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Production of 6-Deoxy-13-cyclopropyl-Erythromycin B by *Saccharopolyspora erythraea* NRRL 18643

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Cyclopropane carboxylic acid was fed to *Saccharopolyspora erythraea* NRRL 18643 (6-deoxyerythromycin producer), resulting in the production of 6-deoxy-13-cyclopropyl-erythromycin B. These studies provide further evidence that deoxyerythronolide B synthase has a relaxed specificity for the starter unit.

The biosynthesis of the erythromycin aglycone, 6-deoxyerythronolide B (**1**), is carried out by deoxyerythronolide B synthase (DEBS), a type I modular polyketide synthase (PKS), in a manner analogous to fatty acid synthesis (Figure 1). This aglycone is derived from a propionyl-CoA starter unit and six (2*S*)-methylmalonyl-CoA extender units[1]. The chain is subsequently cleaved from the PKS and cyclized to form **1**. Previous work using a cell free system has shown that the first two modules of DEBS have a relaxed specificity for the starter unit[2]. Using this *in vitro* system, novel triketide lactones were formed by the incorporation of alternate starter units, acetyl-CoA and butyryl-CoA. *Saccharopolyspora erythraea* has also been shown to be capable of acetate incorporation *in vivo* to form 15-norerythromycin C and 8,8a-deoxyoleandolide[3,4]. Recent results demonstrated that the wild type strain, *S. erythraea* NRRL 2338, can incorporate cyclopropane carboxylic acid and cyclobutane carboxylic acid to form novel erythromycins[5]. Based on these data, it was hypothesized that novel forms of 6-deoxy-erythromycin could be produced by feeding cyclopropane carboxylic acid to *S. erythraea* NRRL 18643, a genetically-engineered strain with a knockout in the C-6 hydroxylase (*ery*F)[6]. This hypothesis was demonstrated experimentally.

Materials and Methods

Microorganism

Saccharopolyspora erythraea NRRL 18643 was maintained as a 20% glycerol stock at −20°C.

Production of 6-Deoxy-13-cyclopropyl-erythromycin B at Flask Scale

S. erythraea NRRL 18643 was plated onto 1/2YPD agar (Difco yeast extract 0.5%, Difco Bacto peptone 0.5%, dextrose 0.25%, MOPS buffer 0.5%, Difco Bacto agar 1.7%, pH adjusted to 7.0 prior to autoclaving) and incubated at 28°C until grown (approximately 4 days). An agar plug (6 mm diameter) was used to inoculate a 250 ml flask containing 25 ml of 1/2YPD broth (Difco yeast extract 0.5%, Difco Bacto peptone 0.5%, dextrose 0.25%, MOPS buffer 0.5%, pH adjusted to 7.0 prior to autoclaving). The culture was incubated on a rotary shaker at 29°C, 225 rpm for 24 hours. At this time, 2.5 ml of the first stage culture was inoculated into a 250 ml flask containing 25 ml of Ery-P medium (dextrose 5%, Nutrisoy flour 3%, $(NH_4)_2SO_4$ 0.3%, NaCl 0.5%, $CaCO_3$ 0.6%, pH adjusted to 7.0 prior to autoclaving) which was then incubated as above for a total of six days. Cyclopropane carboxylic acid was added to the flasks at 24 and 48 hours to a final concentration of 200 ppm at each ad-

Fig. 1a. The erythromycin PKS.

