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# Isolation and Characterization of 7-Hydroxy-6-demethyl-6-deoxy-erythromycin D, a New Erythromycin Analogue, from Engineered *Saccharopolyspora erythraea*

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Erythromycin biosynthesis is mediated by a modular polyketide synthase (PKS).[1,2] Such PKSs feature a separate enzyme active site, as part of discrete protein domains for each biosynthetic step catalyzed, making them ideal targets for directed biosynthesis. Alteration or replacement of individual domains or modules has allowed many new polyketides to be generated with modifications at positions not readily accessible by synthetic organic chemistry.[3,4]

As a starting material for the synthesis of new erythromycin analogues, we set out to generate 6-demethyl-erythromycins by directed biosynthesis. The 6-methyl group of erythromycin is derived from incorporation of a methylmalonyl unit by the acyltransferase in module 4 (AT4) of the 6-deoxyerythronolide B synthase (DEBS) PKS.[5] Recently, REEVES and co-workers[6] successfully altered the substrate specificity of AT4 in DEBS through site-specific mutagenesis to accept malonyl-CoA or methylmalonyl-CoA. The modified DEBS genes were expressed in the heterologous host *Streptomyces lividans*, and consequently, the aglycone 6-demethyl-6-deoxyerythronolide B was produced. RODRIGUEZ *et al.*[7] described a novel gene delivery and expression system that allows the rapid engineering of recombinant PKSs in an erythromycin overproducing strain of *Saccharopolyspora erythraea* K24-1. Here, we report the use of that gene delivery system to introduce the DEBS genes harboring a mutagenized AT4 domain into *S. erythraea* K24-1 to produce 6-demethyl erythromycins. Compound **1** (7-hydroxy-6-demethyl-6-deoxy erythromycin D) was produced, which is demethylated at the 6-position as predicted, but unexpectedly has a hydroxyl group at the 7-position rather than at the 6-position.

In order to express the AT4-modified DEBS in *S. erythraea* K24-1, the genes encoding the engineered PKS (mutation 3 in reference 6) were transferred from the *S. lividans* expression vector to the pSET152 integrating vector under control of the native *eryAp* promoter.[7] The resulting vector pKOS159-42 was introduced into *S. erythraea* K24-1 by conjugation and the culture broths of the antibiotic-producing isolates were screened by LC/MS for the presence of compounds with mass spectra consistent with 6-demethyl-erythromycin analogues. One isolate was selected for scale-up in 10-liter bioreactors, which produced high levels of a compound with *m/z* 690 consistent with the mass of 6-demethyl-erythromycin D.

Each production bioreactor containing 9 liters of production medium was inoculated with 500 ml of an actively growing culture of *S. erythrea* K24-1::pKOS159-42. The bioreactors were operated at 34°C, pH 7.0±0.15, 3 liters per minute (LPM) airflow, and 600 rpm agitation. The dissolved oxygen was maintained above 40% of air saturation. A dextrin suspension (150 g/liter) was fed at a rate of 5.4 ml/hour throughout the cultivation. The culture broth's pH was adjusted to 8 before harvesting by centrifugation on day 6 after inoculation.

The centrifuged broth from two 10-liter bioreactors was passed over a solid-phase extraction column and eluted with methanol. LC/MS analysis of the eluate indicated at least three compounds which produced prominent ions of *m/z* 690. Two of these compounds were present in quantities too low for isolation; the remaining compound (**1**) was isolated through a combination of solid-phase extraction, liquid-liquid extraction, and reversed-phase column chromatography.

Compound **1** has a molecular formula of $C_{35}H_{63}NO_{12}$, as determined by the $^{13}C$ NMR spectrum and high-resolution mass spectral data. Carbon–hydrogen connectivities were established from the multiplicity-edited HSQC spectrum, while gsCOSY and constant time HMBC data allowed tracing of the carbon-oxygen skeleton. The NMR data were consistent with a structure similar to the expected 6-demethyl-erythromycin D, but having a hydroxyl group at the 7-position rather than the 6-position. In particular, the COSY spectrum indicated coupling between H-8 ($\delta$ 2.51) and H-7 ($\delta$ 4.41), between H-7 and H-6a ($\delta$ 2.13), and between H-6a and H-5 ($\delta$ 3.78). Further evidence for the placement of a hydroxyl group at position 7, rather than at position 6, came from HMBC correlations from H-7 to the

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Table 1. The $^{13}C$ and $^{1}H$ NMR data for **1** ($CDCl_3$).

