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Erythrolic acids A–E, Meroterpenoids from a Marine-Derived *Erythrobacter* sp.

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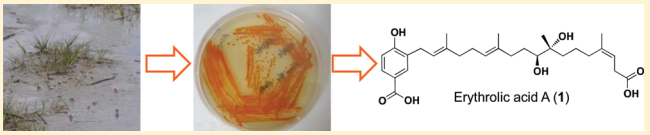
Supporting Information

ABSTRACT: Erythrolic acids A–E (**1**–**5**) are five unusual meroterpenoids isolated from the bacterium *Erythrobacter* sp. derived from a marine sediment sample collected in Galveston, TX. The structures were elucidated by means of detailed spectroscopic analysis and chemical derivatization. The erythrolic acids contain a 4-hydroxybenzoic acid appended with a modified terpene side chain. The side-chain modifications include oxidation of a terminal methyl substituent and in the case of **1**–**4** addition of a two-carbon unit to give terpene side chains of unusual length: C22 for **1** and **2**, C17 for **3**, and C12 for **4**. The relative and absolute configurations of the meroterpenoids were determined by coupling constant, NOE, and Mosher's analysis. In vitro cytotoxicity toward a number of nonsmall cell lung cancer (NSCLC) cell lines revealed only modest activity for erythrolic acid D (**4**) (2.5 μM against HCC44). The discovery of these unusual diterpenes, along with the previously reported erythrazoles, demonstrates the natural product potential of a previously unstudied group of bacteria for drug discovery. The unusual nature of the terpene side chain, we believe, involves an oxidation of a terminal methyl group to a carboxylic acid and subsequent Claisen condensation with acetyl-CoA.



INTRODUCTION

Meroterpenoids are natural products of mixed biosynthetic origin, containing a terpene element in combination with a carbon skeleton derived from other biosynthetic pathways, such as the shikimate or polyketide pathways.[1] There are relatively few meroterpenoids from bacterial sources, with the napyradiomycins, neomarinone, furaquinocin C, and azamerone being among the few examples.[2] We have found that bacteria of the genus *Erythrobacter* are prolific producers of meroterpenoids. *Erythrobacter* are strict aerobic Gram-negative bacteria that are ubiquitous in the marine environment. Many species of *Erythrobacter* are known to be producers of carotenoids, which are responsible for the smooth red-orange color of the colonies growing on agar plates.[3] However, to the best of our knowledge, there are no other reports of natural products from this class of bacteria.

As part of our efforts to search for novel bioactive natural products from marine-derived bacteria, we investigated the secondary metabolism from strain SNB-035 that by 16S rRNA analysis was identified as closely resembling *Erythrobacter* sp. Our studies on this strain reveal that *Erythrobacter* species are prolific producers of unique bioactive natural products. We have previously reported the isolation of two natural products from this bacterial strain, erythrazoles A and B (**6** and **7**), which contain a benzothiazole moiety coupled to a terpene side chain.[4] The molecules we report here are related to **6** and **7**, displaying similar two-carbon homologated terpenes. Bioassay-guided fractionation using the Locus Derepression assay (LDR), which identifies molecules that modulate epigenetic regulation, led to the isolation of five meroterpenoids erythrolic acids A–E (**1**–**5**) containing a hydroxybenzoic acid moiety and in the case of **1**–**4** a two-carbon homologated terpene side chain. Of the analogues, only erythrolic acid D (**4**) was found to have modest cytotoxicity. Although the biological activity of these compounds is modest at best in the assays for which they have been evaluated, the unusual two-carbon homologation provides a terpenoid skeleton that could be further explored.

RESULTS AND DISCUSSION

Marine bacterium SNB-035 was isolated from a sediment sample collected from Trinity Bay, Galveston, TX (29° 42.419′ N, 94° 49165′ W) and isolated on seawater-based acidic Gauze media. Analysis of the 16S rRNA revealed 98% identity to *Erythrobacter citreus*. Large-scale shake fermentation (30 L) was carried out to obtain sufficient material for full chemical and biological analysis of the new analogs. The excreted metabolites were collected using XAD-7 resin and the resulting crude extract purified by a combination of solvent/solvent extraction and reversed-phase chromatography to give fractions enriched in terpenoid metabolites. Final purification by gradient reversed-phase HPLC gave erythrolic acids A (**1**, 2.5 mg), B (**2**, 1.5 mg), C (**3**, 0.7 mg), D (**4**, 1.5 mg), and E (**5**, 0.6 mg) (Figure 1).

The molecular formula of erythrolic acid A (**1**) was determined to be $C_{29}H_{42}O_7$ (calcd for $C_{29}H_{41}O_7$, 501.2852), based on a high-resolution ESI-MS $[M - H]^-$ of 501.2849, indicating nine degrees of unsaturation. The ^{1}H NMR spectrum

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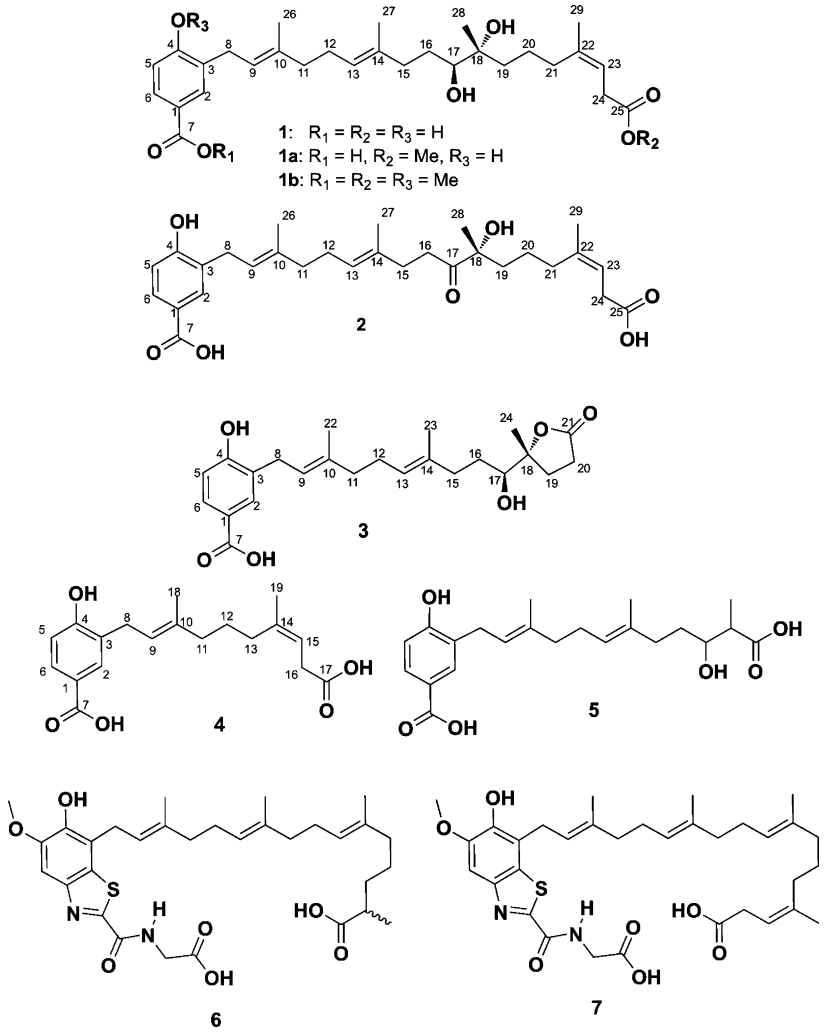