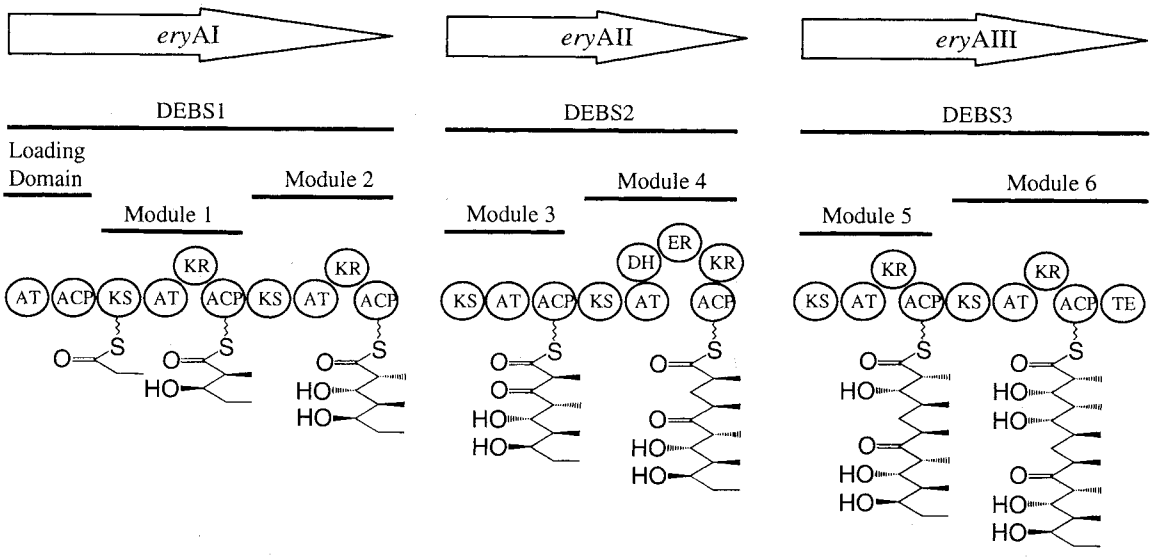

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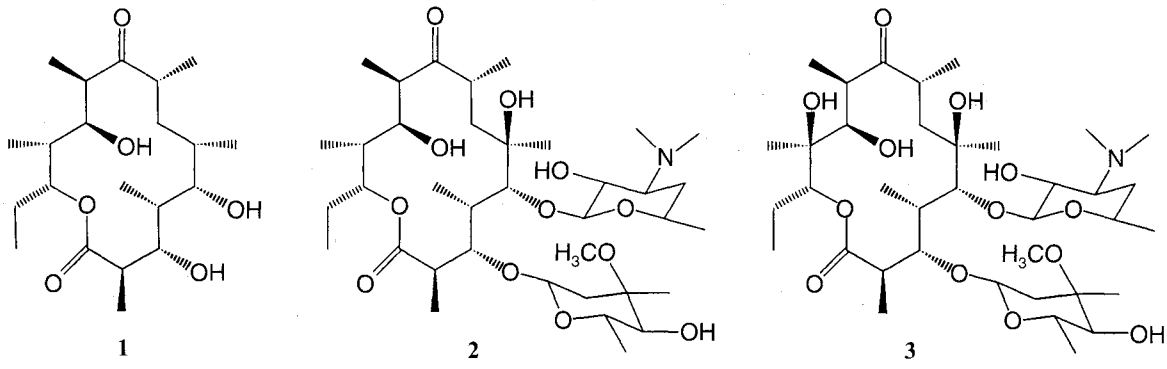
Fig. 1b. Structures of 6-deoxyerythronolide B (**1**), erythromycin B (**2**) and erythromycin A (**3**).



dition. After six days, whole broth was adjusted to pH 9.0 with NaOH. The fermentation was then extracted once with an equal volume of ethyl acetate. The organic phase was reduced to dryness under nitrogen (45°C bath). The residue was resuspended in 1.0 ml methanol for HPLC-MS analysis. A novel peak was detected at 21.5 minutes by HPLC-MS.

Scaleup Procedure

Four milliliters of NRRL 18643 were inoculated into 1 liter of TW medium (cerelose 0.5%, tryptone 0.5%, Difco yeast extract 0.25%, EDTA 0.0036%, pH adjusted to 7.1 prior to autoclaving) in a 2.8 liter fernbach flask and incubated on a rotary shaker at 29°C, 210 rpm for 3 days. Cyclopropane carboxylic acid was added to this culture at 24 hours for a final concentration of 200 ppm. Eight hundred milliliters of this first stage were inoculated into a 14 liter fermentor (New Brunswick) containing 8 liters of modified Ery-P medium (prepared in 7 liters of water: cerelose 1%, Nutrisoy flour 3%, $(NH_4)_2SO_4$ 0.3%, NaCl 0.5%, $CaCO_3$

