Cyclomarins A−C, New Antiinflammatory Cyclic Peptides Produced by a Marine Bacterium (*Streptomyces* sp.)

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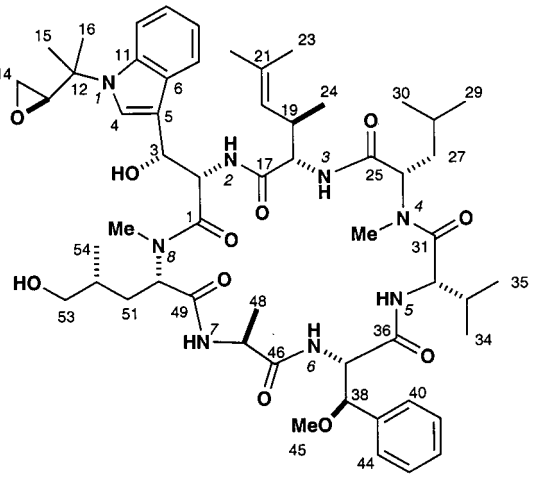
Abstract: Three new cyclic heptapeptides, cyclomarins A−C (**1**−**3**), were isolated from extracts of a cultured marine bacterium collected in the vicinity of San Diego, CA. The major metabolite, cyclomarin A (**1**), contains three common and four unusual (or unique) amino acids. The structures of the new metabolites were determined using 1D and 2D NMR methods, and the stereochemistry was determined from an X-ray crystal structure of the diacetate derivative of **1**. Cyclomarin A (**1**) displays significant antiinflammatory activity in both in vivo and in vitro assays.

Microorganisms collected from marine environments, although poorly known biologically, are emerging as a significant new chemical resource for drug discovery.[1] Over the past several years numerous molecules with interesting bioactivities have been described from this new source.[2] In our research program, we have focused considerable attention on the marine actinomycetes, which although minor members of marine communities, have been shown to produce novel metabolites with antibiotic,[3] antitumor,[4] and antiinflammatory properties.[5] In connection with our continuing interest in the discovery of novel bioactive secondary metabolites, we investigated an interesting estuarine streptomycete, strain CNB-982, isolated from a sediment sample collected in Mission Bay, CA. We initially became interested in this organism when its crude extract displayed modest cytotoxicity activity against HCT-116, a human colon cancer cell line. We discovered that under saline culture conditions, this organism produces a family of novel cyclic heptapeptides, cyclomarins A (**1**), B (**2**), and C (**3**). While



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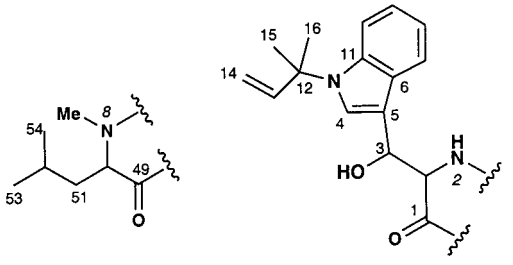

2 **3**

the major metabolite, cyclomarin A, is cytotoxic in vitro toward cancer cells (the mean IC_{50} against a panel of human cancer cell lines is 2.6 μM), cyclomarin A is more interesting for its significant in vitro and in vivo antiinflammatory properties,

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which were revealed during subsequent biotesting. The cyclomarins are defined herein as novel cyclic heptapeptides containing four unusual amino acids.

