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Tropolone Ring Construction in the Biosynthesis of Rubrolone B, a Cationic Tropolone Alkaloid from Endophytic *Streptomyces*

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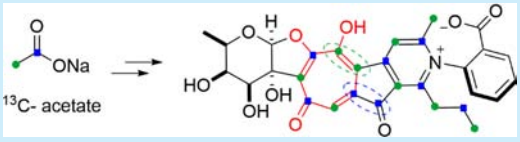
Supporting Information

ABSTRACT: Rubrolones are tropolonoid natural products with a unique carbon skeleton. Extensive secondary metabolite analysis of the endophytic *Streptomyces* sp. KIB-H033 revealed a new class of rubrolone analogue possessing a rare benzoic acid−pyridine inner salt moiety. Precursor feeding with [^{13}C]-acetate revealed a labeling pattern consistent with tropolone moiety construction via type-II PKS chemistry followed by complex oxidative rearrangements. This bacterial biosynthetic route represents a surprising departure from fungal tropolone assembly during stipitatic acid biosynthesis.



Tropolonoids are an important class of aromatic natural products possessing a seven-membered aromatic tropolone ring.[1] Since the isolation of puberulonic acid in 1932,[2,3] an additional 200 natural tropolones have been isolated from plants, fungi, and to a lesser extent, bacteria.[1] Many compounds in this class have potent antibacterial, antifungal, antiplasmodial, and antitumor activities,[1] and the mechanism of tropolone biosynthesis has intrigued researchers for decades. This mystery was finally solved in 2012 by demonstrating that the fungal tropolone stipitatic acid is assembled by a nonreducing polyketide synthase (NR-PKS) possessing a C-methyltransferase domain and that oxidative tailoring facilitates a pinacol rearrangement to afford the seven-membered tropolone ring.[4,5] However, the mechanism of tropolone formation in prokaryotes remains unknown.

In our ongoing efforts to discover new microbial natural products, we have been chemically and biologically screening extract libraries from endophytes of traditional Chinese medicinal (TCM) plants obtained from un- and underexplored ecological niches.[6,7] These screens revealed a purple red pigment producer identified as *Streptomyces* sp. KIB-H033, an endophyte isolated from the TCM plant *Camellia sinensis* that shows 99.0% 16S rRNA gene sequence identity to *Streptomyces echinoruber* strain X-14077. In this paper, we describe the isolation of a new compound rubrolone B (**1**), which is a cationic tropolone alkaloid that differs from the known rubrolone A (**2**),[8,9] by an additional benzoic acid on the pyridine ring to furnish a unique cationic moiety rarely found in natural products (Figure 1).[10]

Rubrolone natural tropolonoids were first isolated from *S. enchinoruber* in 1978[8,9] and comprise a unique carbon skeleton possessing a 2,3,4,6-tetrasubstituted pyridine ring, a cyclopentanone, a deoxysugar, and a tropolone. Although synthetic chemists have devised two total synthetic routes to the aglycon,[11,12] the total chemical synthesis and biosynthetic origin of rubrolone remain unreported. Herein, we describe the isolation and structure elucidation of rubrolone B (**1**) from *Streptomyces* sp. strain KIB-H033 and identify the cardioprotective activity of this compound. Surprisingly, ^{13}C-acetate labeling reveals a new mechanism of tropolone biosynthesis that diverges significantly from the ring-expansion mechanism established for stipitatic acid in fungi.

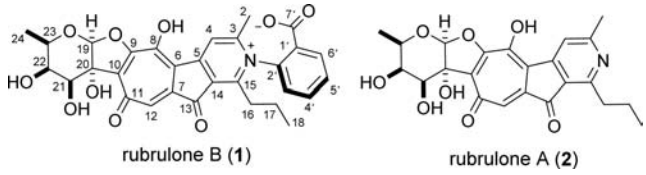


Figure 1. Chemical structures of rubrolones A (**2**) and B (**1**).

Compounds **1** and **2** were isolated and structurally elucidated from fermentation of the endophytic strain *Streptomyces* sp. KIB-H033. Compound **1** was obtained as a purple red amorphous solid with a molecular formula of $C_{30}H_{27}NO_{10}$ according to its HRESIMS peak at m/z 562.1711 $[M + H]^+$ (calcd for $C_{30}H_{28}NO_{10}$, 562.1713). The ^{13}C NMR in combination with analysis of the DEPT and HSQC spectra differentiated 30 carbon atoms into three carbonyl groups, 11 nonprotonated olefinic carbons, six protonated aromatic carbons, one acetal carbon, three oxygenated aliphatic methines, one oxygenated quaternary carbon, two methylene groups, and three methyl groups (Table S1). Furthermore, the ^{1}H NMR spectrum perfectly matched the above ^{13}C NMR data (Table S1), and a six-ring structure was required for **1** to fulfill

