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CYTOTOXIC ALCYONOLIDE CONGENERS FROM AN OKINAWAN SOFT CORAL *CESPITULARIA* SP.

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Abstract – Four new alcyonolide congeners (**1-4**) were isolated from a soft coral *Cespitularia* sp. together with the known alcyonolide (**5**). Their structures were determined by spectroscopic and chemical analyses. Compounds **1-5** showed moderate to weak cytotoxicities against HCT 116 cells.

INTRODUCTION

Soft corals are rich sources of novel secondary metabolites.[1-2] The majority of compounds identified from soft corals fall into the category of diterpenoids, which are of considerable interest due to their cytotoxic and antibacterial activities.[3-6] In the course of our study on the secondary metabolites from Okinawan marine organisms, we investigated a soft coral *Cespitularia* sp. Extracts from the soft coral inhibited the cell division of fertilized sea urchin eggs. This paper describes the isolation, structure elucidation and bioactivities of four new alcyonolide congeners (**1-4**). Alcyonolide (**5**), which is the major constituent of the ethyl acetate extract of the soft coral, was first isolated in 1981 from an Okinawan soft coral *Alcyonium* sp. and its absolute stereochemistry was determined by chemical and physicochemical means.[7] The carbon framework of **5** corresponds to a seco-type variety of xenicins.[8-10]

RESULTS AND DISCUSSION

Samples (3.0 kg, wet weight) of the *Cespitularia* sp. overgrown on a coral reef were collected by hand from the coast of Zamami Island, Okinawa, and extracted with acetone. The acetone extracts were partitioned between water and ethyl acetate. The ethyl acetate extract, which showed 80% inhibition of the first cleavage of fertilized sea urchin eggs at 20×10^{-6} g/L, was partitioned between aqueous MeOH

and hexanes. The aqueous MeOH extract exhibited cytotoxicity against NBT-T2 cells (rat bladder cells, 100% inhibition of the cell division at 1.0 x 10^{-6} g/L). Purification of the aqueous MeOH extract by silica gel column chromatography followed by normal-phase HPLC gave four new compounds [(**1**, 0.00012% of wet soft coral), (**2**, 0.00032%), (**3**, 0.00019%), (**4**, 0.0002%)] and the known alcyonolide (**5**, 0.09%). Alcyonolide (**5**) was unambiguously identified by comparison of its spectral data with those described in the literature.[7]

