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Erythrazoles A–B, Cytotoxic Benzothiazoles from a Marine-Derived *Erythrobacter* sp.

Youcai Hu and John B. MacMillan*

Department of Biochemistry, Division of Chemistry, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390, United States

john.macmillan@utsouthwestern.edu

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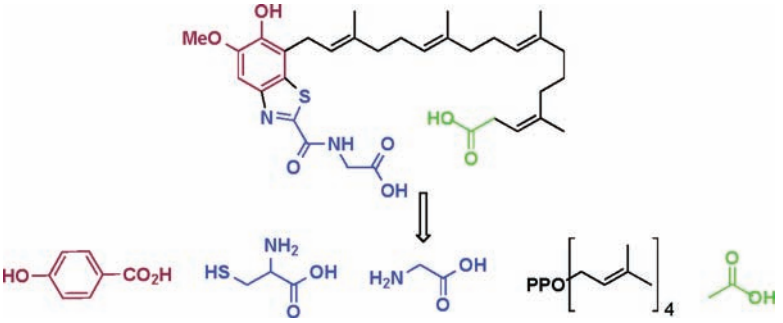
ABSTRACT



Chemical examination of an extract from an *Erythrobacter* sp. isolated from mangrove sediments yielded erythrazoles A (1) and B (2). The erythrazoles are of mixed biosynthetic origin containing a tetrasubstituted benzothiazole, an appended diterpene side chain, and a glycine unit. Erythrazole B is cytotoxic to a panel of non-small cell lung cancer (NSCLC) cell lines, with IC_{50} values of 1.5, 2.5, and 6.8 μM against H1325, H2122, and HCC366, respectively.

Marine bacteria have proven to be a valuable resource of biologically active natural products.[1] A predominant focus of these studies has been on marine-derived actinomycetes, leading to a number of biologically and structurally interesting natural products, such as abyssomycin C,[2] salinosporamide A,[3] marinomycin,[4] and ammosamide A.[5] However other bacteria, such as species of *Bacillus, Pseudomonas*, and *Burkholderia* have proven to be an additional source of biologically and chemically interesting natural products.[6] As part of our efforts to isolate marine-derived bacteria from mangrove sediments, we isolated bacterial strain SNB-035 that by 16S rRNA analysis was identified as closely resembling *Erythrobacter* sp. *Erythrobacter* are Gram-negative bacteria that are known producers of carotenoids, but to our knowledge no other natural products have been reported for this class of bacteria.[7] Our studies on this strain reveal that *Erythrobacter* species are prolific producers of natural products.

Through high-throughput screening efforts, we identified the extract from *Erythrobacter* strain SNB-035 to possess activity in the Locus Derepression Assay (LDR), which identifies molecules that modulate epigenetic regulation, such as DNA methylation and histone acetylation.[8] Ultimately, bioassay guided fractionation led to two other compounds with the ability to modulate epigenetics; however it allowed us to identify erythrazoles A and B (**1**–**2**). Structurally, **1** and **2** possess a benzothiazole moiety, which

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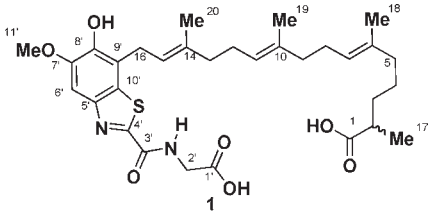
is rare in natural products. Furthermore, **2** arises from four biosynthetic pathways; NRPS, terpene, shikimate, and polyketide. Although combinations of two of these pathways is common, natural prouducts containing all four pathways are seldom observed. Subsequent biological evaluation in a variety of cancer related screening efforts revealed **2** to have low μM cytotoxicity against the non-small cell lung cancer (NSCLC) cell lines H1395, H2122, and HCC366.