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the unsaturation requirement. The existence of one aromatic spin-system, −CHCHCHCH− (C3′/C4′/C5′/C6′), was suggested by the COSY and HSQC signals at δ_H 8.23 (dd, J = 7.8, 1.2 Hz, 1H, H-6′), 7.94 (td, J = 7.7, 1.2 Hz, 1H, H-4′), 7.84 (td, J = 7.7, 1.2 Hz, 1H, H-5′), and 7.80 (brd, J = 7.7 Hz, 1H, H-3′), together with their olefinic carbons at δ_C 134.33 (C-4′, d), 132.37 (C-6′, d), 131.67 (C-5′, d), 129.26 (C-3′, d). Taking the other three quaternary carbons at δ_C 165.09 (C-7′, s), 136.46 (C-2′, s), 128.13 (C-1′, s) into consideration, the HMBC spectrum supported the assignment of an ortho-substituted benzoate unit (Figure 2) by the correlations from

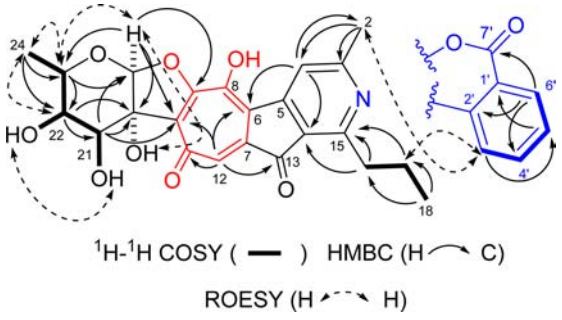


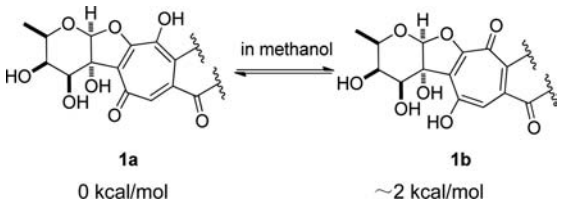
Figure 2. Key COSY, HMBC, and ROESY correlations for **1** and the deduced benzoate moiety.

H-6′ to C-2′, C-4′ and C-7′; H-4′ to C-2′ and C-6′; H-5′ to C-1′ and C-3′; H-3′ to C-1′ and C-5′. The remaining NMR signals were similar to those of **2**, as further COSY and HMBC 2D NMR data analysis of **1** (Figure 2) verified that they were structurally similar. The only difference was the presence of an additional benzoate moiety in **1**. Detailed comparison of NMR shifts demonstrated that the tropolone unit and deoxysugar in **1** were almost identical to **2**, thus excluding the possibility that the benzoate was attached to any of them through either C−O or C−C bonds. Bridging of the benzoate moiety and pyridine ring via a C−N bond was also supported by ROESY correlations from H-3′ to H_3-2 and H_2-17 and by the perturbed NMR chemical shift of the propyl side chains and Me-2 in **1** compared to compound **2** (Table S1).

We next sought to determine the absolute configuration of **1**. The contiguous structural moieties of **1**, including the stereogenic centers in the deoxysugar, were also found in **2**, which possessed physicochemical properties identical to those reported in the literature.[8,9] We therefore assigned the same absolute configurations to the deoxysugar moieties in **1** and **2**, which is consistent with an assumed common biosynthetic origin. However, we still could not conclusively assign the absolute stereochemistry for the iminium−aryl axis using NMR data or other empirical evidence.

To resolve the absolute axial configuration of **1**, we turned to quantum chemical calculations, which require accurate conformation and geometry for meaningful prediction of CD spectra. For instance, tropolones may rapidly interconvert between two keto−enol tautomers in solution.[13] Though rubrolone A (**2**) is more stable in DMSO than methanol, the ^{13}C NMR peaks are irregular (Figure S15) compared to the much clearer peaks of rubrolone B (**1**) in DMSO, presumably due to the stabilizing effect of the internal salt (Figure S5). However, the strong UV absorption of DMSO required CD spectral acquisition in methanol, which resulted in a rapid keto−enol tautomerization between **1a** and **1b** (Scheme 1)

Scheme 1. Energy of Keto−Enol Tautomer 1b Relative to Tautomer 1a Calculated at the B3LYP/6-31G(d,p) Level in Methanol



evidenced by dramatic solvent-dependent color changes (Figure S2). To inform CD spectral interpretation, we applied quantum chemical calculations to estimate a small single point energy difference of ~2 kcal/mol between **1a** and **1b** (Scheme 1 and Figure S1), which suggests a ratio near unity in methanol.