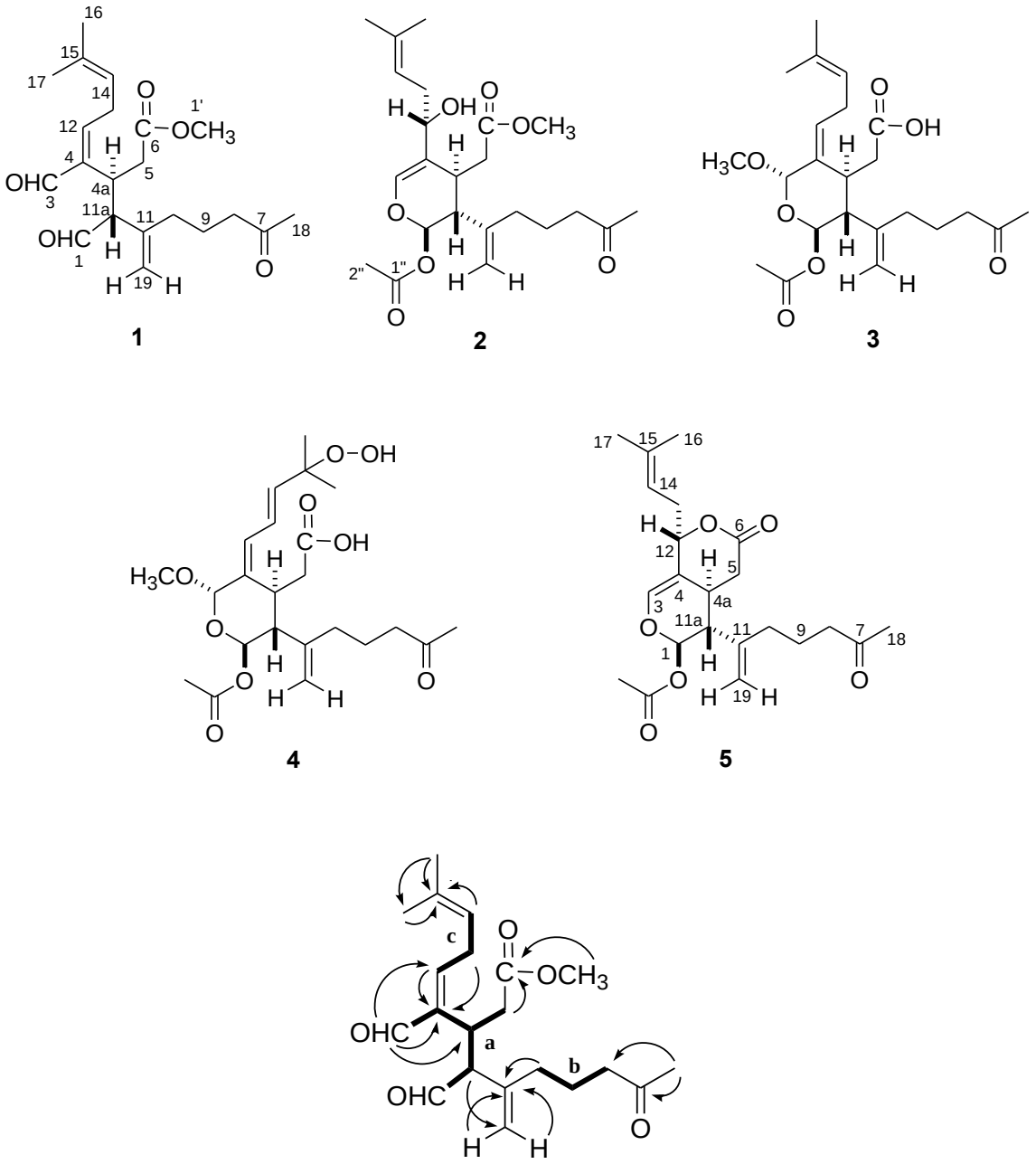

Figure 1. Partial structures (**a**, **b**, and **c**) of **1** based on COSY (bold lines) and some important HMBC correlations (arrows).

Table 1. ^{1}H NMR data ($CDCl_3$, 500 MHz) of compounds **1**-**5**

	δ_H (mult., J/Hz)									
Position	**1**		**2**		**3**		**4**		**5**	
1	9.48	(d, 2.1)	6.11	(dd, 2.7, 0.8)	6.01	(d, 2.1)	6.02	(d, 3.5)	5.94	(d, 8.0)
3	9.25	(d, 2.0)	6.35	(s)	4.87	(s)	4.91	(s)	6.38	(s)
4a	3.68	(dddd, 11.1, 10.0, 4.0, 2.0)	2.77	(m)	3.16	(m)	3.36	(m)	2.72	(m)
5	2.75 2.59	(dd, 15.8, 10.0) (dd, 15.8, 4.0)	2.96 2.81	(dd, 15.1, 3.3) (dd, 15.1, 9.7)	3.12 2.78	(dd, 15.0, 9.4) (dd, 15.0, 5.6)	3.04 2.93	(dd, 15.6, 7.5) (dd, 15.6, 7.5)	2.76 2.29	(dd, 18.6, 6.9) (dd, 18.6, 12.5)
8	2.36	(m)	2.43	(t, 7.2)	2.44	(m)	2.46	(m)	2.44	(t, 7.0)
9	1.63	(m)	1.75	(m)	1.74	(m)	1.72	(m)	1.73	(q, 7.0)
10	1.79	(m)	2.12 2.06	(m) (m)	2.11 2.01	(m) (m)	2.07 2.02	(m) (m)	2.02 1.97	(m) (m)
11a	3.80	(dd, 11.1, 2.1)	2.46	(m)	2.53	(t, 2.1)	2.58	(t, 3.5)	2.20	(t, 8.0)
12	6.46	(t, 7.3)	4.05	(t, 6.5)	5.59	(t, 7.3)	6.17	(d, 11.0)	4.75	(t, 7.5)
13	3.18 3.07	(ddd, 17.2, 7.3, 7.3) (ddd, 17.2, 7.3, 7.3)	2.40 2.15	(m) (m)	2.74 2.70	(m) (m)	6.53	(dd, 15.3, 11.0)	2.52 2.43	(m) (m)
14	5.10	(m)	5.04	(br t, 7.1)	4.98	(m)	5.86	(d, 15.3)	5.08	(t, 7.5)
16	1.73	(s)	1.64	(s)	1.67	(s)	1.34	(s)	1.61	(s)
17	1.69	(s)	1.70	(s)	1.59	(s)	1.36	(s)	1.70	(s)
18	2.11	(s)	2.14	(s)	2.13	(s)	2.15	(s)	2.12	(s)
19a	5.07	(s)	4.94	(s)	4.99	(s)	5.02	(s)	4.91	(s)
19b	4.89	(s)	4.98	(s)	4.86	(s)	4.93	(s)	5.01	(s)
1'	3.60	(s)	3.68	(s)						
2"			2.10	(s)	2.10	(s)	2.11	(s)	2.08	(s)
$OC\underline{H}_3$					3.39	(s)	3.41	(s)		

