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Erythronolides H and I, New Erythromycin Congeners from a New Halophilic Actinomycete *Actinopolyspora* sp. YIM90600

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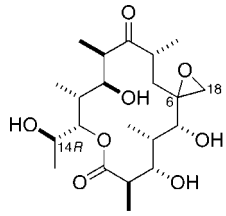
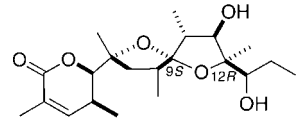
ABSTRACT



Erythronolide H (**2**)



Erythronolide I (**3**)

Erythronolides H and I, novel congeners of the clinically important antibacterial drug erythromycin A, have been isolated from the new halophilic actinomycete *Actinopolyspora* sp. YIM90600. In addition to producing the new erythromycin congeners, *A.* sp. YIM90600 produces erythromycin C in a high titer. The presence of the C-14 hydroxyl moiety and the C-6/C-18-epoxide in erythronolide H and the spiroketal moiety of erythronolide I sheds new insights into structural diversity of erythromycin analog libraries potentially accessible by combinatorial biosynthesis.

Erythromycin A (**1**) and related analogues have been clinically used for more than 50 years and are considered first line therapeutics for the treatment of upper and lower respiratory tract infections. Their antibacterial activity is attributed to inhibition of protein synthesis resulting from binding to the peptidyltransferase site of the bacterial 50S ribosomal subunit.[1] A significant problem with the first-generation macrolide erythromycin A is its facile decomposition under acidic conditions resulting in loss of activity and undesirable gastrointestinal side effects.[2] Efforts to diversify the erythromycin scaffold in order to minimize side effects and in vivo degradation have constituted a major area of research resulting in second generation macrolides such as clarithromycin,[3] azithromycin,[4] and roxithromycin.[5] The development of resistance to second generation macrolides, most notably in the form of ribosomal mutation (erm) or by efflux (mef) mechanisms, has hastened the discovery and development of a third generation of macrolide antibiotics

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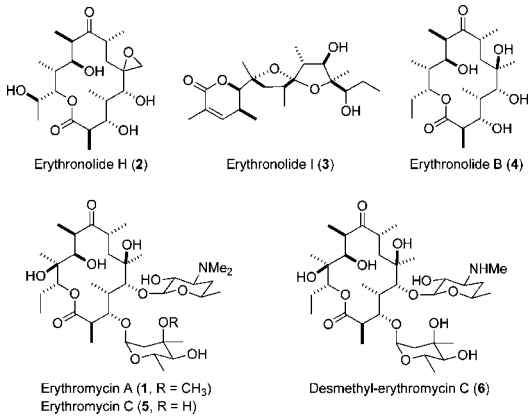
including cethromycin (ABT-773),[6] EP-420,[7] and BAL-19403;[8] these agents constitute recent additions to the repertoire of clinical antibacterial therapeutics. In addition to their use as antibacterial therapeutics, erythromycin derivatives have recently been reported to inhibit HIV-1 replication in macrophages through modulation of MAP kinase activity.[9]

Actinopolyspora sp. YIM90600, obtained from a dried salt lake in Xingjiang province, China, requires 8−30% salt for growth and shows little 16S rRNA gene sequence identity relative to its nearest halophilic neighbor species, *Actinopolyspora halophila*, *Actinopolyspora mortivallis*, and *Actinopolyspora iraqiensi*.[10] Thus, *A.* sp. YIM90600 likely represents a new *Actinopolyspora* species. Moreover, *A.* sp. YIM90600, the culture extracts of which displayed significant antibiotic activity and moderate cytotoxicity, may represent the first strain belonging to the genus *Actinopolyspora* to be examined for its ability to produce bioactive natural products. Here we report the structures of erythronolides H (**2**) and I (**3**), two novel erythromycin congeners, along with known erythromycin biosynthetic intermediates erythronolide B (**4**), erythromycin C (**5**), and demethyl-erythromycin C (**6**).



The *A.* sp. YIM90600 strain was cultivated in a NaCl-based medium for 28 days with vigorous shaking. Solid phase extraction of the broth using HP-20 resin, filtration through cheesecloth, and compound elution from the resin with acetone afforded, after solvent removal under vacuum, a gummy extract that was subjected to fractionation on silica gel. Multiple rounds of silica gel chromatography and C18 reverse phase HPLC afforded compounds **2**−**6**. Structure elucidation of **2** was straightforward due to its structural similarity to known compounds **4**−**6**. High resolution ESI-MS (HRESIMS) analysis of **2** gave an $[M + Na]^+$ ion at *m*/*z* 439.2292 consistent with a molecular formula of $C_{21}H_{36}O_8$ (calcd for $C_{21}H_{36}O_8Na$, 439.2302). Acquisition and analysis of NMR data for **2** (see Supporting Information) and comparison to data acquired for **4**−**6** suggested compound **2** to be a 14-membered macrolactone possessing an erythromycin-like core scaffold. This assignment was confirmed by high resolution 2D NMR spectroscopy. The regiochemistry of hydroxylation was independently assigned on the basis of HMQC and HMBC data (Figure 1). A