In addition, two atropisomers of each keto−enol tautomer were considered for quantum chemical CD calculations of **1** in methanol. A weighted summation of TDDFT calculations (B3LYP/6-31G(d,p)) yielded predicted CD spectra for (*M*)-**1a**, (*M*)-**1b**, (*P*)-**1a**, and (*P*)-**1b** (Figure 3) according to

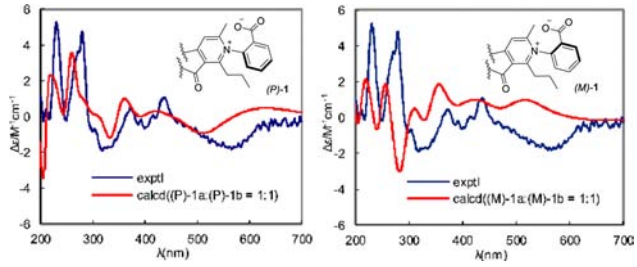


Figure 3. Assignment of the absolute axial configuration of rubrolone B (**1**) by comparing its experimental CD spectrum with the spectra calculated for *P* (left) and *M* (right) atropisomers.

Boltzmann statistics.[14,15] The overall spectra obtained were UV corrected and compared with the measured CD spectrum of **1** in methanol. The experimental spectrum was more consistent with the spectrum calculated for the (*P*)-atropo-diastereomer (Figure 3, left) over the wavelength range examined. The absolute configuration of the iminium−aryl axis in **1** was therefore assigned as the *P* atropisomer. In summary, the new cationic tropolone alkaloid was named rubrolone B with the full absolute stereochemistry depicted.

The tropolone carbon skeleton in stipitatic acid has been shown to originate from acetate and *S*-adenosyl methionine (Scheme 3A),[4] suggesting a common biosynthetic origin with the structurally similar rubrolone aglycon involving a polyketide synthase (PKS) and C-methyltransferase. We therefore fed ^{13}C-labeled acetate and [methyl-^{13}C]-L-methionine to the fermentation media at 24 h (the time point at which **1** could first be detected) and 48 h after inoculation and then isolated and analyzed **1** by ^{13}C NMR to determine the extent and positions of ^{13}C incorporation. Addition of 250 mg L^{-1} of sodium [1-^{13}C] acetate to cultures of *Streptomyces* sp. KIB-H033 yielded ^{13}C enrichments at C-3, C-5, C-7, C-9, C-11, C-13, C-15, and C-17 (Figure 4A), whereas C-2, C-4, C-6, C-8, C-10, C-12, C-14, C-16, and C-18 were enriched (Figure 4B) when sodium [2-^{13}C] acetate was added. No obvious enrichment was observed from C-19 to C-24 (sugar moiety) or from C-1′ to C-7′ (benzoic acid moiety). Significantly, isotope incorporation was not observed when [methyl-^{13}C]-L-methionine was added

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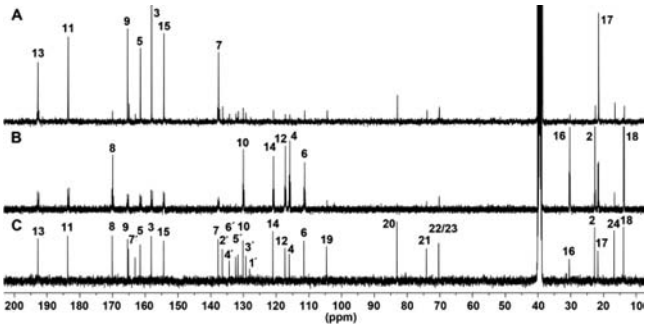
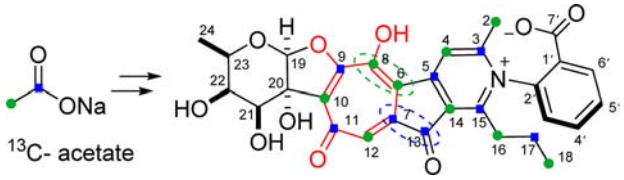


Figure 4. ^{13}C NMR spectra of rubrolone B (**1**) obtained after: (A) feeding with [1-^{13}C] acetate (enhanced signals are numbered); (B) feeding with [2-^{13}C] acetate (enhanced signals are numbered); (C) no feeding with any ^{13}C labeled precursor (all carbon signals are numbered).

to the culture medium at 300 mg L^{-1}. A final feeding experiment with [1,2-^{13}C] acetate and 2D $^{13}C-^{13}C$ COSY analysis determined the distribution of acetate units and confirmed carbon signal assignments (Figure S13). Overall, the labeling experiments reveal a polyketide origin for the tropolone core and fused heterocyclic aglycon, and the absence of methyltransferase involvement implies a distinct mechanism of tropolone formation in bacteria compared to fungi.