Table 2. ^{13}C NMR data ($CDCl_3$, 125 MHz) of compounds **1**-**5**

	δ_C				
C no.	**1**	**2**	**3**	**4**	**5**
1	198.7	92.1	93.4	92.8	92.9
3	195.6	138.8	103.5	103.3	137.7
4	140.5	116.0	130.7	132.2	110.4
4a	32.9	30.2	32.4	32.6	31.1
5	35.8	38.5	39.9	39.9	34.9
6	172.3	173.5	175.6	176.3	169.9
7	208.1	208.8	209.3	209.6	208.1
8	42.7	43.1	43.0	42.8	42.9
9	21.0	21.8	21.9	21.3	21.5
10	34.4	35.1	35.1	34.2	34.9
11	141.9	146.2	146.9	146.1	145.1
11a	60.4	43.6	46.0	47.2	48.8
12	158.5	73.0	132.5	130.7	79.5
13	28.5	34.0	26.0	123.6	35.1
14	119.0	119.9	121.2	141.0	117.9
15	134.8	135.3	133.2	82.0	136.0
16	25.7	18.3	25.8	21.3	18.2
17	18.0	26.0	17.9	24.7	25.9
18	29.9	30.1	30.2	30.1	30.1
19	116.6	112.3	112.8	113.2	113.7
1'	51.5	51.8			
1"		169.6	170.1	170.0	169.3
2"		21.3	21.5	21.7	20.9
$OC\underline{H}_3$			55.2	55.2	

The molecular formula of **1** was determined to be $C_{21}H_{30}O_5$ by HRESIMS [*m/z* 361.2019 $(M-H)^+$, calcd for $C_{21}H_{29}O_5$, 361.2015], which accounted for seven degrees of unsaturation. The IR absorption bands at 1713 and 1674 cm^{-1} indicated the presence of several carbonyl groups. The ^1H and ^{13}C NMR data of **1** are summarized in Tables **1** and **2**. The ^{1} H and ^{13}C NMR data of compound **1** indicated the presence of a ketone (δ_C 208.1), two aldehydes (δ_C 198.7, δ_H 9.48; δ_C 195.6, δ_H 9.25), an ester (δ_C 172.3), two trisubstituted double bonds (δ_C 158.5, δ_H 6.46; δ_C 140.5 and δ_C 134.8; δ_C 119.0, δ_H 5.10), a disubstituted double bond including a terminal methylene (δ_C 141.9; δ_C 116.6, δ_H 5.07, δ_H 4.89), two methines (δ_C 60.4, δ_H 3.80; δ_C 32.9, δ_H 3.68), five methylenes (δ_C 42.7, δ_H 2.36; δ_C 35.8, δ_H 2.75, δ_H 2.59; δ_C 34.4, δ_H 1.79; δ_C 28.5, δ_H 3.18, δ_H 3.07; δ_C 21.0, δ_H 1.63) and four methyls (δ_C 51.5, δ_H 3.60; δ_C 29.9, δ_H 2.11; δ_C 25.7, δ_H 1.73; δ_C 18.0, δ_H 1.69). For the four methyls, one was associated with the ketonic carbonyl based on HMBC correlations ($H_3$18/C7 and $H_3$18/C8) and the NMR chemical shifts, another was assigned to the methyl ester based on an HMBC correlation ($H_3$1'/C6) and the NMR chemical shifts, and the remaining two were part of an isobutenyl group based on HMBC correlations ($H_3$16/C14, $H_3$16/C15, $H_3$17/C14, $H_3$17/C15, $H_3$16/C17 and $H_3$17/C16) and the NMR chemical shifts. The NMR data of **1** showed similarities to those of **5**. The main differences were the presence of two aldehyde groups and the lack of an acetal which exists in **5**. The major spin systems (**a**, **b**, and **c**) were found to be as shown in Figure 1 based on ^1H-^1H COSY and HMBC data. The partial structures (**a**, **b**, and **c**) and other fragments (C6, C7-C18, C11-C19, and C16-C15-C17) were connected using HMBC correlations, giving the planar structure of **1** (Figure 1). The NOE observed between H3/H12 revealed an *E* configuration for C4, 12 double bond. The configurations of C11a and C4a are yet to be determined. Dialdehyde **1** is a precursor-like compound of alcyonolide (**5**) whose carbon framework corresponds to a seco-type variety of xenicins,[10] and assuming a biosynthetic relationship between **1** and **5**, its absolute stereochemistry was tentatively deduced to be as depicted in **1**.