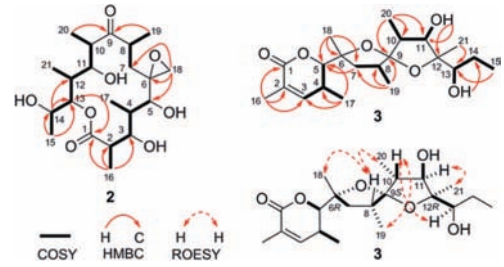


Figure 1. Key COSY and HMBC correlations for **2** and **3** as well as key ROESY correlations for **3**.

spirocyclic epoxide at the C-6 was evident for **2** based on the observation of signals in the NMR spectrum [δ_C 58.8 (s) and δ_C 46.9 (t), δ_H 2.76 and 2.27 (each 1H, d, $J = 7.0$ Hz)], as well as, HMBC correlations of H_2-18 with C-5, C-6, and C-7; these data however fall short of assigning the stereochemistry at C-6. The rest of the stereochemistry of **2** was deduced by considering its biosynthetic origin and comparative analysis of NMR data relative to **4**−**6**. Although the relative configuration of **2** at C-14 could not be determined from spectroscopic data alone, the almost identical coupling constants between H-13 and H-14, as well as, similarities of other NMR data with those of (14*R*)-14-hydroxy TB-010 and (14*R*)-14-hydroxy TE-031 allowed us to assign an *R* configuration to C-14 of **2**.[11]

The molecular formula of $C_{21}H_{34}O_6$, determined by HRESIMS for the $[M + Na]^+$ ion at *m*/*z* 405.2244 (calcd for $C_{21}H_{34}O_6Na$, 405.2244) and NMR data, indicated that **3** is also a C-21 polyketide having five degrees of unsaturation. The ^1H, ^{13}C, and HSQC NMR spectral data (see Supporting Information) were consistent with the presence of one carbonyl group (δ_C 166.7), one trisubstituted double bond (δ_C 146.4, d; δ_C 126.8, s), three oxygenated tertiary carbons

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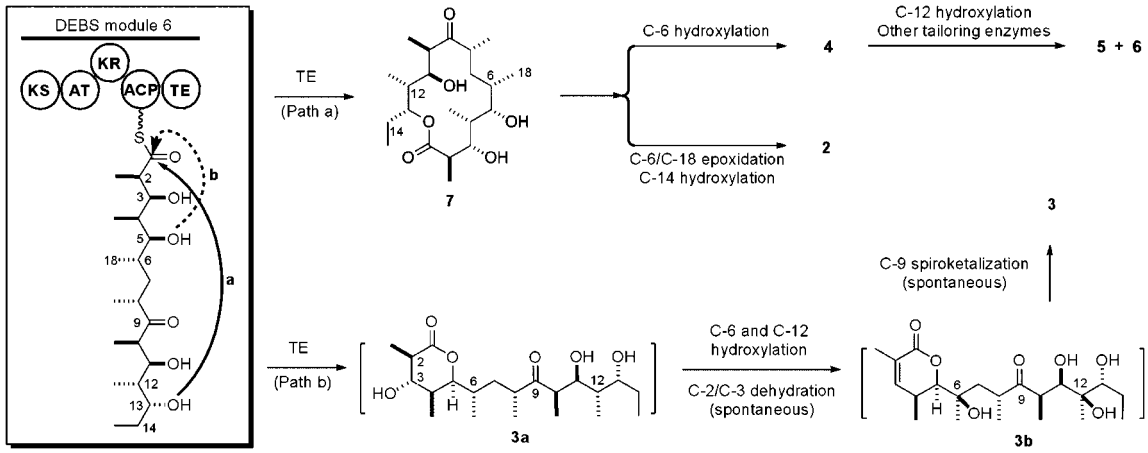
Scheme 1. Proposed Biosynthesis of **2**−**6** in *A.* sp. YIM90600 Modeled on Erythromycin Biosynthesis in *Sacc. erythraea*[12a]



[a] TE-catalyzed release of the full length polyketide intermediate from DEBS module 6 via cyclization paths **a** and **b** affords macrolide **7** and lactone **3a**; both serve as substrates for multiple oxygenases en route to **2**−**6**. ACP, acyl carrier protein; AT, acyl transferase; DEBS, deoxyerythronolide B synthase; KR, ketoreductase; KS, ketosynthase; TE, thioesterase.