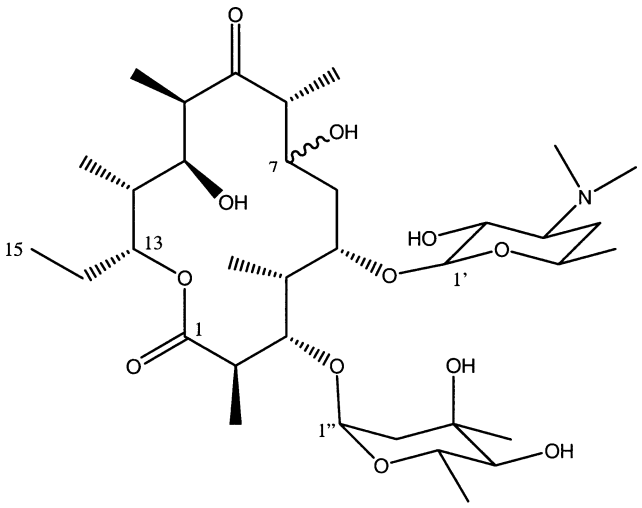
| position | $^{13}C$ | $^{1}H$ |
|---|---|---|
| 1 | 178.1 | -- |
| 2 | 45.0 | 2.99, m |
| 3 | 82.7 | 3.70, overlap |
| 4 | 41.0 | 1.90, overlap |
| 5 | 80.2 | 3.78, overlap |
| 6 | 37.7 | 2.13, overlap |
| | | 1.86, overlap |
| 7 | 66.8 | 4.41, br d, $J$ = 8.0 Hz |
| 8 | 50.0 | 2.51, br q, $J$ = 6.5 Hz |
| 9 | 211.2 | -- |
| 10 | 43.8 | 2.83, q, $J$ = 7.0 Hz |
| 11 | 71.1 | 3.72, overlap |
| 12 | 41.0 | 1.72, m |
| 13 | 77.4 | 5.05, dd, $J$ = 9.5, 4.5 Hz |
| 14 | 25.3 | 1.82, overlap |
| | | 1.51, dqd, 14.0, 7.5, 4.5 |
| 15 | 10.6 | 0.92, t, $J$ = 7.5 Hz, 3H |
| 2-Me | 15.2 | 1.22, d, $J$ = 7.5 Hz, 3H |
| 4-Me | 10.3 | 1.20, d, $J$ = 7.0 Hz, 3H |
| 8-Me | 7.2 | 1.10, d, $J$ = 6.5 Hz, 3H |
| 10-Me | 6.3 | 1.04, d, $J$ = 7.0 Hz, 3H |
| 12-Me | 9.2 | 0.89, d, $J$ = 7.0 Hz, 3H |
| 1' | 105.7 | 4.29, d, $J$ = 7.5 Hz |
| 2' | 70.2 | 3.23, dd, $J$ = 10.0, 7.0 Hz |
| 3' | 65.4 | 2.45, m |
| N-Me | 40.2 | 2.25, s, 6H |
| 4' | 28.1 | 1.67, ddd, $J$ = 12.5, 3.5, 2.5 Hz |
| | | 1.20, overlap |
| 5' | 70.4 | 3.59, m |
| 5'-Me | 21.0 | 1.24, d, $J$ = 6.0 Hz, 3H |
| 1" | 100.4 | 4.94, d, $J$ = 3.5 Hz |
| 2" | 40.6 | 2.16, d, $J$ = 14.0 Hz |
| | | 1.82, overlap |
| 3" | 69.5 | -- |
| 4" | 76.2 | 2.99, overlap |
| 5" | 66.5 | 3.77, overlap |
| 3"-Me | 25.4 | 1.25, s, 3H |
| 5"-Me | 17.7 | 1.31, d, $J$ = 6.0 Hz, 3H |

C-8 methyl carbon ($\delta$ 7.23), and from the C-8 methyl protons to C-7 ($\delta$ 66.77), C-8 ($\delta$ 50.0), and C-9 ($\delta$ 211.2). Complete $^{1}H$ and $^{13}C$ NMR assignments are listed in Table 1. The one- and two-dimensional NMR data for the rest of the molecule are consistent with the structure shown.

