottom chamber and incubated at 37 °C in a CO_2 incubator for 1 h. The cells internalize calcein−AM, and the intracellular esterases cleaved the AM moiety to generate free calcein. Fluorescence of the samples was determined at 485 nm excitation, 520 nm emission, using an ELISA plate reader (BioTek). The number of cells that have invaded through the BME coat was calculated using a standard curve.

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Supporting Information Available: ^1H NMR and attached proton test (APT) spectra of compounds **1**−**5** are available free of charge via the Internet at http://pubs.acs.org.

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