Marine bacterium SNB-035 was isolated from a sediment sample collected from Trinity Bay (Galveston, TX) and isolated on an acidified Gauze media. Analysis of the strain by 16S rRNA revealed a 98% identity to *Erythrobacter citreus*. A large-scale fermentation (30 L) by shake fermentation was carried out to obtain sufficient material for full chemical and biological analysis of the new compounds. The excreted metabolites were collected using XAD-7 resin, and the resulting crude extract was purified by a combination of solvent/solvent extraction (*n*-hexane, DCM, ethyl acetate, and methanol/H_2O) and reversed phase chromatography to give fractions enriched in terpenoid metabolites. Final purification by gradient reversed phase HPLC gave erythrazole A (**1**, 1.0 mg) and B (**2**, 0.6 mg).



Erythrazole A (**1**) was isolated as a light yellow glass, $[\alpha]_D$ +3.3 (*c* 0.12 MeOH); UV (MeOH) λ_{max}(log ε) 210 (4.1), 254 (4.1), 332 (3.8). High-resolution ESI-MS (HRESIMS) analysis of **1** gave an $[M + H]^+$ at *m*/*z* 587.2792 consistent with a molecular formula of $C_{31}H_{42}N_2O_7S$ (calcd for $C_{31}H_{43}N_2O_7S$, 587.2791) and 12 degrees of unsaturation. Analysis of the ^{13}C and HSQC NMR spectra of **2** revealed five methyl carbons, nine methylene carbons, five methine carbons, and twelve sp^2 quaternary carbons. A combination of the four methyl singlets from δ_H 1.09 to 1.83 ppm and the number of sp^2 carbons from δ_C 121 to 138 ppm were highly indicative of a terpenoid chain (Table 1). This was confirmed by the analysis of the $^1H-^1H$ COSY and gHMBC spectra of **1** (Figure 1). HMBC correlations from the H17 methyl doublet (δ_H 1.09) to C1 (δ_C 182.8), C2 (δ_C 40.4), and C3 (δ_C 34.2), as well as COSY correlations from H2/H3, H3/H4, and H4/H5 established C1−C5. COSY correlations from H7/H8, H8/H9, H11/H12, and H12/H13 and key HMBC correlations from H18 (δ_H 1.50) to C5 (δ_C 40.3), C6 (δ_C 135.6), and C7 (δ_C 125.5); from H19 (δ_H 1.51) to C9 (δ_C 40.5), C10 (δ_C 135.8), and C11 (δ_C 125.0); and from H20 (δ_H 1.83) to C13 (δ_C 40.7), C14 (δ_C 137.9), and C15 (δ_C 121.7) provided assignment of a fragment terminating in a carboxylic acid. The three double bonds in **1** were all assigned to have the *E* configuration based on the chemical shift of vinyl methyl groups, C18 (δ_C 15.6), C19 (δ_C 16.1), and C20 (δ_C 16.5).[9] The downfield chemical shift of the methylene doublet of H16 (δ_H 3.60) and HMBC correlations to the C14/C15 olefin and to three additional sp^2 carbons indicate the terpene chain is attached to an aromatic ring.

The remaining structural assignment of **1** required significant interpretation of NMR data, as we still had to account for $C_{11}H_9N_2O_5S$ and eight degrees of unsaturation. The UV spectrum of **1** showed a λ_{max} at 254 and 332 nm, which indicated a heteroaromatic fused ring system. Based on the UV, remaining molecular formula, and chemical shift values, we deduced **1** to contain a benzothiazole. As there is only one aromatic proton in **1**, we relied on key HMBC correlations to assign the tetrasubtituted benzothiazole moiety, including from H18 (δ_H 3.60) on the terpene side chain to C8′ (δ_C 145.5), C9′ (δ_C 120.0), and C10′ (δ_C 131.8); from H15 (δ_H 5.24) to C9′; and from H6′ (δ_H 7.47) to C5′ (δ_C 147.4), C7′(δ_C 150.1), C8′, and C10′.