The antibacterial activity of compound **1**, tested on *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Enterococcus faecalis*, and *Haemophilus influenzae* (Table 2), was marginal against microorganisms that are sensitive to erythromycin A. This is not surprising since **1** lacks the 12-OH and the 3′-O–$CH_3$ that contribute substantially to antibiotic activity.

The hydroxylation of 6-deoxyerythronolide B to erythonolide B is the first step in the pathway to convert 6-dEB into erythromycins and is catalyzed by the cytochrome P450 oxidase EryF.[8] The unexpected hydroxylation of

Fig. 1. Structure of 7-hydroxy-6-demethyl-6-deoxy erythromycin D.




**1**

Table 2. Antibacterial activity of erythromycin A and 7-hydroxy-6-demethyl-6-deoxy erythromycin D.

| Microorganism | MIC (μg/ml) | |
|---|---|---|
| | Erythromycin A | 1 |
| ***S.pneumoniae*** | | |
| ATCC6301 | 0.025 | 6.25 |
| ATCC700671 | 0.049 | 12.5 |
| ATCC700676 | 6.25 | 12.5 |
| ATCC700677 | 6.25 | 6.25 |
| ATCC700905 | 3.12 | 12.5 |
| ATCC700906 | >12.5 | >12.5 |
| ATCC49619 | 0.049 | 6.25 |
| ***S. aureus*** | | |
| ATCC6538p | 0.098 | >12.5 |
| ATCC33591 | >12.5 | >12.5 |
| ATCC14154 | >12.5 | >12.5 |
| ATCCBAA-39 | >12.5 | >12.5 |
| ATCCBAA-44 | >12.5 | >12.5 |
| ATCC29213 | 0.20 | >12.5 |
| ***S. epidermis*** | | |
| ATCC12228 | 0.20 | >12.5 |
| ***E. faecalis*** | | |
| ATCC51575 | >12.5 | >12.5 |
| ***Haemophilus Influenzae*** | | |
| ATCC9006 | 1.56 | >12.5 |
| ATCC49766 | 6.25 | >12.5 |

compound **1** at carbon C-7 rather than at C-6 presumably occurs due to an altered binding configuration of the substrate, 6-nor-erythronolide B, in the active site of the EryF oxidase. PETKOVIC *et al.*[9] recently reported the production of 6-demethyl erythromycin D, possessing the natural C-6 hydroxyl function, from a different engineered strain of *S. erythraea*. Although the alterations to the PKS (DEBS) genes are different from those reported here, this should not affect the specificity of EryF.

## Experimental Section

### General Experimental Procedures

HRESIMS were obtained by manual peak matching versus internal standards by high-resolution mass spectrometry using an Applied Biosystems Mariner TOF spectrometer configured with a Turbo-Ionspray source in positive ion mode. $^{1}$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) were recorded with a Bruker DRX 400 spectrometer equipped with a Nalorac 3-mm MDG-400B probehead. Chemical shifts were referenced to $\delta$ 7.26 and 77.0 for $^{1}$H and $^{13}$C NMR spectra, respectively. For each compound, $^{1}$H, $^{13}$C, COSY, HMBC, and multiplicity-edited HSQC experiments were carried out.

### Strain and Expression Vector

*S. erythraea* K24-1 was described previously.[7] The AT4-modified DEBS gene, which has the two AT4 amino acids adjacent to His-201 mutated (mutation 3 in reference 6), was introduced into *S. erythraea* K24-1 by conjugation as described previously.[7] Briefly, the engineered PKS genes were transferred from the *S. lividans* expression vector to the pSET152 integrating vector under control of the *eryAp* promoter by conventional cloning procedures resulting in the vector pKOS159-42, which was introduced into *S. erythraea* K24-1 by conjugation. Rapamycin-resistant transconjugants were confirmed to produce compounds with antibacterial activity against *M. luteus* using an agar plate bioassay.