The cyclomarins were isolated from the culture by direct extraction of the whole broth with ethyl acetate. While the producing organism is not an obligate halophile and can be grown in a culture medium prepared from distilled, as opposed to salt, water, cyclomarins A−C are produced only when salt is added to the culture medium. The condensed solvent residue was fractionated by Sephadex LH-20 column chromatography (3:1:1 hexane/toluene/methanol), and fractions containing **1**−**3** were further purified by reversed-phase C-18 HPLC (85:15 methanol/water) to yield cyclomarin A (**1**) (fine white crystals, mp 164−7 °C) and two minor metabolites, cyclomarins B (**2**) and C (**3**). The molecular formula of **1** was determined to be $C_{56}H_{82}O_{11}N_8$ by high-resolution FAB mass spectrometry [[M − H_2O + H] *m/z* 1025.6062, calcd 1025.6057, required for $C_{56}H_{80}O_{10}N_8$] coupled with ^{1}H and ^{13}C NMR data. UV absorption bands at 293, 287, and 222 nm showed the presence of indole and benzenoid chromophores, while the infrared spectrum showed amide carbonyl bands (1644 cm^{-1}), aromatic absorptions (1512, 1453, cm^{-1}), and NH and OH (3300−3500 cm^{-1}) functionalities.

The peptide nature of cyclomarin A was readily inferred from its ^{1}H NMR and ^{13}C NMR spectral features. The ^{1}H NMR spectrum of **1** in $CDCl_3$ (Table 1) contained 82 proton resonances, seven of which were assigned as either OH or NH due to a lack of correlation with carbon atoms in the HMQC experiment. Seven proton resonances between δ 4 and 5 were indicative of amino acid α-protons. These data, combined with the observation of seven carbonyl signals between δ 168.5 and 172.5 in the ^{13}C NMR spectrum, suggested that **1** was a heptapeptide. Additional carbon NMR data showed the presence of 14 methyl groups, 4 methylenes, 25 methine carbons, and 6 quaternary carbons, all of which were consistent with the spectral features generally observed for cyclic peptides.

Extensive NMR analysis showed the identity of seven amino acids, four of which were either uncommon or unique. The three common amino acids, alanine, valine, and *N*-methylleucine, were readily defined by double quantum-filtered COSY and TOCSY experiments. *N*-Methylleucine and *N*-methylhydroxyleucine, the latter an uncommon amino acid, had several overlapping signals in the ^{1}H NMR spectrum, thus heteronuclear multiple bond correlation NMR experiments (HMBC) were required to distinguish between the two. The unusual amino acid β-methoxyphenylalanine was also identified and its assignment confirmed using COSY and HMBC NMR experiments.

Cyclomarin A was also shown to contain the novel amino acid 2-amino-3,5-dimethylhex-4-enoic acid, which was also identified using 2D heteronuclear NMR methods. The presence of three methyl groups made identification of this amino acid fairly straightforward. The two olefinic methyl groups (C-22 and C-23) showed HMBC correlations to one another and to both olefinic carbons (C-21 and C-20), while the third methyl group (C-24) showed correlations only to C-20 and the α- and β-carbons, C-18 and C-19, respectively. Further, the C-18 α-proton correlated to C-20, while the C-19 β-proton showed correlations to both C-20 and C-21. These HMBC correlations, along with comprehensive NOE, COSY, and TOCSY data, allowed unambiguous assignment of this novel amino acid.

The remaining amino acid, *N*-(1,1-dimethyl-2,3-epoxypropyl)-β-hydroxytryptophan, was also assigned using 2D heteronuclear NMR experiments. While three isolated spin systems defining an indole ring, an epoxyisoprene unit, and the α- and β-carbons of the amino acid, were readily apparent from COSY and TOCSY NMR data, connection of these fragments required several key HMBC correlations involving the indolic proton at C-4. This proton exhibited correlations to C-3, C-5, and C-6, allowing the amino acid carbons to be established. The same proton also showed correlations to C-11 and C-12, thus allowing the epoxyisoprenyl functionality to be established at the indole nitrogen.