Figure 1. Structures of natural products from *Erythrobacter* sp. strain SNB-035.

of **1** (Table 1) showed signals for a 1,3,4-trisubstitued benzene ring with protons at δ_H 7.76 ppm (d, J = 2.0 Hz, H-2), 7.69 ppm (dd, J = 8.4, 2.0 Hz, H-6), and 6.77 ppm (d, J = 8.4 Hz, H-5); three vinyl methyl singlets at δ_H 1.73, 1.71, 1.61; three olefinic ^{1}H (δ_H 5.35, 5.34, and 5.21); and 12 allylic methylene protons. Analysis of ^{13}C NMR and HSQC spectra revealed 29 carbons corresponding to two carbonyls (δ_C 170.6 and 176.7), one oxygenated quaternary sp^2 carbon (δ_C 161.2), 11 sp^2 carbons (δ_C 115.4−140.3), two oxygenated sp^3 carbons (δ_C 75.6 and 78.3), and nine sp^3 carbons (δ_C 16.4−41.1). In the HMBC spectrum of **1**, both of the proton signals at δ_H 7.76 and 7.69 showed correlations to the carbonyl carbon at δ_C 170.6 and the oxygenated quaternary carbon at δ_C 161.2, indicating that compound **1** is a 3-substituted 4-hydroxybenzoic acid derivative, with a 22-carbon side chain. A combination of ^{1}H NMR, ^{13}C NMR, ^{1}H−^{1}H COSY, and gHMBC data (Table S1, Supporting Information) allowed us to assemble an unusual 22-carbon terpene fragment terminating in a carboxylic acid. A few key HMBC correlations (Figure 2) for the terpene portion of **1** were from the H_3-26 singlet (δ_H 1.71, s) to C-9 (δ_C 123.5), C-10 (δ_C 137.6), and C-11 (δ_C 41.0) and from the H_3-27 singlet (δ_H 1.61) to C-13 (δ_C 125.6), C-14 (δ_C 136.3), and C-15 (δ_C 38.1). The saturated portion of the terpene was assigned based on HMBC correlations from the H_3-28 singlet (δ_H 1.07) to C-17 (δ_C 78.3), C-18 (δ_C 75.6), and C-19 (δ_C 39.2) and from the H_3-29 singlet (δ_H 1.73) to C-21 (δ_C 33.5), C-22 (δ_C 140.3), and C-23 (δ_C 118.2). COSY correlations between H-15/H-16 and H-16/H-17 fully establish the saturated spin system. A COSY correlation between H-23 (δ_H 5.35) and H-24 (δ_H 3.01) combined with HMBC correlations from both H-23 and H-24 to the carbonyl carbon at δ_C 176.7 established the side chain, with the unusual two carbon homologation to a C22 terpene.

The downfield chemical shift of the H_2-8 doublet (δ_H 3.31) and HMBC correlations from H-8 to C-2, C-3, and C-4 indicate the terpene side chain is located at C-3 of the benzoic acid ring. The two carboxylic acids in **1** were further confirmed by conversion of **1** to the bis-methyl ester (**1a**) with TMS-CHN_2, giving rise to two new methyl singlets at δ_H 3.65 and 3.84 ppm in the ^{1}H NMR. The configuration of the double bonds in **1** was deduced as 9*E*,13*E*,23*Z* on the basis of the chemical shifts[5] of C-26 (δ_C 16.4), C-27 (δ_C 16.4), and C-29 (δ_C 23.7) and confirmed by ROESY correlations between H-26/H-8, H-9/H-11, H-27/H-12, H-13/H-15, H-29/H-23, and H-24/H-21.

The absolute configuration of C-17 in **1** was determined by application of the modified Mosher method on its trimethylated product **1b**, which was generated via exhaustive treatment of **1** with TMS-CHN_2 (Figure 3).[6] Treatment of **1b**, in separate experiments, with *R*-(−)- and *S*-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) produced

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Table 1. NMR Data for Erythrolic Acids A (1) and B (2) in CD_3OD

	1		2	
no.	δ_H, mult (J in Hz)	δ_C	δ_H, mult (J in Hz)	δ_C
1		122.6 C		122.8 C
2	7.76, d (2.0)	132.6 CH	7.75, d (1.9)	132.7 CH
3		129.3 C		129.4 C
4		161.2 C		161.2 C
5	6.77, d (8.4)	115.4 CH	6.75, d (8.5)	115.4 CH
6	7.69, dd (8.4, 2.0)	130.5 CH	7.68, dd (8.5, 1.9)	130.6 CH
7		170.6 C		170.7 C
8	3.31, m, overlap	29.0 CH_2	3.32, m	29.1 CH2
9	5.34, m	123.5 CH	5.33, dt	123.6 CH
10		137.6 C		137.3 C
11	2.07, t (7.3)	41.0 CH_2	2.05, m	40.9 CH_2
12	2.15, q (7.3)	27.8 CH_2	2.12, q (7.3)	27.7 CH_2
13	5.21, t (7.3)	125.6 CH	5.17, dt	125.9 CH
14		136.3 C		135.3 C
15a	2.23, m	38.1 CH_2	2.17, m	34.3 CH_2
15b	1.98, m			
16a	1.72, m	30.6 CH_2	2.67 dt	36.7 CH_2
16b	1.34, m			
17	3.26, dd (10.9, 1.6)	78.3 CH		216.9 C
18		75.6 C		80.5 C
19a	1.51, m	39.2 CH_2	1.67, m	40.4 CH_2
19b	1.45, m		1.54, m	
20a	1.53, m	22.6 CH_2	1.46, m	22.8 CH_2
20b	1.44, m		1.21, m	
21	2.04, t (7.0)	33.5 CH_2	2.02, d (7.0)	32.9 CH_2
22		140.3 C		139.6 C
23	5.35, m	118.2 C	5.32, dt	118.8 C
24	3.01, d (7.2)	34.5 CH_2	2.92, d (7.2)	34.6 CH_2
25		176.7 C		176.7 C
26	1.71, s	16.4 CH_3	1.72, s	16.4 CH_3
27	1.61, s	16.4 CH_3	1.61, s	16.4 CH_3
28	1.07, s	22.2 CH_3	1.23, s	25.6 CH_3
29	1.73, s	23.7 CH_3	1.68, s	23.6 CH_3