(δ_C 82.1, 82.3, 113.8), six methines, three of which are oxygenated (δ_C 84.8, 86.4, 77.3), two methylene moieties (δ_C 40.0, 23.6), and seven methyl groups (δ_C 11.5, 17.2, 13.7, 24.0, 13.0, 11.5, 21.9). Two resonances in the ^{1}H NMR spectrum (δ_H 3.05, d, J = 5.5 Hz, OH-11; δ_H 2.98, br, OH-13) lacking any carbon HSQC correlations were assigned to the two OH groups accounting for all 34 protons consistent with the molecular formula. The presence of two hydroxyl groups supported by this data is consistent with three heterocyclic moieties accounting for the degree of unsaturation displayed by **3**.

Consistent with the UV−vis data, **3** possesses a six-membered α,β-unsaturated lactone, as assigned on the basis of ^{1}H−^{1}H COSY correlations involving H-3/H-4 and H-4/H-5,H$_3$-17, and long-range ^{1}H−^{13}C HMBC correlations from the vinylic methyl group (H$_3$-16, δ_H 1.90) to C-1 (δ_C 166.7), C-2, and C-3 (δ_C 146.4) and from the secondary methyl group (H$_3$-17, δ_H 1.07) to C-3, C-4 (δ_C 31.1), and C-5 (δ_C 84.8). Further analysis of the ^{1}H−^{1}H COSY data revealed carbon connectivities of C-7/C-8/C-19, C-20/C-10/C-11, and C-13/C-14/C-15. Strong HMBC correlations of methyl signals of H$_3$-18, H$_3$-19, H$_3$-20, and H$_3$-21 with corresponding carbons support the assigned fragment connectivity deduced from COSY spectrum via three oxygenated tertiary carbons C-6, C-9, and C-12, respectively. Analysis of COSY correlations of OH-11 with H-11 and of OH-13 with H-13 as well as HMBC cross peaks of OH-11 with C-11 and C-12, and of OH-13 with C-14 confirmed the location of the two hydroxyl groups (Figure 1). These findings are consistent with the C-9 spiroketal (δ_C 113.8, s), which, along with the lactone moiety, satisfy the degree of unsaturation originally determined on the basis of initial MS and 1D NMR data.

ROESY spectra were used to establish the relative stereochemistry of **3**. Although we have not yet determined its absolute stereochemistry, we envision **3** to possess the same stereochemistry as **1** on the basis of its biosynthetic origin (Scheme 1). Thus, as shown in Figure 1, the *S* configuration was assigned to the spiroketal carbon C-9, consistent with ROESY correlations of H$_3$-19 with H-10, and of H$_3$-20 with H-8. Similarly, ROESY cross-peaks of H$_3$-18 with H-8 and of H$_3$-21 with H-10 confirmed the *R* configuration at both C-6 and C-12 in **3**. The configurations of all other asymmetric centers in **3** appear to be identical to those of the corresponding centers in **4**, **5**, and **6**, as would be expected from their common biosynthetic origin (Scheme 1).

Erythromycin biosynthesis has been extensively investigated in *Saccharopolyspora erythraea*, featuring the deoxyerythronolide B synthase (DEBS)-catalyzed biosynthesis of 6-deoxyerythronolide B (**7**) from the acyl CoA precursors and subsequent C-6 and C-12 hydroxylations as well as other tailoring steps, converting **7** into **6**, **5**, and **1**.[12] Modeled on the established paradigm for erythromycin biosynthesis, both **2** and **3** could therefore be viewed as shunt metabolites of **5** and **6** in *A.* sp. YIM90600. Thus, as depicted in Scheme 1, DEBS-catalyzed synthesis is envisioned to afford the full length polyketide intemediate as an acyl-*S*-ester that is covalently linked to the acyl carrier protein domain of DEBS module 6. However, in contrast to erythromycin biosynthesis in *Sacc. erythraea*, release of the polyketide intermediate from DEBS in *A.* sp. YIM90600 is envisioned to proceed in two paths: (i) macrolactonization by the C-13 hydroxyl group affording **7** (path a) as has been demonstrated previously in *Sacc. erythraea* and (ii) nucleophilic attack by the C-5 hydroxyl group affording lactone **3a** (path b), reminiscent of an engineered DEBS.[13]

C-6 and C-12 hydroxylations of **7** are catalyzed by two P-450 enzymes EryF and EryK, respectively, followed by