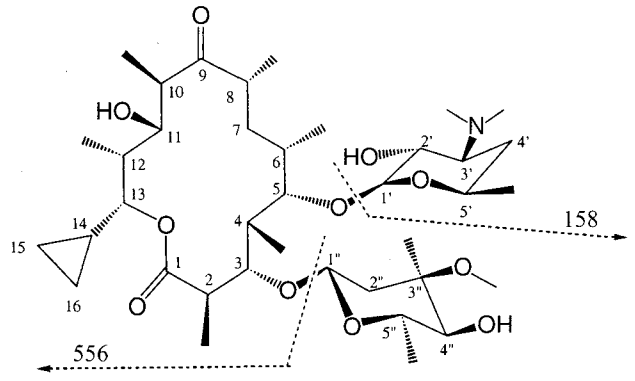
0.6%, $MgSO_4$ 0.05%, corn steep solids 0.5%, pH adjusted to 7.0, autoclaved at 121°C, 99 minutes; 1 liter of a 5% dextrose solution was autoclaved separately at 121°C, 30 minutes then aseptically added to the fermentor for a final volume of 8 liters). The fermentor was run at 28°C, 8 liters air/minute, 800 rpm, with pH controlled between 6.9 and 7.3 for 163 hours.

Isolation

After 163 hours, 16 liters of whole broth from two fermentors was adjusted to pH 9.0 using NaOH. Broth was then extracted with an equal volume of ethyl acetate. The organic phase was concentrated using a rotary evaporator, then dissolved in 500 ml methylene chloride and extracted with an equal volume of pH 9.0 water. The methylene chloride phase was dried *in vacuo*, yielding 10 grams of an oil. Nine grams of this material was dissolved in 50 ml of dichloromethane and loaded onto a Biotage flash 40 M prep system equipped with a silica cartridge. The column was sequentially eluted with 500 ml of each of the following solvents: dichloromethane, dichloromethane - methanol (99 : 1), dichloromethane - methanol (90 : 10), dichloromethane - methanol - ammonia (80 : 20 : 0.2), and methanol. Each mobile phase combination was collected as a single fraction (500 ml). All macrolides eluted in the fraction corresponding to dichloromethane - methanol - ammonia (80 : 20 : 0.2), yielding 522 mg dry solid. This material was further purified by normal phase HPLC using a 5 μm YMC diol column (20×250 mm) with a mobile phase gradient of A - B 100 : 0 to 80 : 20 over 60 minutes at 20 ml/minute where: A=(hexane with 0.1% diethylamine) and B=(9 : 1 chloroform - methanol with 0.1% diethylamine). Fractions corresponding to 42~47 minutes were combined, and the solvent was removed *in vacuo*. Three separate injections yielded a total of 47 mg.

This material was further purified by reverse phase HPLC using a Phenomenex 5 μm hexyl-phenyl column (21.2×250 mm) with a mobile phase gradient of A - B 70 : 30 to 65 : 35 over 100 minutes at 20 ml/minute where: A=(0.05 M aqueous ammonium acetate with 0.1% TFA) and B=(acetonitrile - tetrahydrofuran 4 : 1). Fractions corresponding to 42~57 minutes were combined and saturated with sodium bicarbonate. The product was extracted with dichloromethane and dried *in vacuo*. Two separate injections yielded a total of 19 mg. This material was further purified by reverse phase HPLC using a 5 μm Phenomenex C8 Luna column (21.2×250 mm) with a mobile phase gradient consisting of (0.05 M aqueous ammonium acetate with 0.1% TFA)-methanol 40 : 60 to 35 : 65 over 50 minutes at 20 ml/minute. Fractions corresponding to 20~25 minutes were combined and saturated with sodium bicarbonate. The product was extracted with dichloromethane and dried *in vacuo* yielding 11 mg purified material.

Fig. 2. Proposed structure and fragmentation for 6-deoxy-13-cyclopropyl-erythromycin B (**4**).