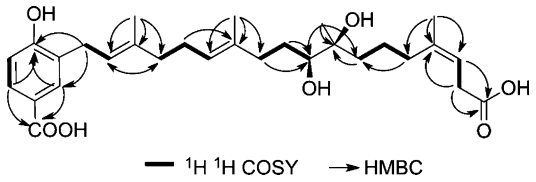

Figure 2. Key ^{1}H–^{1}H COSY and HMBC correlations for compound **1**.

the corresponding *S*- and *R*-Mosher esters **1d** and **1e**, respectively. Interpretation of the ^{1}H NMR chemical shift differences ($\Delta\delta = \delta_S - \delta_R$) between **1d** and **1e**, established the absolute configuration of C-17 as *S* by applying Mosher ester analysis (Figure 3). To assign the relative configuration of the 17,18-diol, the dimethyl ester **1a** was transformed into acetonide **1c** by treatment with dimethoxypropane in acetone with catalytic pyridinium *p*-toluenesulfonic acid. 1D NOE experiments (Figure 4 and Table S2, Supporting Information) on **1c** showed NOE enhancements on the C-28 methyl singlet and CH_3-30α by irradiating H-17, enhancement of H-17 and CH_3-30β upon irradiating CH_3-30α, and enhancement of H-17 and CH_3-30α on irradiation the methyl singlet H-28. Full analysis of the NOE data and the confirmation of the five-membered ring acetonide[7] indicated the *erythro* configuration for the 17, 18-diol in **1c**, thus establishing the absolute configuration of C-18 as *R*. This configuration was also supported by the ^{13}C chemical shifts of C-17 and C-18 (δ_C 78.3 and 75.6).[8]

The molecular formula of erythrolic acid B (**2**) was determined to be $C_{29}H_{40}O_7$ on the basis of a HRESIMS $[M - H]^-$ of 499.2692 (calcd for $C_{29}H_{39}O_7$, 499.2696), two hydrogens less than **1**. The ^{1}H and ^{13}C NMR spectra for **1** and **2** (Table 1) showed very similar signals, with the exception that the H-17 doublet at δ_H 3.26 for **1** was missing for **2**, and the carbon signal at δ_C 78.3 (C-17) for **1** was replaced by a new carbonyl signal at δ_C 216.9 for **2**. The data indicated that the hydroxyl group at C-17 in **1** has been oxidized to a ketone in **2**. This assignment was supported by HMBC correlations from both H_3-28 (δ_H 1.23) and H_2-16 (δ_H 2.67) to the C-17 carbonyl signal at δ_C 216.9. The full structure of **2** was further confirmed by COSY and HMBC experiments (Table S1, Supporting Information). Similarly, the stereochemistry of the double bonds in **2** were determined as 9*E*, 13*E*, and 23*Z* based on the chemical shifts of C-26 (δ_C 16.4), C-27 (δ_C 16.4), and C-29 (δ_C 23.6)[5] and ROESY correlations. Biosynthetically, compound **2** is likely to be derived from **1** by oxidation at C-17. Hence, we assigned the stereochemistry of C-18 in **2** as *R*, the same as in **1**.

Erythrolic acid C (**3**) was determined to have a molecular formula of $C_{24}H_{32}O_6$ based on HRESIMS $[M + H]^+$ of *m*/*z*

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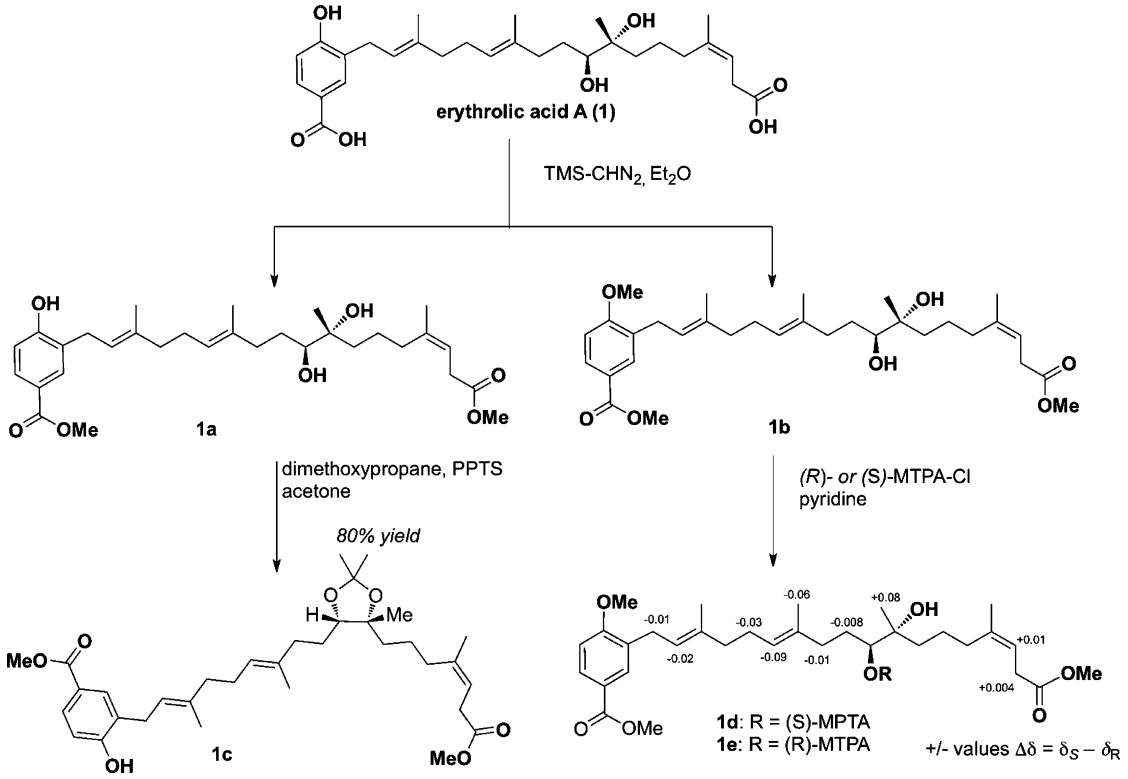