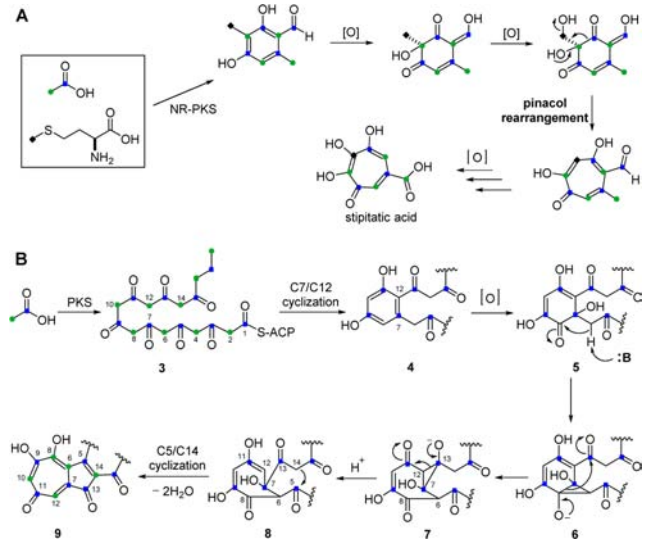
The incorporation of acetate units into an aromatic structure suggests carbon chain assembly by a possible type-II PKS involving a butyryl-ACP starter unit[16−18] followed by seven malonyl-CoA chain extensions. Interestingly, the head-to-tail incorporation of intact acetate units expected from PKS chemistry was not completely observed in the labeling pattern of the rubrolone B aglycon (Scheme 2). Instead, [1-^{13}C] acetate

Scheme 2. Summary of ^{13}C-Acetate Incorporation into Rubrolone B (1), Highlighting the Unusual Labeling Pattern of the Tropolone Ring (in Red)



generated consecutive ^{13}C incorporations between C-7 and C-13 and [2-^{13}C] acetate yielded incorporations between C-6 and C-8. These findings indicate unusual tricyclic aromatic ring construction with complex oxidative rearrangements occurring after the typical C-7/C-12 cyclization of the poly-β-ketoacyl chain intermediate **3** (Scheme 3B).[19−21] The [2-^{13}C] acetate enriched C-8 in the tropolone ring of **1** is oxidized, suggesting the action of oxygenases during tropolone ring formation. Specifically, we propose that the ACP-bound intermediate **4** undergoes dearomatization by oxidation at C-7 and C-8 to generate **5**. The cyclopropane-containing intermediate **6** would then be generated by C-6 and C-8 cyclization (Scheme 3B), chemical precedent for which has been established from tropolone synthetic studies.[22,23] Rearrangement of **6** to another cyclopropane intermediate **7** would be followed by cleavage of the cyclopropane ring between C-12 and C-13. Subsequent C5/C14 cyclization and dehydrations would afford the unique tropolone ring in a manner consistent with the observed labeling pattern.

Scheme 3. (A) Mechanism of Tropolone Ring Formation in Fungi Stipitatic Acid. (B) Proposed Mechanism of Tropolone Ring Formation in Rubrolones



Both **1** and **2** were tested for cytotoxic activity against three selected human cancer cell lines (A-549, HL60, and MCF-7) and normal cardiomyocytes. These compounds exhibited no cytotoxic activity at 100 μM but at 10 μM significantly increased the viability of neonatal rat cardiomyocytes exposed to H_2O_2-induced injury (Table S2), suggesting potential cardioprotection.[24−26]

In summary, our discovery of compound **1** has expanded the novel aromatic tropolone skeleton of the rubrolones to include a unique inner salt. Specifically, ortho arylation of a pyridine ring with benzoic acid yields a pyridine inner salt rarely observed in natural products. Furthermore, in contrast to the majority of endophyte natural product research involving fungi,[27,28] these apparently cardioprotective agents are produced by an endophytic actinomycete, *Streptomyces* sp. KIB-H033, highlighting the potential of neglected endophytic actinomycetes to provide novel bioactive natural products. Interestingly, the internal salt in compound **1** minimizes tautomerization and therefore simplifies NMR spectra, which facilitated critical isotopic labeling experiments. The labeling pattern observed from ^{13}C labeled acetate-feeding studies was consistent with type-II PKS-catalyzed assembly of the tropolone aglycon with a butyryl starter unit. Surprisingly, the labeling pattern also revealed a bacterial mechanism of tropolone ring formation that diverges significantly from the mechanism employed by fungi during stipitatic acid biosynthesis (Scheme 3), highlighting the elegance and versatility of natural product biosynthetic pathways. To the best of our knowledge, this is the first indication that a type-II PKS may be responsible for the synthesis of the tropolone aromatic ring system, and this report sets the stage for elucidating rubrolone biosynthetic pathways.

■ ASSOCIATED CONTENT

Supporting Information

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Experimental details, HRMS and 1D and 2D NMR spectra, and related computational data (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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