Analysis

HPLC-MS data was acquired using a Hewlett-Packard 1050 liquid chromatograph interfaced to a VG Platform II mass spectrometer equipped with an APcI source. The following chromatographic conditions were used: Waters Symmetry 5 μm C18: 2.1×150 mm HPLC column with a mobile phase gradient of 10 mM ammonium acetate-acetonitrile 70 : 30 to 50 : 50 in 30 minutes, at a flow rate of 0.22 ml/minute, 6 μl injection volume.

The following NMR experiments were acquired on a Bruker DMX500 using a 5 mm z-gradient inverse broadband probe: proton, carbon, DEPT, gradient COSY, gradient HMQC (Heteronuclear Multiple Quantum Correlation spectroscopy), and gradient HMBC (Heteronuclear Multiple Bond Correlation spectroscopy). HR-MS data supporting molecular formula assignments were obtained through M-Scan, Inc., West Chester, PA, using a VG Analytical AutoSpec E spectrometer operated with an LSIMS (Cs^+ ion bombardment) ion source.

Mean Minimum Inhibitory Concentration Assays

MIC's (run in triplicate) were determined according to NCCLS standard methodology.

Table 1. NMR assignment for **4**.

(All ppm values are relative to TMS)

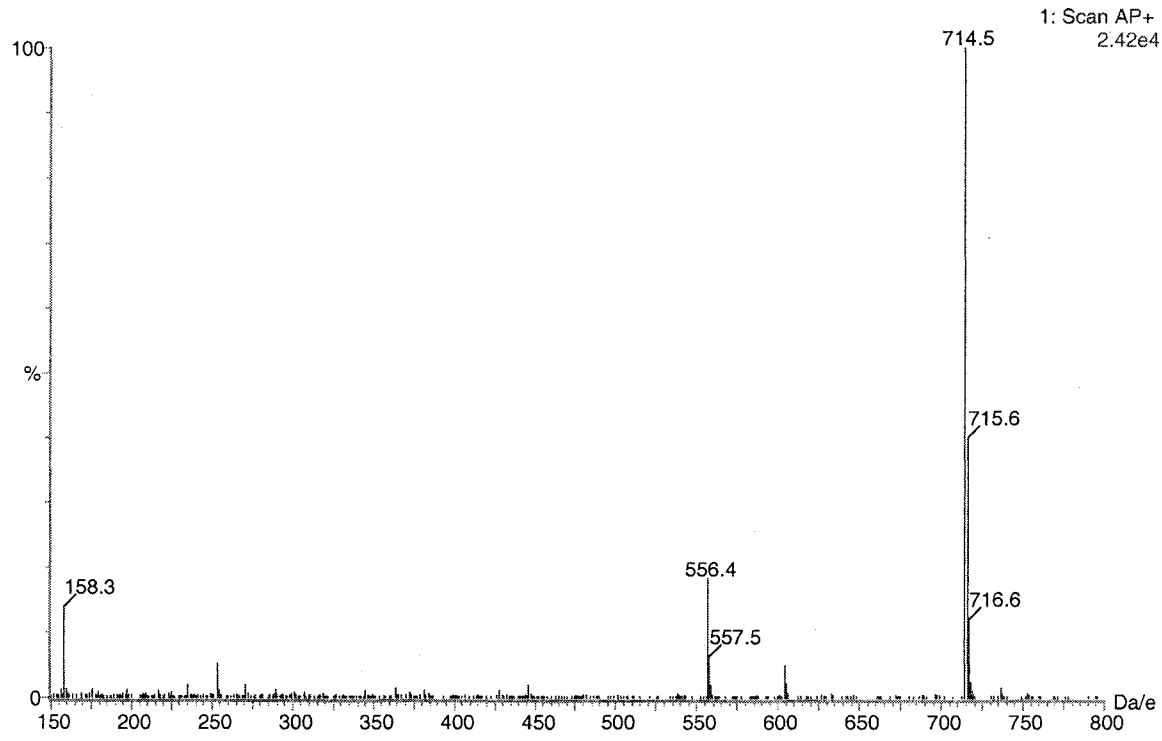
Atom	^{13}C - ppm	# of attached H	^{1}H - ppm	Atom	^{13}C - ppm	# of attached H	^{1}H - ppm
1	177.5	0		8Me	17.16	3	1.2
2	45.39	1	2.94	10Me	8.33	3	1.09
3	80.51	1	3.66	12Me	10.15	3	1.04
4	43.56	1	1.77	1'	103.94	1	4.36
5	85.74	1	3.52	2'	70.4	1	3.55
6	37.2	1	1.58	3'	66.38	1	3.37
7	34.78	2	1.82/1.26	4'	31.47	2	2.03/1.51
8	45.39	1	2.64	5'	68.22	1	3.62
9	217.38	0		3'NMe (2)	40.23	3	2.86
10	42	1	2.92	5'Me	21.32	3	1.34
11	70.53	1	3.52	1"	97.96	1	4.88
12	41.86	1	1.93	2"	35.59	2	2.40/1.60
13	79.97	1	4.52	3"	73.02	0	
14	13.53	1	1.22	4"	78.35	1	3.06
15	4.82	2	0.63/0.44	5"	66.09	1	3.97
16	3.44	2	0.63/0.33	3"Me	49.71	3	3.31
2Me	15.5	3	1.26	3"OMe	21.83	3	1.28
4Me	10.2	3	1.2	5"Me	18.55	3	1.32
6Me	20.17	3	1.25				