Figure 3. Stereochemical determination of **1**.

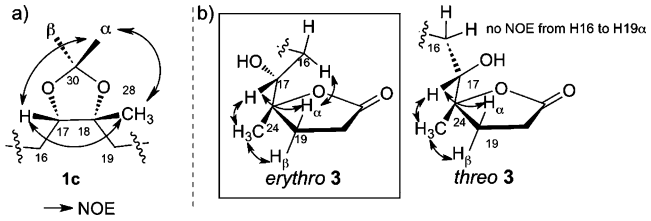

Figure 4. (a) NOE correlations for **1c**. (b) Potential *erythro* and *threo* configurations for C17/C18 of **3**. Observed NOE correlations establish *erythro* configuration.

417.2285 (calcd for $C_{24}H_{33}O_6$, 417.2277), indicating nine degrees of unsaturation. The ^{1}H NMR spectrum of **3** (Table 2) showed typical signals for a prenylated benzoic acid, including a 1,3,4-trisubstitued benzene ring based on ^{1}H NMR signals at δ_H 7.76 (d, J = 2.0 Hz, H-2), 7.71 (dd, J = 8.3, 2.0 Hz, H-6), and 6.78 (d, J = 8.3 Hz, H-5), as well as two triplet signals for olefinic protons at δ_H 5.35 and 5.21. By analysis of ^{1}H−^{1}H COSY, HSQC and gHMBC spectra for **3** (Table S3, Supporting Information), a 17-carbon terpene fragment was established. The key COSY correlations included those for H-8 (δ_H 3.31)/H-9 (δ_H 5.35), H-11 (δ_H 2.08)/H-12 (δ_H 2.15)/H-13 (δ_H 5.21), H-15 (δ_H 2.22, 2.02)/H-16 (δ_H 1.59, 1.37)/H-17 (δ_H 3.51), and H-19 (δ_H 2.34, 1.87)/H-20 (δ_H 2.60).

HMBC correlations from H-22 to C-9, C-10 and C-11, from H-23 to C-13, C-14, and C-15, from H-24 to C-17, C-18, and C-19 clearly indicated the presence of a terpene chain. Moreover, the COSY correlations between H-19 and H-20 as well as HMBC correlations from both H-19 and H-20 to the C-21 carbonyl carbon at δ_C 179.9 revealed that the side chain terminated in a carboxylic acid or ester. The benzoic acid, two double bonds, and terminal carbonyl only account for eight of the nine degrees of unsaturation, indicating the presence of an additional ring. The downfield shift of the terminal carbonyl carbon at δ_C 179.9 and the C-18 oxygenated quaternary carbon at δ_C 90.5, allowed for formation of the five-membered lactone ring. As a result, the planar structure of compound **3** was established.

The stereochemistry of the double bonds in **3** were determined as 9*E*,13*E* based on the chemical shifts of C-22 (δ_C 15.9) and C-23 (δ_C 15.9).[5] The relative configuration of C-17/C-18 in **3** was established by a 2D ROESY experiment, with the H_3-24 singlet having NOE correlations with H-19β and H-17, and H-19α showing NOE correlations with H-16 and H-17. However, there was no apparent NOE correlation between H-16 and H-24 (Figure 4 and Table S3, Supporting Information). If the relative configuration with *threo*, we should not observe an NOE between H-16 and H-19α. Thus, the NOE data are consistent with C-17/C-18 having the *erythro* configuration. Based on the similarity of **1** and **3**, we assume the absolute configuration is 17*S*,18*R*.

The molecular formula of erythrolic acid D (**4**) was determined to be $C_{19}H_{24}O_5$ on the basis of the HRESIMS which gave an $[M + Na]^+$ of m/z 355.1510 (calcd for $C_{19}H_{24}O_5Na$, 355.1521), indicating eight degrees of unsaturation. The ^{1}H NMR spectrum of **4** (Table 2) showed signals for a prenylated benzoic acid, including ^{1}H NMR signals for a 1,3,4-trisubstitued benzene ring at δ_H 7.76 (d, J = 2.2 Hz, H-2), 7.71 (dd, J = 2.2, 8.4 Hz, H-6), and 6.78 (d, J = 8.4 Hz, H-5), as well as two triplet signals for olefinic protons at δ_H 5.35 and 5.30. Comparison of the NMR spectra for **4** with **1** and **3** clearly indicated the presence of a monoterpenoid unit with a terminal carboxylic acid. The COSY correlations for H-8 (δ_H 3.32)/H-9 (δ_H 5.35), H-11 (δ_H 2.06)/H-12 (δ_H 1.56)/H-13 (δ_H 2.04), and H-15 (δ_H 5.30) /H-16 (δ_H 2.98) (Table S4, Supporting Information) and HMBC correlations from H-18 (δ_H 1.72) to C-9, C-10 and C-11, from H-19 (δ_H 1.73) to C-13,

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Table 2. NMR Data for Erythrolic Acids C (3), D (4), and E (5) in CD_3OD