### Media

*S. erythraea* K24-1::pKOS159-42 was cultivated in seed (SE-V1) and production (SE-0.5F1) media. SE-V1 contained corn starch 16 g, corn dextrin 10 g, soy meal flour 15 g, sodium chloride 2.5 g, corn steep liquor 5 g, ammonium sulfate 1 g, soy bean oil 6 g, and calcium carbonate 4 g in 1 liter of deionized water. SE-0.5F1 contained corn starch 17.5 g, corn dextrin 16 g, soy meal flour 16.5 g, sodium chloride 3.5 g, corn steep liquor 6 g, ammonium sulfate 1 g, soy bean oil 3 g, and calcium carbonate 4 g in 1 liter of deionized water. The media were prepared by adding all components to 90% final volume with mixing. Then, the media were brought to final volume and autoclaved at 121°C for 90 minutes. After sterilization SE-0.5F1 was transferred to a sterile fermenter and adjusted to pH 7.0 for fermentation. All media were supplemented with 1 ml/liter Antifoam B (JT Baker) unless otherwise noted to prevent excessive foaming. SE-V1 was supplemented with 30 mg/liter apramycin sulfate.

### Culture Conditions

*S. erythraea* K24-1::pKOS159-42 was maintained as a cell bank consisting of 1 ml vials frozen at −80°C and was used to initiate the bioreactor seed train. A primary seed culture (1°S) was started by inoculating 50 ml SE-V1 (in 250 ml flasks, Bellco #2540) with 1 cell bank vial and was cultivated for 2 days at 34°C and 245 rpm (1″ orbital shaker). A secondary seed culture (2°S) was prepared by transferring 50 ml of 1°S to 500 ml SE-V1 (in 2.8-liter Fernbach flasks, Bellco #2554) and cultivating for 2 days at 34°C and 245 rpm. Finally, a Biostat CT (B. Braun) 10-liter fermenter containing 9 liters of SE-0.5F1 was inoculated with the secondary seed culture. The production fermenter was operated at 34°C, pH 7.0±0.15, 3 LPM airflow, and 600 rpm agitation. The pH was automatically controlled with 2.5 N sodium hydroxide or 2.5 N sulfuric acid. The dissolved oxygen was maintained above 40% of air saturation by manual control of airflow (3～5 LPM) and agitation (600～750 rpm). Foaming was controlled by automatic addition of Antifoam B. At 24 hours after inoculation, a dextrin feed was started. The dextrin feed was prepared as a 150 g/liter dextrin suspension (requires mixing) and was fed at a rate of 5.4 ml/hour. The feed was maintained until harvest on day 6 after inoculation.

### Isolation of 7-Hydroxy-6-demethyl-6-deoxy Erythromycin D

Whole broth from two 10-liter fermentations of *S. erythrea* K24-1::pKOS159-42 was adjusted to pH 8 and clarified by centrifugation. Erythromycins were captured by passing the supernatant over a column of HP20 sorbent (Mitsubishi), washing with water, and eluting with methanol. The resulting solid-phase extraction product was chromatographed on Diaion HP-20SS sorbent (Supelco) using a stepped gradient of 50～100% MeOH. LC/MS analysis of several later-eluting fractions indicated compounds having masses consistent with 6-demethyl-erythromycin D. These fractions were pooled and further fractionated by two rounds of isocratic $C_{18}$ chromatography

(Bakerbond, 40 $\mu$m), first in 67% MeOH/5 mM $NH_4OAc$, and then in 63% MeOH/5 mM $NH_4OAc$, giving pools enriched in **1**. Compound **1** was further purified by partitioning between ethyl acetate and saturated aqueous sodium bicarbonate, followed by $C_{18}$ chromatography with a stepped gradient of 50～100% MeOH, to yield 10 mg.

Minimal inhibitory concentrations (MICs) of **1** against *S. pneumoniae*, *S. aureus*, *S. epidermis*, *E. faecalis*, and *H. influenzae* were determined using a microdilution technique (NCCLS document M7-A5).[10] Compounds were dissolved to 5 mg/ml in dimethylsulfoxide, diluted to 25 $\mu$g/ml, and then further serially diluted 2-fold. An equal volume of cells ($A_{600}$=0.1) and drug dilution were mixed to give final drug concentrations between 0.006 and 12.5 $\mu$g/ml. Cultures were grown overnight at 35°C in 96-well plates, and MIC values were determined by visual inspection.

7-Hydroxy-6-demethyl-6-deoxy Erythromycin D (**1**): Yellow solid; 10 mg; UV (DAD, 50% MeCN) end absorption; IR (film) $\nu_{max}$ 3509, 2974, 2938, 1738, 1711, 1456, 1373, 1243, 1182, 1115, 1050, 1005 $cm^{-1}$.

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