Table 2. Mean minimum inhibitory concentration in μg/ml (run in triplicate).

Organism	Erythromycin B (**2**)	Erythromycin A (**3**)	6-Deoxy-erythromycin A	6-Deoxy-13-cyclopropyl-erythromycin B (**4**)
Staphylococcus aureus 1116	≦0.2	≦0.2	1.56	1.56
Staphylococcus aureus 1117[a]	>100	>100	>100	>100
Staphylococcus aureus 0052	≦0.2	≦0.2	0.39	0.78
Staphylococcus aureus 1120[a]	>100	>100	>100	>100
Staphylococcus aureus 0129[a]	>100	>100	>100	>100
Staphylococcus aureus 1032[a]	100	100	50	100
Streptococcus hemolyticus 1006	100	>100	100	100
Streptococcus agalactiae 1023[a]	>100	>100	>100	>100
Streptococcus agalactiae 1024	≦0.2	≦0.2	≦0.2	≦0.2
Streptococcus pyogenes 0203	≦0.125	≦0.125	≦0.125	≦0.125
Streptococcus pyogenes 1079	>64	>64	>64	>64
Streptococcus pyogenes 1064	8	8	16	8
Streptococcus pyogenes 1064 + Ery[c]	8	16	16	8
Streptococcus pneumoniae 1016	0.0625	0.0312	0.125	0.5
Streptococcus pneumoniae 1046	>100	>100	>10	>100
Streptococcus pneumoniae 1095	>10	>10	>10	>10
Streptococcus pneumoniae 1175	>10	>10	>10	>10
Streptococcus pneumoniae 1175 + Ery[c]	10	>10	>10	>10
Haemophilus influenzae 1100	0.2	2	not tested	32
Haemophilus influenzae 0131[b]	4	4	32	32
Moraxella catarrhalis 0040	≦0.125	≦0.125	≦0.125	0.25
Moraxella catarrhalis 1055	2	2	4	8
Escherichia coli 0266	100	100	>100	>100

[a]: Multiply resistant strains generated at Pfizer or hospital isolates. [b]: Hospital isolate. [c]: Resistance induced by adding erythromycin.

Fig. 3. Mass spectral data for **4**.



Results and Discussion

Structure Elucidation

The isolated material was characterized as 6-deoxy-13-cyclopropyl-erythromycin B (**4**, Figure 2) by mass spectroscopy and NMR spectroscopy (Table 1). The mass spectrum (Figure 3) contained a base peak of *m/z* 714.5, consistent with the proposed structure **4**. In addition, the peak at *m/z* 556.4 is consistent with the loss of the neutral sugar cladinose, a common fragment in the erythromycin series. Also consistent with the proposed structure is the signal at *m/z* 158.3 which is characteristic of the basic sugar desosamine. High resolution MS ($M+H^+=714.4748$) was consistent with the formula $C_{38}H_{67}NO_{11}$ to within 6 ppm.