	3		4		5	
no.	δ_H, mult (J in Hz)	δ_C	δ_H, mult (J in Hz)	δ_C	δ_H, mult (J in Hz)	δ_C
1		122.4		122.6		122.5
2	7.76, d (2.0)	132.4	7.76, d (2.2)	132.6	7.75, d (2.1)	132.5
3		129.3		129.4		129.1
4		161.1		161.2		160.9
5	6.78, d (8.3)	115.1	6.78, d (8.4)	115.4	6.78, d (8.5)	115.2
6	7.71, dd (2.0, 8.3)	130.2	7.71, dd (8.4, 2.2)	130.4	7.71, dd (2.1, 8.5)	130.4
7		170.2		170.5		170.4
8	3.31, m, overlap	28.6	3.32, d (7.6)	29.1	3.32, m, overlap	28.7
9	5.35, t (7.0)	123.2	5.35, t (7.3)	123.5	5.34, t (7.5)	123.4
10		137.1		137.4		137.1
11	2.08, t (7.4)	40.5	2.06, t (7.5)	40.6	2.07, t (7.6)	40.8
12	2.15, q (7.5)	27.3	1.56, tt (8.1, 7.5)	27.2	2.13, q (7.2)	27.5
13	5.21, t (7.3)	125.7	2.04, t (8.1)	32.4	5.19, t (6.4)	125.5
14		135.5		140.3		135.6
15α	2.22, m	36.9	5.30, t (7.6)	118.1	2.15, t (7.9)	36.9
15β	2.02, m				1.98, m	
16α	1.59, m	30.5	2.98, dd (7.6, 1.2)	34.3	1.56, m	34.2
16β	1.37, m				1.49, m	
17	3.51, dd (10.6, 2.1)	76.1		176.6	3.75, m	73.1
18		90.5	1.72, br s	16.2	2.40, dt (6.7)	47.0
19α	2.34, m	29.2	1.73, q (1.2)	23.7		179.4
19β	1.87, m					
20	2.60, t (8.5)	30.1			1.72,s	16.1
21		179.9			1.60, s	15.9
22	1.72, s	15.9			1.14, d (7.2)	12.4
23	1.62, s	15.9				
24	1.32, s	22.7				

C-14 and C-15, from both H-15 (δ_H 5.30) and H-16 (δ_H 2.98) to C-17 (δ_C 176.7) and from H-8 (δ_H 3.31) to C-2, C-3 and C-4 establish an unusual 12-carbon terpene fragment located at C-3 of the benzoic acid ring. Similarly, the stereochemistry of the double bonds in **4** was determined as 9*E*,14*Z* on the basis of the chemical shifts of C-18 (δ_C 16.2) and C-19 (δ_C 23.7).[5]

The molecular formula of erythrolic acid E (**5**) was determined to be $C_{22}H_{30}O_6$ on the basis of HRESIMS with an $[M + Na]^+$ of m/z 413.1936, (calcd for $C_{22}H_{30}O_6Na$, 413.1940). Analysis of ^1H, COSY, HSQC and HMBC data (Table S5, Supporting Information) allowed for the identification of a 4-hydroxybenzoic acid unit and a sesquiterpene side chain. The key COSY correlations for H-15 (δ_H 2.15, 1.98)/H-16 (δ_H 1.56, 1.49)/H-17 (δ_H 3.75)/H-18 (δ_H 2.40)/H-22 (δ_H 1.14) as well as key HMBC correlations from H-22 to C-17 (δ_C 73.1), C-18 (δ_C 47.0), and C-19 (δ_C 179.4) and from H-18 to C-17, C-19 and C-22 (δ_C 12.4) are highly indicative of a sesquiterpene terminating in a carboxylic acid and hydroxylated at C-17. The double bonds in **5** were assigned as 9*E*,13*E* based on the chemical shifts of C-20 (δ_C 16.1) and C-21 (δ_C 15.9).[5] Due to lack of material we were unable to establish the relative and absolute configuration of C-17/C-18. Compound **5** is the only one of the erythrolic acids isolated to have a traditional terpene backbone, albeit with a terminal methyl group oxidized to a carboxylic acid. This is similar to the terpene framework of erythrazole A (**6**). As described below, the presence of the terminal carboxylic acid at C-19 in **5** could provide insight into the biosynthesis of the other erythrolic acids.

Compounds **1**–**4** contain a C22, C17, or C12 terpene side chain, two more carbons than a regular diterpene, sesquiterpene, and monoterpene, respectively, which is uncommon in natural products. Mycophenolic acid (MPA) is one of the limited examples of two-carbon homologated terpenes.[8] The biosynthesis of MPA has been shown to be derived from 6-farnesyl-5,7-dihydroxy-4-methylphthalide by oxidative cleavage of an olefin, thereby reducing the terpene by three-carbons in a "cleavage pathway" (Figure 5).[9,10] However, we believe the unusual terpene side chains in the erythrolic acids are generated by an alternative "homologation pathway". The isolation of **5**, which contains a standard length terpene chain, but with oxidation of a terminal methyl group to a carboxylic acid, led us to speculate that **5** could be a precursor for a two-carbon homologation to give **3**. Specifically in our proposed pathway to **1**, C22 terpene **9** may arise from Claisen condensation of acetate with diterpene **8** (Figure 5). Terpene **8** would arise from oxidation of a terminal methyl group and reduction of the olefin. Following the Claisen condensation, subsequent elimination of H_2O would result in α,β-unsaturated acid **10**, which would isomerize to give the requisite C22 terpene.[1] Although we have attempted feeding studies with sodium [1-^{13}C] acetate (100 mg/L), sodium [1,2-^{13}C] acetate (100 mg/L), [U−$^{13}C_6$] glucose (100 mg/L) and sodium [3-^{13}C] pyruvate, our incorporation rates were extremely low, and we have been unsuccessful in determining which of the two pathways is responsible for the two additional carbons.

The biological activity of the purified compounds was evaluated in the Locus Depression (LDR) assay in cytotoxicity assays against three nonsmall cell lung cancer (NSCLC) cell lines and for direct inhibition of HDAC 2. Erythrolic acid D (**4**) was the only molecule that showed activity, with an IC_{50} value of 2.4 μM against the NSCLC cell line HCC44 and 3.4 μM