Table 1. 1D and 2D NMR Data of **1** in CD_3OD

no.	δ_H, mult. (*J* in Hz)	δ_C	COSY	HMBC
1		182.8		
2	2.36, dq (7.0)	40.4	3, 17	1, 3, 17
3α	1.55, m	34.2	4, 3*β*	
3*β*	1.32, m		3α	
4	1.36, tt (7.4)	26.4	5, 3α	2, 6
5	1.91, t (7.4)	40.3	4	3, 4
6		135.6		
7	5.00, t (7.4)	125.5	8, 18	
8	1.90, q (7.4)	27.2	7, 9, 18	6, 7, 9, 10
9	1.84, t (7.4)	40.5	8	7, 8, 10, 11, 19
10		135.8		
11	5.02, t (7.0)	125.0	12, 19	12, 13
12	2.09, q (7.0)	26.9	11, 13, 19	10, 11, 13, 14
13	2.03, t (7.0)	40.7	12	11, 12, 14, 15, 20
14		137.9		
15	5.24, t (7.3)	121.7	16, 20	13, 16, 9′
16	3.60, d (7.3)	28.8	13, 15, 20	14, 15, 8′, 9′, 10′
17	1.09, d (7.0)	17.4	2	1, 2, 3
18	1.50, s	15.6	7, 8	5, 6, 7
19	1.51, s	16.1	11, 12	9, 10, 11
20	1.83, s	16.5	14, 15	13, 14, 15
1′		175.2		
2′	4.03, s	43.6		1′, 3′
3′		161.5		
4′		161.2		
5′		147.4		
6′	7.47, s	103.7	11′	5′, 7′, 8′, 10′
7′		150.1		
8′		145.5		
9′		120.0		
10′		131.8		
11′	3.98, s	56.4		7′

The ^{13}C chemical shifts of C7′ and C8′ at δ_C 150.1 and 145.5, respectively, suggested the *ortho*-dioxygen substituted pattern. An HMBC correlation from the methyl singlet H11′ (δ_H 3.98) to C7′ indicated the −OMe was placed at C7′. The ^1H chemical shift of the H11′ protons ruled out the possibility of a thioether or a methylamine at C7′. The presence of an −OH at C8′ was confirmed by treatment of **1** with CH_2N_2 in Et_2O and obtaining a product with three new methyl singlets at 3.60, 3.63, and 3.87 ppm, with the signal at 3.87 representing the newly formed −OMe at C8′.[10] Moreover, utilizing the ^{13}C chemical shifts of C4′(δ_C 161.2), C5′, and C10′, we could assign the regiochemistry of the benzothiazole ring such that the nitrogen atom is attached to C5′, the sulfur atom is attached to C10′, and both the nitrogen and sulfur atoms are attached to C4′. This is based on comparison to literature values of previously reported benzothiazoles with similar substitution patterns (Figure S1).[11] In all examples, the sulfur bearing carbon (C10′) is shifted upfield to around ∼135 ppm, whereas the nitrogen bearing carbon (C5′) is downfield ∼150 ppm. This data match our assignment of the benzothiazole ring. To complete the structural assignment of **1**, we deduced the presence of a terminal glycine based on HMBC correlations from the H2′ methylene pair (δ_H 4.03) to C1′ (δ_C 175.3) and to C3′ (δ_C 161.6). Recording the NMR data of **1** in DMSO-d_6 (Table S2) indicated the presence of an NH proton at δ_H 8.17 (t, J = 4.0 Hz), which showed a COSY correlation with H2′ at δ_H 3.52 (d, J = 4.0 Hz) and a weak HMBC correlation with C4′(δ_C 158.1) on the benzothiazole ring. As a result, the assignment of the entire backbone of **1** was completed. Erythrazole A (**1**) represents the first example of a benzothiazole containing diterpene. As discussed below, benzothiazole containing natural products are quite rare.