The ^{13}C NMR (Table 2) and HRESIMS [*m/z* 445.2193 $(M+Na)^+$, calcd for $C_{23}H_{34}O_7Na$, 445.2202] data revealed that **2** had a formula of $C_{23}H_{34}O_7$. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) resembled those of alcyonolide (**5**). The major difference was the appearance of a methoxy group [δ_H 3.68 (3H, s), δ_C 51.8 (q)] in **2**, but no methoxy group in **5**. The methoxy group was part of the methyl ester based on an HMBC correlation between $H_3$1' and C6. The major spin systems (C1-C11a-C4a-C5, C8-C9-C10 and C12-C13-C14) were revealed based on ^1H-^1H COSY data. These partial structures and other fragments (C3-C4, C6, C1', C16-C15-C17, C7-C18, C11-C19 and C1"-C2") were connected using HMBC correlations of H1/C11, H1/C1", $H11_a$/C11, H3/C1, H3/C4, H3/C12, H5/C4, H5/C6, $H_2$9/C11, $H_3$16/C14, $H_3$16/C17, $H_3$17/C14, $H_3$17/C16, $H_3$18/C7, $H_3$18/C8, $H_2$19/C10, $H_2$19/C11, $H_3$2"/C1", and $H_3$1'/C6, giving the planar structure of **2**. The relative stereochemistry of **2** was established based on the NOE data.

The NOE correlations between $H4_a$ /H1 and $H4_a$/$H19_a$ confirmed that these protons were on the same face of the molecule. Methanolysis of alcyonolide (**5**) (55 °C, overnight) afforded **2**. The ^{1}H NMR data and HPLC retention time (t_R) of the methanolysate of **5** were identical to those of **2** and the $[\alpha]_D^{27}$ +78 (*c* 0. 18 $CHCl_3$) of the methanolysate was comparable to that of **2**. Thus, the absolute structure of compound **2** was established.

Analysis of compound **3** by HRESIMS [*m/z* 445.2202 $(M+Na)^+$, calcd for $C_{23}H_{34}O_7Na$, 445.2202] gave a $C_{23}H_{34}O_7$ molecular formula. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 2) also resembled those of alcyonolide (**5**). The IR (ν_{max} 1732, 1715 cm^{-1}) and ^{13}C NMR data (δ_C 209.3, 175.6, 170.1) indicated the presence of a ketone, a carboxyl (or an ester), and an ester (or a carboxyl). The main differences were the presence of a methoxy group [δ_H 3.39 (3H, s), δ_C 55.2 (q)] and an acetal [δ_H 4.87 (1H, s), δ_C 103.5 (d)], and as well as the lack of the oxygenated methine in **3**. The methoxy group was part of the acetal based on an HMBC correlation between O$C\underline{H}_3$ and C3. The major spin systems (C1-C11a-C4a-5, C8-C9-C10, and C12-C13-C14) were revealed based on ^{1}H-^{1}H COSY data. These partial structures and other fragments (C3-C4, C6, C7-C18, C16-C15-C17, C11-C19, and C1”-C2”) were connected using HMBC correlations of H1/C1”, H3/C1, H3/C4, H3/C12, H5/C4, H5/C6, $H_2$10/C11, $H_3$16/C14, $H_3$16/C15, $H_3$16/C17, $H_3$17/C14, $H_3$17/C15, $H_3$18/C7, $H_3$18/C8, $H19_a$/C11a, $H19_b$/C11, $H19_b$/C10, $H_3$2”/C1”, establishing the entire carbon framework and the structure of the acetal moieties. Furthermore, a C6 carbonyl carbon was determined to be a carboxyl carbon on the basis of a characteristic chemical shift (δ_C 175.6) of C6, the IR spectrum, and the molecular formula. The relative stereochemistry of **3** was determined by the NOESY spectrum. The NOE correlations between H1/H4a, H1/$H19_a$/ and H1/O$C\underline{H}_3$ confirmed that these protons were on the same face of the molecule. The NOE correlations between H12 /H3 and H12/O$C\underline{H}_3$ suggested an *E* configuration for the C4, 12 double bond of **3**. Treatment of alcyonolide (**5**) with methanol (55 °C, overnight) afforded **3** in low yield. The ^{1}H NMR data and the HPLC retention time of the methanolysate of **5** were identical to those of **3**, which confirmed the structure of **3**.