Of the seven amino acids, 2-amino-3,5-dimethylhex-4-enoic acid and *N*-(1,1-dimethyl-2,3-epoxypropyl)-β-hydroxytryptophan have not been previously described, although similar *N*-prenyltryptophan amino acids have been observed in the ilamycins.[6] The amino acid β-methoxyphenylalanine is a well-known synthetic building block, but it is a rare constituent of natural products, having been found only in the discokiolides, cyclic depsipeptides from the marine sponge *Discodermia kiiensis*.[7] A related amino acid, β-methoxytyrosine, was observed in callipeltin A, a cyclic depsipeptide from a marine sponge of the genus *Callipelta*.[8]

Sequencing of the amino acid residues in **1** was accomplished using 2D NMR heterocorrelation methods. Two-bond HMBC correlations were evident from the amide protons to the carbonyls of the adjacent amino acids. For the *N*-methyl groups, three-bond correlations to their own α-carbons and to the carbonyls of the adjacent amino acids were evident. In addition, both two- and three-bond correlations from the α-protons to carbonyls were apparent for most (but not all) of the amino acids. ROESY NMR data, which demonstrated numerous peptide proton proximities, confirmed the assigned sequence.

Cyclomarin B (**2**), a minor component of the mixture (2% total cyclomarin mixture), analyzed for the molecular formula $C_{56}H_{82}O_{10}N_8$ by HRFABMS. The structure of **2** was assigned on the basis of its molecular formula as well as analysis of ^{1}H, ^{13}C, and COSY NMR data. The only significant difference in the ^{1}H NMR spectrum of **2** in comparison with **1** was H-53, which was shifted upfield to δ 0.74 (from δ 3.12) and integrated for three protons instead of two. The ^{13}C NMR shift for C-53 was also shifted upfield (δ 23.1 vs δ 66.3). In the proton COSY spectrum, the methine proton H-52 showed coupling only with the two methyl doublets at δ 0.74. These results indicated that C-53 was not hydroxylated, thus a second unit of *N*-methylleucine was present rather than *N*-methylhydroxyleucine.

Cyclomarin C (**3**), also a minor constituent of the mixture (3%), analyzed for $C_{56}H_{82}O_{10}N_8$ by HRFABMS. The ^{1}H NMR shifts for **3** were consistent with those of **1** except for protons H-13, H-14a, and H-14b. In **3**, the signals for these protons were characteristic for a terminal vinyl group. The downfield shift observed for C-13 (δ 143.6 vs δ 57.6) and C-14 (δ 111.2 vs δ 45.3) in the ^{13}C NMR spectrum also reflected the presence of a terminal alkene rather than an epoxide.

While the absolute configurations of the common amino acids comprising cyclomarin A (alanine, valine, and methylleucine) were determined by acid hydrolysis followed by chiral TLC analysis using standards, it was necessary to undertake a single-crystal X-ray diffraction study to determine the configurations of the remaining stereocenters in **1**. Cyclomarin A itself provided

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Table 1. ^{1}H and ^{13}C NMR Data for Cyclomarins A−C (**1**−**3**, $CDCl_3$)