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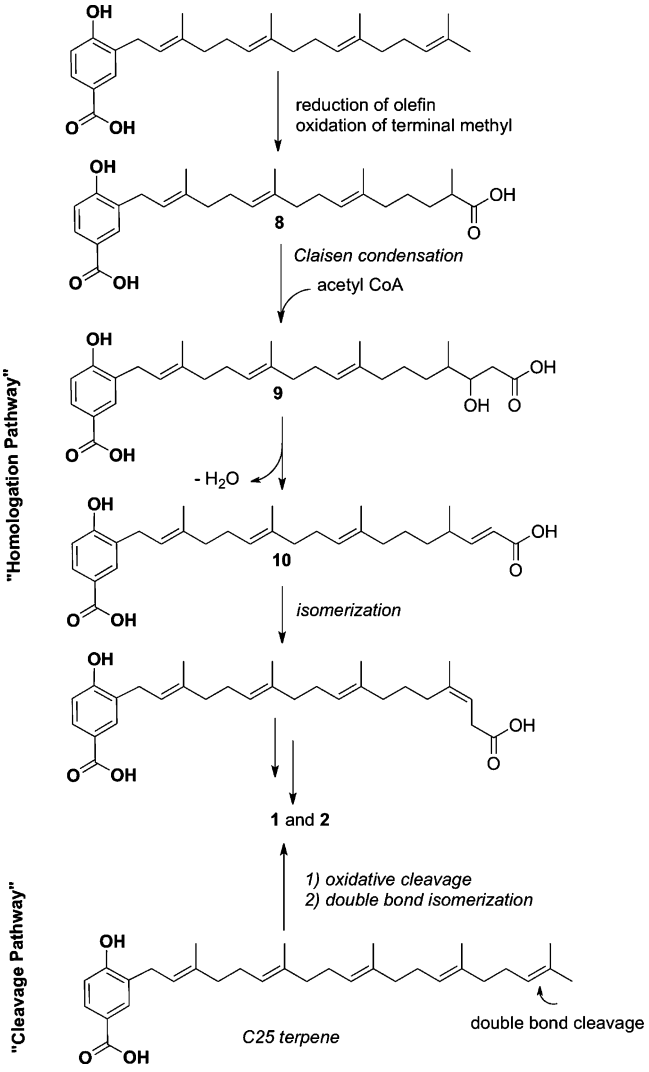

Figure 5. Two biosynthetic pathways to C22 terpene.

against HCC366. Compound **4** is the only erythrolic acid derivative to lack oxygenation along the terpene side chain, much the same as erythrazole B (**7**), which also exhibits comparable cytotoxicity. It is possible that the C19 vinyl methyl plays a role in the biological activity.

EXPERIMENTAL SECTION

General Procedures. The optical rotations were recorded with a polarimeter equipped with a halogen lamp (589 nm). ^{1}H and 2D NMR spectral data were recorded at 600 MHz in CD_3OD or $CDCl_3$, and chemical shifts were referenced to the corresponding solvent residual signal. ^{13}C NMR spectra were acquired at 100 MHz, and chemical shifts were referenced to the corresponding solvent residual signal. High resolution ESI-TOF mass spectra were provided by The Scripps Research Institute, La Jolla, CA. Low-resolution LC/ESI-MS data were measured using via ESI-MS with a reversed-phase C_{18} column (Phenomenex Luna, 150 mm × 4.6 mm, 5 mm) at a flow rate of 0.7 mL/min. Preparative HPLC was performed on an Agilent 1200 series instrument with a DAD detector, using a phenyl-hexyl column (Phenomenex Luna, 250 × 10.0 mm, 5 μ). ODS (50 μm) was used for column chromatography.

Collection and Phylogenetic Analysis of Strain SNB-035. The marine-derived bacterium, strain SNB-035, was isolated from a sediment sample collected from Trinity Bay, Galveston, TX (29° 42.419′ N, 94° 49165′ W). Bacterial spores were collected via stepwise centrifugation as follows: 2 g of sediment was dried over 24 h in an incubator at 35 °C and the resulting sediment added to 10 mL sH_2O containing 0.05% Tween 20. After a vigorous vortex for 10 min, the sediment was centrifuged at 2500 rpm for 5 min (4 °C). The supernatant was removed and transferred into a new tube and centrifuged at 18000 rpm for 25 min (4 °C) and the resulting spore pellet collected. The resuspended spore pellet (4 mL sH_2O) was plated on a acidified Gauze media, giving rise to individual colonies of SNB-035 after two weeks. Analysis of the 16S rRNA sequence of SNB-035 revealed 98% identity to *Erythrobacter citreus*.

Cultivation and Extraction. Bacterium SNB-035 was cultured in 30 2.8 L Fernbach flasks each containing 1 L of a seawater-based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g $CaCO_3$, 40 mg $Fe_2(SO_4)_3{\cdot}4H_2O$, 100 mg KBr) and shaken at 200 rpm at 27 °C. After seven days of cultivation, sterilized XAD-7-HP resin (20 g/L) was added to adsorb the organic products, and the culture and resin were shaken at 200 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The acetone soluble fraction was dried in vacuo to yield 4.1 g of extract.

Isolation. The extract (4.1 g) was partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and MeOH/H_2O. The EtOAc soluble layer (342 mg) was fractionated by flash column chromatography on ODS (50 μm, 50 g), eluting with a step gradient of MeOH and H_2O (30:70−100:0), and 25 fractions (Fr.1−Fr.25) were collected. Fractions 16−18 were combined and purified by reversed-phase HPLC (Phenomenex Luna, phenyl-hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 mm, UV = 210 nm) using a gradient solvent system from 20% to 88% CH_3CN (0.1% TFA) over 30 min to afford erythrolic acid A (**1**, 2.5 mg, t_R = 20.7 min), erythrolic acid B (**2**, 1.5 mg, t_R = 22.3 min). Fractions 7−9 were combined and purified with the same HPLC column using a gradient from 25% to 68% CH_3CN (0.1% TFA) over 29 min to afford erythrolic acid C (**3**, 0.7 mg, t_R = 24.3 min), erythrolic acid D (**4**, 1.5 mg, t_R = 19.5 min), and erythrolic acid E (**5**, 0.6 mg, t_R = 20.7 min).

Erythrolic acid A: white solid (**1**, 2.5 mg); $[\alpha]_D$ + 2.0 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 257 (3.6), 211 (3.9); ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) see Table 1. ESI-MS *m*/*z* 525.2 $[M + Na]^+$, 501.2$[M - H]^-$; HRESIMS *m*/*z* 501.2849 $[M - H]^-$ (calcd for $C_{29}H_{41}O_7$, 501.2852).

Erythrolic acid B: white solid (**2**, 1.5 mg); $[\alpha]_D$ +4.1 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 259 (3.8), 217 (4.1), 211 (4.1); ^{1}H NMR (600 MHz, CD_3OD) and ^{13}C NMR (100 MHz, CD_3OD) see Table 1; ESI-MS *m*/*z* 523.2 $[M + Na]^+$, 499.2 $[M - H]^-$; HRESIMS *m*/*z* 499.2692 $[M - H]^-$ (calcd for $C_{29}H_{39}O_7$, 499.2696).