The molecular formula of **4** was determined to be $C_{23}H_{34}O_9$ by HRESIMS [*m/z* 477.2095 $(M+Na)^+$, calcd for $C_{23}H_{34}O_9Na$, 477.2101]. The IR (ν_{max} 1732, 1714 cm^{-1}) and ^{13}C NMR data (δ_C 209.6, 176.3, 170.0) indicated the presence of a ketone, a carboxyl (or an ester), and an ester (or a carboxyl). The ^{13}C NMR chemical shifts were almost identical to those of **3** except for those of C13-C17 (Table 2). In contrast to **3**, compound **4** contained an oxygenated quartenary carbon and two sp^2 methine carbons instead of a methylene, a sp^2 quarternary and a sp^2 methine carbons. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) and the molecular formula revealed the presence of a hydroperoxy group and a *trans* disubstituted double bond [C13-C14; δ_C 123.6 (d), δ_H 6.53 (dd, *J* = 15.3, 11.0 Hz); 141.0 (d), δ_H 5.86 (d, *J* = 15.3 Hz)] in

compound **4**. A positive iodine-starch test further supported the presence of the hydroperoxy group in **4**.[11] The hydroperoxy group was placed at C15 [δ_C 82.0 (s)] based on HMBC correlations from H16 to C14, C15 and C17. Extensive analysis of the 1D and 2D NMR data led to the structure of **4**. Compound **4** must be formed by the ene reaction between **3** and the singlet oxygen.

Compounds **1**-**5** showed activity against HTC116 cells (human colorectal cancer cells) with IC_{50} values of 26.1 x 10^{-6}, 157 x 10^{-6}, 12.9 x 10^{-6}, 209 x 10^{-6} and 4.09 x 10^{-6} g/L, respectively. Alcyonolide (**5**) also exhibited cytotoxicity against NBT-T2 cells (100% inhibition at 1.0 x 10^{-7} g/L).

CONCLUSION

Four new diterpenes **1**-**4** and the known alcyonolide (**5**) were isolated from the soft coral *Cespitularia* sp. Alcyonolide was the major constituent of the ethyl acetate extract of the soft coral. The carbon frameworks of compounds **1**-**5** correspond to a seco-type variety of xenicins and dialdehyde **1** is a precursor-like compound of alcyonolide. Compounds **1**-**5** showed moderate to weak cytotoxicities against HCT 116 cells depending on the dose. We could not exclude possibility that methoxy groups in compounds **1**, **2**, **3**, and **4** could be derived from methanol used in isolation process. Further chemical and biological studies on alcyonolide and its derivatives are in progress in our laboratory.

EXPERIMENTAL

GENERAL ASPECTS

Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra of the methanol solutions were measured on a JASCO V-550 spectrophotometer. IR spectra were recorded on a JASCO FT/IR-300 spectrometer. The ^1H, ^{13}C, and 2D NMR spectra were recorded on a Bruker Avance III 500 spectrometer, and ^1H and ^{13}C chemical shifts were referenced to the solvent peaks (δ_H 7.26 and δ_C 77.24 in $CDCl_3$). Mass spectra were measured on an LTQ Orbitrap hybrid mass spectrometer. Open column chromatography was performed on Kieselgel 60 (70-230 mesh, Merck). HPLC was performed using a COSMOSIL-packed ODS HPLC column (C18, 10 x 250 mm) or COSMOSIL Si60 HPLC column (5SL, 10 x 250 mm). Analytical TLC was performed using Kieselgel 60 F_{254} DC-fertigplatten (Merck). All solvents used were reagent grade.

Animal Material. The soft coral was collected at low tide from the coast of Zamami Island, Okinawa, Japan in March, 2011, and identified as *Cespitularia* sp. A voucher specimen was deposited at the University of the Ryukyus (Specimen no. 110312).