	cyclomarin A (**1**)		cyclomarin B (**2**)		cyclomarin C (**3**)	
no.	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	170.8		170.6		170.5	
2	53.3	4.58 (t, 3)	52.5	4.55 (t, 4)	52.7	4.56 (t, 4)
3	68.7	5.31 (d, 2.5)	68.3	5.29 (d, 4.4)	68.4	5.30 (d, 4.4)
4	123.4	7.34 (s)	123.0	7.24 (s)	123.0	7.30 (s)
5	112.1		111.9		111.2	
6	127.0		127.0		126.7	
7	119.3	7.58 (d, 8)	119.3	7.62 (d, 8)	118.6	7.49 (d, 8)
8	119.8	7.10 (ddd, 1, 7, 7)	119.7	7.13 (dd, 7, 7)	119.4	7.04 (dd, 7, 7)
9	122.1	7.17 (dd, 8, 8)	121.0	7.18 (dd, 8, 8)	121.4	7.18 (dd, 8, 8)
10	111.8	7.74 (d, 8.5)	113.4	7.70 (d, 8)	114.2	7.52 (d, 8)
11	135.9		135.9		135.7	
12	58.1		57.8		57.6	
13	57.7	3.22 (dd, 3, 4)	57.6	3.20 (dd, 3, 4)	143.6	6.06 (dd, 10, 17)
14a	45.4	2.90 (t, 4)	45.3	2.89 (dd, 3, 4)	111.2	5.22 (d, 10)
14b		2.76 (dd, 2.5, 4.5)		2.75 (m)		5.17 (d, 17)
15	23.1	1.57 (s)	24.2	1.56 (s)	24.9	1.56 (s)
16	24.4	1.66 (s)	24.2	1.63 (s)	24.9	1.70 (s)
17	172.4		172.5		172.5	
18	58.1	4.08 (t, 10)	57.9	4.10 (t, 5)	57.9	4.08 (t, 5)
19	35.5	1.66 (m)	35.4	1.65 (m)	35.4	1.63 (m)
20	124.7	4.77 (d, 10)	124.7	4.77 (d, 10)	124.7	4.78 (d, 10)
21	134.4		134.4		134.4	
22	25.7	1.26 (s)	25.6	1.26 (s)	27.7	1.24 (s)
23	20.8	1.73 (s)	20.7	1.73 (s)	19.9	1.72 (s)
24	18.5	0.64 (d, 7.5)	18.7	0.64 (d, 6.8)	18.4	0.64 (d, 6.8)
25	168.6		167.9		168.3	
26	58.7	4.83 (t, 3.5)	58.5	4.78 (t, 10)	58.4	4.83 (t, 10)
27a	38.9	2.25 (4.5, 10.5, 13.5)	38.8	2.23 (m)	38.8	2.23 (m)
27b		1.05 (m)		1.08 (m)		1.06 (m)
28	25.0	1.40 (m)	24.9	1.30 (m)	25.6	1.42 (m)
29	23.4	0.82 (d, 6)	23.1	0.78 (d, 6.8)	23.3	0.82 (d, 6.8)
30	23.5	0.87 (d, 6)	23.4	0.85 (d, 6.3)	23.4	0.87 (d, 6.3)
31	170.8		171.4		171.4	
32	55.3	4.36 (t, 8.5)	55.2	4.35 (t, 8.5)	55.2	4.36 (t, 8.5)
33	30.7	2.20 (m)	30.7	2.20 (m)	30.7	2.20 (m)
34	19.3	1.06 (d, 6.5)	19.2	1.05 (d, 6.8)	18.6	1.05 (d, 6.8)
35	19.9	0.94 (d, 6.5)	19.9	0.92 (d, 6.8)	19.2	0.94 (d, 6.5)
36	169.7		169.5		169.5	
37	55.9	4.90 (t, 5)	55.8	4.89 (t, 5)	55.8	4.89 (t, 5)
38	80.1	5.08 (d, 5.5)	79.6	5.05 (d, 5)	79.8	5.07 (d, 5)
39	135.1		134.1		135.0	
40−44	127−128	7.24−7.26 (m)	127−128	7.24−7.26 (m)	127−128	7.24−7.26 (m)
45	57.7	3.37 (s)	57.6	3.37 (s)	57.6	3.36 (s)
46	171.5		171.8		171.5	
47	50.6	4.88 (m)	50.4	4.83 (m)	50.5	4.88 (m)
48	21.2	1.31 (d, 7.5)	22.1	1.29 (d, 7.3)	20.7	1.30 (d, 7.3)
49	168.8		168.2		168.7	
50	59.2	4.81 (t, 8)	58.8	4.77 (m)	59.2	4.78 (m)
51a	33.1	2.33 (7.5, 11, 14.5)	37.9	2.05 (m)	33.0	2.28 (m)
51b		0.72 (4, 6.5, 14.5)		0.83 (m)		0.67 (m)
52	32.8	1.43 (m)	24.6	1.50 (m)	33.2	1.42 (m)
53a	66.3	3.18 (m)	23.1	0.74 (d, 6.8)	66.2	3.18 (dd, 5, 11)
53b		3.26 (m)		−		3.23 (dd, 4, 11)
54	17.6	0.76 (d, 7)	18.4	0.74 (d, 6.8)	17.7	0.75 (d, 6.8)
NH-2		6.72 (d, 4)		6.88 (d, 3)		6.80 (d, 3)
NH-3		8.05 (d, 10)		8.03 (d, 10)		8.05 (d, 10)
NH-5		7.95 (d, 8)		7.94 (d, 8)		7.93 (d, 8)
NH-6		7.13 (d, 4.5)		7.12 (d, 5)		7.12 (d, 5)
NH-7		8.16 (d, 10.5)		7.97 (d, 10)		8.17 (d, 10)
NMe-4	29.6	2.83 (s)	29.4	2.82 (s)	29.4	2.82 (s)
NMe-8	29.3	2.73 (s)	29.3	2.73 (s)	29.2	2.71 (s)