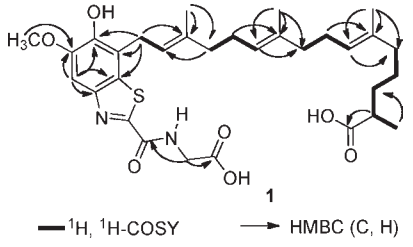


Figure 1. Key correlations for structural assignment of **1**.

To determine the absolute configuration at C2 in **1**, we turned to Kusumi's phenylglycine methyl ester (PGME) method.[12] Derivitization with *S*-**3** or *R*-**3** gave the diamides **4a** and **4b** (Figure 2). To our surprise the ^1H NMR spectrum ($CDCl_3$) of **4a** revealed a 1.0:0.6 ratio of diastereomers (based on integration of the H17 methyl doublet), indicating that erythrazole A is a mixture of enantiomers. The 1.0:0.6 ratio was verified by resolving **1** via chiral HPLC (Chiracel OD). Unfortunately, due to overlapping ^1H NMR signals of the diagnostic protons in **4a** and **4b**, we are unable to assign the absolute configuration with confidence.

Erythrazole B (**2**) was isolated as a light yellow glass, UV (MeOH) λ_{max}(log e) 210 (4.09), 254 (3.99), 334 (3.7). The molecular ions identified in negative ESI-MS at m/z 611 $[M - H]^-$ and positive ESI-MS at m/z 635 $[M + Na]^+$ allowed the deduction of its molecular weight of 612 Da, 26 Da more than **2**. High-resolution ESI-MS gave an $[M + H]^+$ at m/z 613.2951 consistent with a molecular formula of $C_{33}H_{44}N_2O_7S$ (calcd for $C_{33}H_{45}N_2O_7S$, 613.2947) and 13 degrees of unsaturation. Based on this molecular formula and the similarity of the UV, ^1H and ^{13}C NMR of **1** and **2** (Table S1), we could deduce that the benzothiazole moiety was intact and that **2** differed from **1** only in the length and substitution of the terpene side chain. This was confirmed by the analysis of the $^1H-^1H$ COSY and gHMBC spectra of **2** to establish an unusual 22 carbon terpene fragment terminating in a carboxylic acid. A few key HMBC correlations for the terpene portion of **2** were from C1 (δ_C 180.5) to H2 (δ_H 2.95) and from C3 (δ_C 121.2), C4 (δ_C 137.3), and C5 (δ_C 32.4) to the methyl singlet H19 (δ_H 1.69). COSY correlations from H5/H6 and H6/H7 established the modified portion of the terpene chain. The four double bonds in **2** were assigned as 3*Z*, 8*E*, 12*E*, and 16*E*, respectively, based on the chemical shift of the vinyl methyls, C19 (δ_C 23.5), C20 (δ_C 15.9), C21 (δ_C 15.9), and C22 (δ_C 16.5).[9] This is an unusual derivitazation of a terpene. Previous examples of C12 or C7 terpenes (e.g., mycophenolic acid) have been observed, with the unusual chain length arising from an oxidative cleavage of a C15 to a C12 or C7 terpene.[13] Due to the location and geometry of the C3 olefin in **2**, we believe **2** arises from a two-carbon homologation of **1**, presumably via acetate addition and subsequent elimination of H_2O to give the olefin at C3.

Benzothiazoles are a prevalent heterocyclic moiety in pharmaceuticals, such as in the diabetic drug zopolrestat[14] and the fatty acid oxidation inhibitor CVT-3501.[15]

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(10) An alternative structure could have been a benzoxazole ring with a −SH at C8′; however upon treatment with CH_2N_2 we would have formed a S-Me group displaying a methyl singlet around δ_H 2.5 ppm, thus ruling out this possibility.
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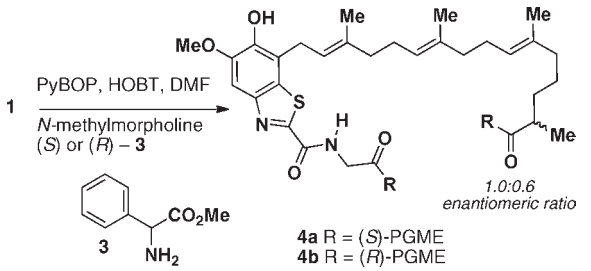

Figure 2. Stereochemistry of C2 in **1**.