ISOLATION

Samples (3.0 kg, wet weight) of *Cespitularia* sp. overgrown on a coral reef were collected by hand from the coast of Zamami Island, Okinawa, and extracted with acetone (5 L) twice. After filtration, the extracts were concentrated *in vacuo* to give an acetone extract. The acetone extract was partitioned between H_2O (200 mL) and EtOAc (200 mL x 2). The EtOAc extract (42.9 g) completely inhibited the first cell division of fertilized sea urchin eggs at 20 x 10^{-6} g/L. The active EtOAc extract was suspended in MeOH-H_2O (9:1, 300 mL) and then extracted with hexanes (300 mL) to remove non-polar fatty materials. The aqueous MeOH phase was concentrated in vacuo to give the aqueous MeOH extract (31.6 g), which exhibited cytotoxicity against NBT-T2 cells (rat bladder cells, 100% inhibition of the cell division at 1.0 x 10^{-6} g/L). The cytotoxic extract was first chromatographed on silica gel MeOH to give 15 fractions. The seventh fraction was determined to be the known alcyonolide (2.68 g). The ninth fraction (1.67 g) was subjected to further separation by CC on silica gel using the gradient solvent mixture of hexanes-EtOAc-MeOH to afford ten fractions. The first fraction (7.7 mg) was purified by HPLC on silica gel using hexanes-EtOAc (5:2) to give four fractions and fraction 3 was compound **1** (3.8 mg). The third fraction (472 mg) from the first column was further chromatographed on silica gel using hexanes with increasing proportions of EtOAc to yield five fractions. The second fraction (295 mg) was separated by CC on silica gel using hexanes with increasing proportions of EtOAc to give three fractions. Fraction 2 (199 mg) was subjected to further separation by CC on silica gel using the gradient solvent mixture of hexanes-EtOAc to furnish two fractions. Fraction 1 (99 mg) was fractionated by HPLC on silica gel using hexanes-EtOAc (5:6) to yield compound **3** (5.8 mg), compound **5** (46.4 mg), compound **2** (9.6 mg), and compound **4** (6.0 mg).

Compound 1: Colorless oil; $[\alpha]_D^{25}$ +25 (*c* 0. 12 $CHCl_3$); FT IR ν_{max} (film) 3407, 2929, 1713, 1674, 1635, 1437 and 1167 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Tables 1 and 2; HRESIMS *m/z* 361.2019 $(M-H)^+$ (calcd for $C_{21}H_{29}O_5$, 361.2015).

Compound 2: Colorless oil; $[\alpha]_D^{29}$ +76 (*c* 0. 10 $CHCl_3$); FT IR ν_{max} (film) 3387, 2980, 2934, 1713, 1582, 1408 1232 and 1191 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Tables 1 and 2; HRESIMS *m/z* 445.2193 $(M+Na)^+$ (calcd for $C_{23}H_{34}O_7Na$, 445.2202).

Compound 3: Colorless oil; $[\alpha]_D^{22}$ +63 (*c* 0. 39 $CHCl_3$); FT IR ν_{max} (film) 3501, 2931, 1732, 1715, 1669, 1435, 1227 and 1151 cm^{-1}; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Tables 1 and 2; HRESIMS *m/z* 445.2202 $(M+Na)^+$ (calcd for $C_{23}H_{34}O_7Na$, 445.2202).

Compound 4: Colorless oil; $[\alpha]_D^{27}$ –22 (*c* 0. 12 $CHCl_3$); FT/IR (film) ν_{max} 3387, 2981, 2937, 2835, 1732, 1714, 1647, 1418, 1363, 1231cm^{-1} ; UV λ_{max} 257 ($\log\varepsilon$ 3.9) nm; ^1H and ^{13}C NMR ($CDCl_3$) data are listed in Tables 1 and 2; HRESIMS *m/z* 477.2095 $(M+Na)^+$ (calcd for $C_{23}H_{34}O_9Na$, 477.2101).

Methanolysis of 5. Alcyonolide (**5**) (20.0 mg) was dissolved in MeOH (1.0 mL) and the solution was heated at 55 °C for 12 h. The reaction mixture was concentrated and the crude product was purified by HPLC [COSMOSIL 5SL, hexanes/EtOAc (5:6)] to give compound **3** (t_R = 12 min, 0.5 mg, 2.5%) and compound **2** (t_R = 16 min, 2.4 mg, 12%): $[\alpha]_D^{29}$ +78 (*c* 0. 18 $CHCl_3$).

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