crystals which were too fine for X-ray diffraction analysis, thus several derivatives were prepared, including bromobenzoyl, phenylbenzoyl, and tosyl derivatives. Somewhat surprisingly, the diacetate derivative ultimately provided the best crystal. The X-ray derived structure for cyclomarin A diacetate is shown in Figure 1.[9]

The X-ray analysis defines the relative stereochemistry at all centers, and the absolute configuration shown was selected to agree with the known amino acid centers. *Cis*-peptide bonds and transannular hydrogen bonds set the conformation of the macrocyclic ring (Figure 1). The C1−N8 peptide bond forms the end of a sharp turn at one end of the molecule. The reverse turn is one not usually seen since the peptide bond at C1−N8 is *cis*. The turn results in the side chains of two unusual amino

(9) A rectangular (0.50 × 0.52 × 0.15 mm^3) crystal was used for data collection on a Bruker 1K CCD detector mounted on a 3 kW Mo sealed tube generator. Frames (1321 frames, 0.3° ω, 30 s/f) were integrated to yield a total of 11208 reflections ($2\theta_{max}$ = 41.66°) of which 6249 were symmetry independent (R_{int} = 6.25%) and 4612 were greater than $4\sigma(F)$. The structure was phased by direct methods and refined by full-matrix least−squares on F^2 using anisotropic displacement parameters for all nonhydrogen atoms. Refinement converged at R_1 = 7.9% and GOF = 1.096 for 793 variable parameters. Archival material has been deposited with the Cambridge Crystallographic Data File and in the Supporting Information.

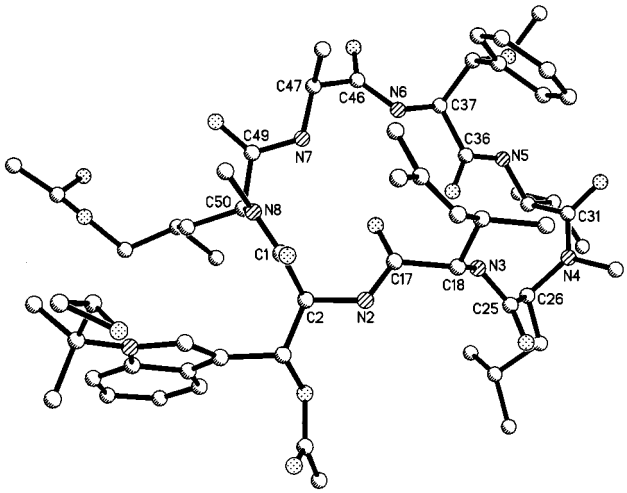

Figure 1. Perspective drawing of the X-ray crystal structure of the diacetate of cyclomarin A (**1**). Hydrogens have been omitted for clarity and selected atoms are labeled. Nitrogens are indicated with diagonal lines and oxygens are indicated by dots.

acids, 2-amino-3,5-dimethylhex-4-enoic acid and *N*-(1,1-dimethyl-2,3-epoxypropyl)-β-hydroxytryptophan, being projected in the same direction. The turn is set up by a hydrogen bond (3.00 Å) between the C17 carbonyl and N7−H. An additional hydrogen bond, from the C36 carbonyl to N3−H (2.84 Å), coupled with another *cis*-peptide bond at C31−N4 sets the conformation of the other end of the ring.