2D NMR determined the line assignment described in Table 1. The number of observed carbons, the respective number of attached protons and the inferred number of oxygen, nitrogen and hydroxyl groups give rise to a calculated molecular formula of $C_{38}H_{67}NO_{11}$ with a total mass consistent with the mass spectral data[8]. The two most pertinent assignments, *i.e.*, the presence of a cyclopropyl group at the 13 position, and the absence of the hydroxyl group at the 6 position, are described in detail below.

The methylene proton and carbon chemical shifts of a cyclopropyl group are quite unique, both being much more up field when compared to other cyclic hydrocarbons. The observed chemical shift values of 3.44 and 4.83 ppm in the carbon spectra and their corresponding proton shifts of 0.33/0.63 ppm and 0.44/0.63 ppm in the proton spectra for the methylenes (positions 16 and 15, respectively) are easily identified in the gr-HMQC spectrum and are consistent with the presence of a cyclopropyl group. The gr-COSY correlates the methylenes to the cyclopropyl methine at position 14 (1.22 ppm), which in turn shows a correlation to the methine proton (4.52 ppm) at position 13 of the macrolide ring, indicating that the cyclopropyl moiety is attached to the ring at this position. In addition, the gr-HMBC data identifies the position of attachment as being two bonds away from the carboxy carbon at position 1 and adjacent to an oxygen as determined by carbon chemical shift (79.97 ppm).

An extensive spin system was observed in the gr-COSY spectrum consisting of six methines, one methylene, and four methyls [2(2-Me), 3(O), 4(4-Me), 5(O), 6(6-Me), 7, 8(8-Me)]. Gr-HMBC correlations from positions 1′ and 1″ to methines in this spin system and from the carbon at position 1 to the 2-Me protons led to the assignments listed in

Table 1. In this spin system the only carbons adjacent to oxygen (as determined by chemical shift) are those at the point of attachment of the two sugars, indicating that the hydroxyl group is not present at position 6. Consistent with this assignment, the chemical shifts of the C6 carbon (37.20 ppm) and the C6 hydrogen (1.58 ppm) indicate that there is no hydroxyl group attached at this position.

Antibacterial Activity

Antibacterial activity (MIC) of **4** was compared to structurally similar erythromycins. Results of these assays are shown in Table 2.

Discussion

The incorporation of cyclopropane carboxylic acid to form 6-deoxy-13-cyclopropyl-erythromycin B (**4**) provides another example of the relaxed specificity of the DEBS starter unit in vivo. Previously, the loading domain of the wild type erythromycin producer has been shown to accept acetate to form low levels of 15-nor-erythromycin C[3]. Similar results were seen when DEBS was expressed in *Streptomyces coelicolor*[9]. These results are not surprising given the similarity to the propionate-derived erythromycin starter unit. The incorporation of a larger starter unit, butyryl CoA, to produce a novel triketide lactone has only been shown in an *in vitro* system[2]. The formation of the novel 6-deoxy-erythromycin B derivative reported herein clearly demonstrates that the DEBS loading domain is relaxed with respect to larger starting units *in vivo* as well.

The novel macrolide had biological activity (Table 2) similar to 6-deoxy-erythromycin A, but was less effective than erythromycins A and B against some pathogens. 6-deoxy-13-cyclopropyl-erythromycin B was less active than erythromycins A and B against *Staphylococcus aureus* 1116, *Staphylococcus aureus* 0052, *Streptococcus pneumoniae* 1016, *Streptococcus pneumoniae* 1175, *Haemophilus influenzae* 1100, *Haemophilus influenza* 0131, *Moraxella catarrhalis* 0040, and *Moraxella catarrhalis* 1055. This difference may be attributed to the lack of the C-6 hydroxyl group, rather than the novel C-13 cyclopropyl group.

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