Erythrolic acid C: white solid (**3**, 0.7 mg); $[\alpha]_D$ +13.3 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 257 (3.3), 210 (3.9); ^{1}H NMR and 2D NMR data (600 MHz, CD_3OD) see Table 2; ESI-MS *m*/*z* 439.2 $[M + Na]^+$ 415.2 $[M - H]^-$; HRESIMS *m*/*z* 439.2110 $[M + Na]^+$ ($C_{24}H_{32}NaO_6$, calcd 439.20966), 417.2285 $[M + H]^+$(calcd for $C_{24}H_{33}O_6$, 417.2277).

Erythrolic acid D: white solid (**4**, 1.5 mg); UV (MeOH) λ_{max} (log ε) 256 (3.9), 214 (4.0); ^{1}H NMR and 2D NMR data (600 MHz, CD_3OD) see Table 2; ESI-MS *m*/*z* 355.1 $[M + Na]^+$, 331.1 $[M - H]^-$; HRESIMS *m*/*z* 355.1510 $[M + Na]^+$ ($C_{19}H_{24}NaO_5$, calcd 355.1521), 333.1692 $[M + H]^+$(calcd for $C_{19}H_{25}O_5$, 333.1702).

Erythrolic acid E: white solid (**5**, 0.6 mg); $[\alpha]^{20}_D$ +12.5 (*c* 0.04, MeOH); UV (MeOH) λ_{max} (log ε) 259 (3.6), 210 (4.1); ^{1}H NMR and 2D NMR data (600 MHz, CD_3OD) see Table 2; ESI-MS *m*/*z* 413.1.2 $[M + Na]^+$ 389.2 $[M - H]^-$; HRESIMS *m*/*z* 413.1939 $[M + Na]^+$ ($C_{22}H_{30}NaO_6$, calcd. 413.1940), 391.2116 $[M + H]^+$ (calcd for $C_{22}H_{31}O_6$, 391.2120).

Methyl Esters of Compound 1. Solutions of **1** (1.5 mg) in dry methanol (0.5 mL) were treated with 30 μL of trimethylsilyldiazomethane (2 M in diethyl ether) at room temperature for 35 min. The reaction mixture was analyzed by LC−MS and purified by RP-HPLC (Phenomenex Luna, phenyl-hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 mm, UV = 210 nm) using a gradient solvent system from 50% to 99% CH_3CN (0.1% FA) over 30 min to afford dimethylerythrolic acid A (**1a**, 0.8 mg, t_R = 17.6 min) and trimethylerythrolic acid A (**1b**, 0.6 mg, t_R = 20.1 min).

Dimethylerythrolic acid A (1a): yellow solid; ^{1}H NMR (600 MHz, CD_3OD) δ 7.74 (1H, d, *J* = 1.8, H-2), 7.70 (1H, dd, *J* = 1.8, 8.4

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Hz, H-6), 6.79 (1H, d, J = 8.4 Hz, H-5), 5.34 (1H, t, J = 7.0 Hz, H-9), 5.29 (1H, t, J = 7.1 Hz, H-23), 5.21 (1H, t, J = 6.9 Hz, H-13), 3.84 (3H, s, 7-$COOCH_3$), 3.65 (3H, s, 25-$COOCH_3$), 3.31 (2H, m, overlap, H-8), 3.26 (1H, d, J = 10.4 Hz, H-17), 3.05 (2H, d, J = 7.1 Hz, H-24), 2.24/1.98 (2H, m, H-15), 2.15 (2H, q, J = 7.1 Hz, H-12), 2.07 (2H, d, t = 7.1 Hz, H-11), 2.04 (2H, d, t = 7.0 Hz, H-21), 1.71/1.34 (2H, m, H-16), 1.73 (3H, s, H-29), 1.70 (3H, s, H-26), 1.62 (3H, s, H-27), 1.51/1.45 (2H, m, H-19), 1.53/1.44 (2H, m, H-20), 1.07 (3H, s, H-28); ESI-MS m/z 553.2 $[M + Na]^+$, 529.2 $[M - H]^-$.

Trimethylerythrolic acid A (1b): yellow solid; ^{1}H NMR (600 MHz, CD_3OD) δ 7.87 (1H, br d, J = 8.5 Hz, H-6), 7.78 (1H, br s, H-2), 6.99 (1H, d, J = 8.5 Hz, H-5), 5.34 (1H, t, J = 7.0 Hz, H-9), 5.29 (1H, t, J = 7.1 Hz, H-23), 5.21 (1H, t, J = 6.9 Hz, H-13), 3.90 (3H, s, 4-OCH_3), 3.85 (3H, s, 7-$COOCH_3$), 3.65 (3H, s, 25-$COOCH_3$), 3.31 (2H, m, overlap, H-8), 3.25 (1H, d, J = 10.4 Hz, H-17), 3.05 (2H, d, J = 7.1 Hz, H-24), 2.24/1.98 (2H, m, H-15), 2.15 (2H, q, J = 7.1 Hz, H-12), 2.06 (2H, d, t = 7.1 Hz, H-11), 2.03 (2H, d, t = 7.0 Hz, H-21), 1.71/1.34 (2H, m, H-16), 1.73 (3H, s, H-29), 1.70 (3H, s, H-26), 1.62 (3H, s, H-27), 1.51/1.45 (2H, m, H-19), 1.53/1.44 (2H, m, H-20), 1.07 (3H, s, H-28); ESI-MS m/z 567.2 $[M + Na]^+$.