Cyclomarin A is one of the most potent antiinflammatory agents we have encountered. The compound possesses significant topical antiinflammatory activity in the phorbol ester (PMA)-induced mouse ear edema assay, showing 92% inhibition of edema at the standard testing dose of 50 μg/ear.[10] At the same screening dose, the antiinflammatory drug indomethacin shows 72% inhibition. More importantly, in the same assay cyclomarin A also shows promising in vivo activity (45% reduction in edema at 30 mg/kg ip administration), indicating the compound may be a potential drug candidate.

Several of the amino acids which compose cyclomarin A appear to be products of unusual biosynthetic pathways. Experiments designed to elucidate the biosynthesis of cyclomarin A are underway.

Experimental Section

General. Proton NMR spectra were recorded at 500 or 300 MHz, while ^{13}C NMR spectra were recorded at 100 MHz. All spectra were recorded in chloroform-*d*, and chemical shifts were referenced to either the corresponding solvent signal or tetramethylsilane: 0.0 ppm/77.0 ppm. The numbers of attached protons on carbon atoms were determined through DEPT experiments, and all carbon assignments made were consistent with the DEPT results. 2D HMBC and HMQC experiments were optimized for $^{n}J_{CH} = 8.0$ Hz and $^{1}J_{CH} = 150.0$ Hz, respectively. HPLC separations were accomplished using a Rainin DYNAMAX 60 Å C-18 column (250 × 10 mm) at a flow rate of 2.5 mL/min with refractive index detection.

Cultivation, Extraction, and Isolation. In an exemplary fermentation, *Streptomyces* sp., isolate CNB-382, was cultured in 11 1-L flasks at room temperature for 7−8 days. The fermentation broth was composed of 3:1 seawater/deionized water with 1% starch, 0.4% yeast extract, and 2% peptone. On the eighth day, the fermentation broths, including cells, were harvested by extraction with ethyl acetate. The combined extracts were concentrated under reduced pressure to afford a light brown residue (650 mg). This residue was purified by LH-20 chromatography and C-18-HPLC to provide cyclomarins A (57 mg), B (1 mg), and C (1 mg).

Cyclomarin A (1): white crystalline solid; $[\alpha]^{20}_{D} = -51.7°$ (c = 0.48, $CHCl_3$); UV (MeOH) λ_{max} 222 (22 900), 287 (1000), and 293 (11 200); IR υ (neat) 3400−3300, 3030, 2962, 2928, 2871, 1644, 1512, 1453, and 748 cm^{-1}; HRFABMS obsd $[M]^+$ m/z 1025.6062, calcd 1025.6057 for $C_{56}H_{80}O_{10}N_8$; EI-MS (rel int) 814 (1), 731 (1), 459 (2), 368 (3), 313 (4), 282 (16), 229 (35), 186 (21), 144 (69), 121 (100), and 116 (33).

Cyclomarin B (2): white solid; $[\alpha]^{20}_{D} = -30.4°$ (c = 0.9, $CHCl_3$); UV (MeOH) λ_{max} 220 (32 900), 275 (3200), and 230 (13 200); IR υ (neat) 3400−3300, 2962, 2925, 2871, 1644, 1508, 1455, 1248, 1094, and 752 cm^{-1}; HRFABMS obsd $[M]^+$ m/z 1026.6181, calcd 1026.6154 for $C_{56}H_{82}O_{10}N_8$.