However in natural products, benzothiazoles are relatively rare, with only a few examples in the literature, including firefly luciferin, which was isolated in the late 1940s.[16,17] Microbially derived benzothiazole containing natural products include rifamycins P and Q[18] and thiazinotrienomycin F and G.[19] There are few biosynthetic investigations of benzothiazoles, but it has been suggested that the motif arises from condensation of cysteine with a benzoquinone.[20] The presence of high levels of coenzyme Q and other ubiquinone analogs in the fermentation broth would suggest that **1** and **2** are from a shunt pathway of ubiquinone biosynthesis.

A plausible biosynthetic sequence begins with prenylation of 4-hydroxybenzoic acid (**5**) with geranylgeranyl pyrophosphate to generate intermediate **6**, followed by a series of well precedented steps found in ubiquinone biosynthesis (decarboxylation, hydroxylation, methylation, oxidation) to generate quinone **7**. Condensation of cysteine with **7** followed by an oxidative cyclization would provide benzothiazine **8**, followed by a decarboxylation, addition of H_2O, and rearrangement to give the benzothiazole containing aldehyde **9** (Figure 3). Subsequent oxidation of the aldehyde to the carboxylic acid and condensation with glycine would provide the entire benzothiazole moiety **10**. Efforts are currently underway to study the biosynthesis of the erythrazoles using labeled precursors and genome sequencing to elucidate the biosynthetic gene cluster.

Although the erythrazoles were in the active fraction of the LDR assay, when tested as the pure compounds, they showed no activity up to a 20 μM concentration. However, when these molecules were examined for cytotoxic activity against a panel of cancer cell lines, we found that **2** had an IC_{50} of 1.5 μM against the NSCLC cell line H1395, 2.5 μM against H2122, and 6.8 μM against HCC366. **1** had no cytotoxicity up to 20 μM against these three NSCLC cell lines. The difference in bioactivity for **1** and **2** is surprising as they only differ in two carbons. We are further exploring the biological activity of these molecules to understand this difference.

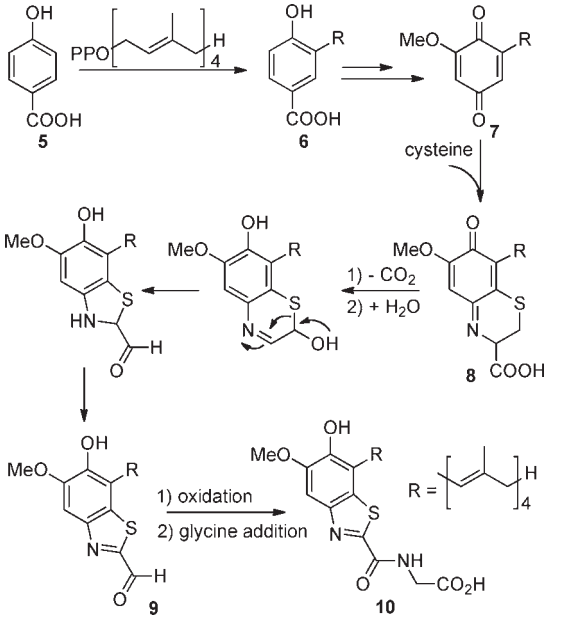

Figure 3. Proposed biosynthetic pathway to benzothiazole moiety.

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Supporting Information Available. General procedures, bioassay protocols, 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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