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other tailoring steps to afford **6**, **5**, and **1** in *Sacc. erythraea*.[12] Homologues of EryF and EryK in *A.* sp. YIM90600 presumably are responsible for the formation of **4**, **5**, and **6**. Since **1** was not detected under any of the conditions examined, the homologue of EryG, an *O*-methyltransferase that catalyzes the conversion of **5** to **1** in *Sacc. erythraea*, is most likely absent in *A.* sp. YIM90600. Alternatively, C-6/C-18 epoxidation and C-14 hydroxylation of **7** could afford **2**. While such activities are not known for erythromycin biosynthesis in *Sacc. erythraea*, an analogous epoxidation, catalyzed by OleP, has been proposed for oleandomycin biosynthesis in *Streptomyces antibioticus*;[14] C-14 hydroxylation of erythromycins has also been reported from biotransformation and metabolism studies.[11]

The β-hydroxy lactone **3a** produced in path b likely undergoes spontaneous C-2/C-3 dehydration to produce tetraol **3b** although it is not clear if this occurs before or after C-6 and C-12 hydroxylations. Following installation of the C-6 and C-12 hydroxyl moieties, **3b** likely undergoes spontaneous spiroketalization at C-9 to render **3** in a fashion analogous to the known acid-catalyzed inactivation of **1**.[15] C-6 and C-12 hydroxylations in both paths a and b are most likely catalyzed by the same enzymes, likely homologues of the EryF and EryK P-450s.[12] It is particularly intriguing that the enzyme specific for C-6 hydroxylation appears to recognize both the 14-membered macrolide such as **7** and the linear polyketide such as **3a** as substrates but the enzyme specific for C-12 hydroxylation, while acting on **7** and **3a** as supported by the isolation of **3**−**6**, does not oxidize C-12 during biosynthesis of **2**.

The discovery of **2** and **3** provides new insights into erythromycin biosynthesis. First, a homologous TE domain from *A.* sp. YIM90600 presumably is responsible for cyclizations resulting in both 14- or 6-membered lactones. This finding supports earlier studies with the DEBS TE domain from *Sacc. erythraea*; these features have been previously demonstrated by engineered DEBSs[13] but are now reconfirmed by the natural erythromycin biosynthetic machinery in *A.* sp. YIM90600. Second, EryF and EryK for erythromycin biosynthesis in *Sacc. erythraea* are generally considered to act preferentially on macrolide substrates.[12] The isolation of **3** suggests that homologues of these enzymes can act equally well on linear substrates such as **3a**. Contrasting this apparent substrate promiscuity is that **2** lacks C-12 hydroxylation, indicating that the EryK homologue may require the C-6 hydroxyl group for substrate recognition. Third, C-14 oxidation in **2** is likely carried out by an adventitious enzyme[11] in *A.* sp YIM90600 as there are no known C-14 oxygenases within the erythromycin biosynthetic gene cluster in *Sacc. erythraea*.[12] Alternately, the EryK homologue present in this new halophilic *A.* sp. YIM90600 strain may simply process the C-6/C-18 epoxide variant of **7** in a way that leads to hydroxylation at C-14 instead of C-12. Such a scenario is reminiscent of the PicK P-450 enzyme that is known to be involved in the production of four antibiotics in *Streptomyces venzuelae* by hydroxylating the C-10 and/or C-12 positions of 12- and 14-membered macrolides.[16]

Perhaps the most interesting insight into erythromycin biosynthesis by *A.* sp. YIM90600 is gleaned by considering the C-6/C-18 epoxide of **2**, which is without precedent among publicly disclosed erythromycin analogues. The presence of this epoxide raises the possibility that it may result from the same P-450 enzymes responsible for C-6 hydroxylation in **4**−**6**, presumably an EryF homologue although EryF is not yet known to be bifunctional in this manner. Oxygenases with such dual activities are exceedingly rare,[17] and OleP is the only P-450 enzyme to our knowledge that has been implicated to possess such dual activities, catalyzing both hydroxylation at C-8 and epoxidation at C-8/C-8a in oleandomycin biosynthesis.[14]

Erythromycin A is readily inactivated via acid catalyzed conversion to its enol ether and 10,11-anhydrospiroketal forms.[15] Semisynthetic approaches to circumvent this problem have been described but remain challenging due to the complexity of the erythromycin scaffold.[18] Compound **2** therefore represents a novel aglycone[12] for chemical and/or enzymatic glycosylation approaches[19] to new erythromycin congeners with enhanced stability and/or altered modes of action. The C-14 hydroxyl and C-6/C-18 epoxide both represent new opportunities for macrolide modifications and new analog design to further expand the size and diversity of erythromycin analog libraries.

Finally, *A.* sp. YIM90600 strain produces large quantities (300 mg/L) of **5** even under suboptimal fermentation conditions. The high titer of **5** suggests that *A.* sp. YIM90600 may prove to be a superior erythromycin producer following further medium optimization and engineering efforts and thus, may satisfy erythromycin production/engineering needs not currently met by more well-established producers.

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Supporting Information Available: Detailed experimental procedures, NMR spectra (1D and 2D) for compounds **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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