Cyclomarin C (3): white solid; $[\alpha]^{20}_{D} = -19.7°$ (c = 1.0, $CHCl_3$); UV (MeOH) λ_{max} 220 (16 400); IR υ (neat) 3400−3295, 2962, 2926, 2871, 1644, 1508, 1455, 1238, 1094, and 752 cm^{-1}; HRFABMS obsd $[M]^+$ m/z 1049.6195, calcd 1049.6052 for $C_{56}H_{82}O_{10}N_8Na$.

Cyclomarin A Diacetate (4). Cyclomarin A (**1**, 26 mg, 0.025 mmol) was dissolved in pyridine (1.0 mL) and acetic anhydride (200 μL). DMAP (2 mg) was added, and the reaction mixture was stirred overnight at room temperature. The reaction mixture was concentrated in vacuo and purified by reversed-phase C-18 HPLC (conditions) to provide the diacetate derivative as a white crystalline solid (23 mg, 82%): ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.4, 171.7, 171.1, 170.7, 170.6, 169.7, 168.7, 168.1, 167.3, 135.9, 135.1, 134.4, 128.8, 128.3 (2), 127.9 (2), 127.2, 124.8, 124.7, 122.4, 120.4, 113.9, 107.8, 80.1, 72.1, 69.0, 59.0, 58.3, 58.2, 57.8, 57.7, 57.5, 55.9, 55.5, 54.7, 50.7, 45.4, 38.9, 35.2, 32.1, 30.9, 29.6, 29.1, 28.4, 25.8, 24.5, 23.3, 23.1, 22.6, 20.8, 20.6, 20.0, 19.4, 18.9, 18.4, 15.3; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, 1H, J = 9.3 Hz), 8.02 (d, 1H, J = 8.7 Hz), 7.96 (d, 1H, J = 9.0 Hz), 7.27−7.15 (m, 8H), 7.14 (s, 1H), 7.13−7.09 (m, 3H), 6.76 (d, 1H, J = 4.8 Hz), 6.48 (d, 1H, J = 9.3 Hz), 5.06 (d, 1H, J = 5.1 Hz), 4.96 (dd, 1H, J = 10.2 and 2.1 Hz), 4.90 (t, 1H, J = 4.8 Hz), 4.83−4.72 (m, 3H), 4.48 (dd, 1H, J = 10.2 and 2.1 Hz), 4.42 (t, 1H, J = 8.5 Hz), 4.08 (t, 1H, J = 10.2 Hz), 3.37 (s, 3H), 3.24 (dd, 1H, J = 6.0 and 10.0 Hz), 3.19 (dd, 1H, J = 3.0 and 4.0 Hz), 2.99 (dd, 1H, J = 7.0 and 10.0 Hz), 2.90 (t, 1H, J = 4.0 Hz), 2.86 (s, 3H), 2.75 (dd, 1H, J = 3.0 and 4.5 Hz), 2.57 (s, 3H), 2.38 (ddd, 1H, J = 7.5, 11.0, and 14.5 Hz), 2.28 (m, 1H), 2.17 (m, 1H), 2.13 (s, 3H), 2.01 (m, 1H), 1.92 (s, 3H), 1.72 (s, 3H), 1.65 (m, 1H), 1.61 (s, 3H), 1.50 (s, 3H), 1.49 (m, 1H), 1.24 (s, 3H), 1.23 (d, 3H, J = 7.5 Hz), 1.16 (m, 1H), 1.10 (d, 3H, J = 6.9 Hz), 1.08 (m, 1H), 1.02 (d, 3H, J = 6.9 Hz), 0.99 (d, 3H, J = 6.9 Hz), 0.64 (d, 3H, J = 6.9 Hz), 0.18 (d, 3H, J = 6.9 Hz), −1.01 (ddd, 1H, J = 2.0, 7.0, and 9.0 Hz).

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Supporting Information Available: Spectral data for cyclomarins A−C and crystal data for the diacetate derivative (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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