Preparation of Acetonide 1c. Compound **1a** (0.6 mg) was treated with 2,2-dimethyoxypropane (0.6 mL) and pyridinium p-toluenesulfonate (3.0 mg) and then stirred at room temperature for 12 h under a N_2 atmosphere. The reaction solution was evaporated in vacuo and purified by RP-HPLC (Phenomenex Luna, phenyl-hexyl, 250 × 10.0 mm, 2.5 mL/min, 5 mm, UV = 210 nm) using a gradient solvent system from 60% to 99% CH_3CN (0.1% FA) over 25 min to yield acetonide **1c** (0.4 mg, t_R = 20.0 min): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.80 (1H, dd, J = 2.1, 8.3 Hz, H-6), 7.79 (1H, d, J = 2.1, H-2), 6.80 (1H, d, J = 8.3 Hz, H-5), 5.32 (1H, t, J = 7.3 Hz, H-9), 5.32 (1H, t, J = 7.3 Hz, H-23), 5.11 (1H, t, J = 6.9 Hz, H-13), 3.87 (3H, s, 7-$COOCH_3$), 3.67 (3H, s, 25-$COOCH_3$), 3.66 (1H, dd, J = 9.1, 3.5 Hz, H-17), 3.36 (2H, d, J = 7.1 Hz, H-8), 3.02 (2H, d, J = 7.1 Hz, H-24), 2.17/1.98 (2H, m, H-15), 2.13 (2H, m, H-12), 2.09 (2H, m, H-11), 2.02 (2H, m, H-21), 1.59/1.47 (2H, m, H-16), 1.76 (3H, s, H-26), 1.73 (3H, s, H-29), 1.60/1.43 (2H, m, H-20), 1.58 (3H, s, H-27), 1.49/1.14 (2H, m, H-19), 1.40 (3H, s, 30β-CH_3), 1.34 (3H, s, 30α-CH_3), 1.17 (3H, s, H-28); ^{1}H NMR (600 MHz, CD_3OD) and 2D NMR data (in both $CDCl_3$ and CD_3OD) see Table S2 (Supporting Information); ESI-MS m/z 593.3 $[M + Na]^+$.

Preparation of MTPA Esters 1d and 1e. Compound **1b** was divided into two portions, and each was dissolved in 400 μL of dry pyridine in a 2 mL vial. The samples were then treated with 3 μL of (R)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) and 3 μL of (S)-MTPA-Cl at room temperature, respectively. After 12 h, the crude ester mixtures were purified by RP-HPLC with a Luna C-18 column (250 × 10 mm) using 30% MeCN–H_2O to 100% MeCN as a linear gradient for 30 min (flow rate 2.5 mL/min) to give (S)-Mosher ester (**1d**) and (R)-Mosher ester (**1e**), respectively. ESI-MS (m/z) 761 $[M + H]^+$ (see the Supporting Information for NMR data).

Biological Assays. Cell lines were cultured in 10 cm dishes in NSCLC cell-culture medium: RPMI/L-glutamine medium, 1000 U/mL of penicillin, 1 mg/mL of streptomycin, and 5% fetal bovine serum. Cell lines were grown in a humidified environment in the presence of 5% CO_2 at 37 °C. For cell viability assays, HCC44, H1395, H2122, and HCC1395 cells (60 mL) were plated individually at a density of 750 and 500 cells/well, respectively, in 384 well microtiter assay plates. After the assay plates were incubated overnight under the growth conditions described above, purified compounds were dissolved and diluted in DMSO and subsequently added to each plate with final compound concentrations ranging from 40 mM to 1 nM and a final DMSO concentration of 0.5%. After an incubation of 96 h under growth conditions, Cell Titer GloTM reagent was added to each well (10 mL of a 1:2 dilution in NSCLC culture medium) and mixed. Plates were incubated for 10 min at room temperature and luminescence was determined for each well using a multimodal plate reader. Relative luminescence units were normalized to the untreated control wells (cells plus DMSO only).

■ ASSOCIATED CONTENT

Supporting Information

General procedures, chemical derivatization, data tables, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Dewick, P. M *Medicinal Natural Products: A Biosynthetic Approach*, 3rd ed.; John Wiley and Sons: West Sussex, 2009.

(2) (a) Shiomi, K.; Nakamura, H.; Iinuma, H.; Naganawa, H.; Isshiki, K.; Takeuchi, T.; Umezawa, H.; Iitaka, Y. *J. Antibiot.* **1986**, *39*, 494–501. (b) Ishibashi, M.; Funayama, S.; Anraku, Y.; Komiyama, K.; Omura, S. *J. Antibiot.* **1991**, *44*, 390–395. (c) Funayama, S.; Ishibashi, M.; Komiyama, K.; Omura, S. *J. Org. Chem.* **1990**, *55*, 1132–1133. (d) Hardt, I. H.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **2000**, *41*, 2073–2076. (e) Cho, J. Y.; Kwon, H. C.; Williams, P.; Jensen, P. R.; Fenical, W *Org. Lett.* **2006**, *8*, 2471–2474.

(3) Denner, E. B.; Vybiral, D.; Koblizek, M.; Kampfer, P.; Busse, H. J.; Velimirov, B. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 1655–1661.

(4) Hu, Y.; MacMillan, J. B. *Org. Lett.* **2011**, *13*, 6580–6583.

(5) Maxwell, A.; Rampersad, D. *J. Nat. Prod.* **1989**, *52*, 614–618.

(6) Ohtani, I.; Kusumi, T.; Kashman, Y.; Kakisawa, H. *J. Am. Chem. Soc.* **1991**, *113*, 4092–4096.

(7) Nakanishi, K.; Schooley, D. A.; Kpreeda, M.; Miura, I. *J. Am. Chem. Soc.* **1972**, *94*, 2865–2867.

(8) (a) Deouet, D.; Cauret, L.; Brosse, J. C. *Eur. Polym. J.* **2003**, *39*, 671–686. (b) Kwon, H. C.; Espindola, A. P. D. M.; Park, J.-S.; Prieto-Davo, A.; Rose, M.; Jensen, P. R.; Fenical, W. *J. Nat. Prod.* **2010**, *73*, 2047–2052.

(9) (a) Clutterbuck, P. W.; Percival, W.; Oxford, A. E.; Raistrick, H.; Smith, G. *Biochem. J.* **1932**, *26*, 1441–1458. (b) Canonica, L.; Kroszczynski, W.; Ranzi, B. M.; Rindone, B.; Santaniello, E.; Scolastico, C. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2639–2643. (c) Muth, W. L.; Nash, C. H. III. *Antimicrob. Agents Chemother.* **1975**, *8*, 321–327.

(10) (a) Geris, R.; Simpson, T. J. *Nat. Prod. Rep.* **2009**, *26*, 1063–1094. (b) Colombo, L.; Gennari, C.; Potenza, D.; Scolastico, C.; Aragozzini, F.; Gualandris, R. *J. Chem. Soc., Perkin Trans. 1* **1982**, 365–373.