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Novel Erythromycins from a Recombinant *Saccharopolyspora erythraea* Strain NRRL 2338 pIG1

I. Fermentation, Isolation and Biological Activity

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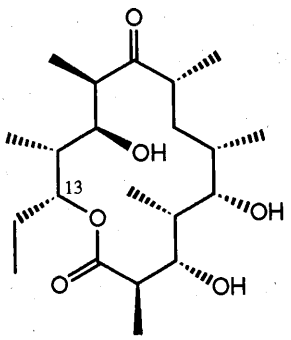
In a previous report, a plasmid, pIG1, which contained the loading domain from the *Streptomyces avermitilis* polyketide synthase (PKS), promoters from *Streptomyces coelicolor* and the DEBS1-TE truncated PKS from *Saccharopolyspora erythraea*, was integrated into the *S. erythraea* chromosome, effectively replacing the natural erythromycin loading domain with the avermectin loading domain. In this paper, we report the feeding of short-chained fatty acids to this recombinant strain, and its parent, NRRL 2338. Both strains incorporated exogenously supplied fatty acids to produce novel, biologically active, C-13 substituted erythromycins.

Polyketides are a large and diverse family of natural products, many of which are commercially significant for human and veterinary applications. The antibacterial macrolides such as erythromycin[1] and tylosin[2], and semisynthetic derivatives of erythromycin, such as azithromycin[3] and clarithromycin[4], are important examples. The avermectin class dominates the livestock endectocide sector in which doramectin[5] and ivermectin[6] are leading products. The actinomycete derived polyketides can be divided into two types, according to whether the enzyme activities are located in domains on multifunctional polypeptides (Type 1) or are located on monofunctional proteins that associate as a multienzyme complex (Type 2). This has led to a classification of the polyketide natural products as either modular (*e.g.*, erythromycin) or aromatic (*e.g.*, tetracycline), respectively.

Early work on the elucidation of the organisation of the gene cluster responsible for the production of erythromycin was pioneered by the groups of KATZ[7] and HUTCHINSON[8,9]. The first biosynthetic intermediate in the sequence is 6-deoxyerythronolide B (6-DEB; Fig. 1). Using a number of simple fatty acid building blocks, 6-DEB is biosynthesised by the stepwise chain assembly on the polyketide synthase (PKS) multienzyme complex of *Saccharopolyspora erythraea*. This intermediate, 6-DEB, undergoes a sequence of hydroxylations, glycosylations and methylations to give the isolated

Fig. 1. Structure of 6-deoxyerythronolide B (6-DEB).



natural product, erythromycin A.

The first direct evidence for the modular organisation of the erythromycin pathway in *S. erythraea* was provided by the groups of KATZ[10] and LEADLAY[11]. They showed that 6-DEB was biosynthesised by three genes (DEBS1, DEBS2, DEBS3) that coded for large multifunctional multi-domain proteins. At the *N*-terminus of DEBS1 is a sequence that encodes for the loading of the biosynthetic starter unit, propionate. Subsequent work by LEADLAY and STAUNTON[12] and MACNEIL[13] has established a similar modular organisation for rapamycin and avermectins, respectively.

A mutant *S. erythraea* strain NRRL 2338 pIG1 has been generated by substitution of the loading domain at the *N*-terminus of DEBS 1 with the loading domain from *S. avermitilis*, the avermectin producer. As the avermectin loader naturally accepts isobutyric acid and 2-methylbutyric acid, fermentation of this construct yielded C-13-isopropyl and C-13-*sec*-butyl erythromycins, as demonstrated by LEADLAY[14]. Our previous work[5] on the substrate feeding of other carboxylic acids to an *S. avermitilis* mutant demonstrated that the avermectin loading domain was capable of accepting a large range of substrates to produce novel C-25 substituted avermectins. For example, doramectin was produced from the feeding of cyclohexane carboxylic acid.

In the present work, we sought to extend the precursor-directed biosynthetic technology, used to discover doramectin, to the production of novel C-13 substituted erythromycins. This paper describes the feeding of exogenous fatty acids to the recombinant *S. erythraea* organism NRRL 2338 pIG1, and its wild-type parent, NRRL 2338.

Materials and Methods

Microorganism

A plasmid vector, pIG1, was constructed incorporating the loading domain from the *S. avermitilis* PKS, *act*I promoters from *S. coelicolor* and the DEBS1-TE thioesterase truncated PKS from *S. erythraea*[14]. This pIG1 vector was used to successfully transform cells of *S. erythraea* NRRL 2338. Through a single crossover, this plasmid DNA was integrated into the chromosome, effectively replacing the natural erythromycin loading domain with the avermectin loading domain.

Chemicals

Unless otherwise indicated, the carboxylic acids were obtained from Lancaster Synthesis Ltd. Methylthiolactic acid was prepared as follows. A 20 litre vessel was charged with sodium hydroxide pellets (1.685 kg, 41.47 mol) and distilled water (2.7 litres) and cooled with an ice water mix. Thiolactic acid (2 kg, 18.85 mol) was added at such a rate so that the temperature held between 60°C and 70°C. Dimethyl sulphate (2.377 kg, 18.85 mols) was added at a temp of 80°C and 90°C and the reaction held at this temperature for 40 minutes with constant stirring. Water (1 litre) was added and the mixture stirred in an ice bath. Concentrated H_2SO_4 (700 ml) was added with the temperature not exceeding 26°C. The reaction mixture was then cooled further and the crystalline solid filtered off. This was then extracted into methylene chloride, washed with solvated brine (2.5 litres) and dried over $MgSO_4$.The methylene chloride was then stripped on a rotary evaporator to yield a yellow oil. (1187 g). This crude material was then distilled under high vacuum whereby the main fraction yielded a clear oil (942 g) which was shown by NMR to be methylthiolactic acid as the racemic mixture.

Thiophene 3-*N*-acetyl cysteamine ester was prepared as follows. To a solution of 3-thiophene carboxylic acid (2.794 g, 21.8 mmol) in dried dichloromethane (90 ml) at 0°C were added DMAP (4-dimethylaminopyridine; 0.266 g, 2.18 mmol), NAc (*N*-Acetyl cysteamine; 2.598 g, 21.8 mmol) and DCC (*N*,*N*-dicyclohexylcarbodiimide; 8.996 g, 32.6 mmol). The mixture was stirred for 10 minutes at 0°C and then at room temperature overnight (16 hours). A white precipitate formed and was collected by filtration. The crude product was recrystallized from dichloromethane and diethyl ether to yield 4.076 g of white crystals (81.6% yield). The ^{13}C and ^{1}H NMR spectra were consistent with the structure.

Fermentation

Recombinant Culture:

A slant culture of *S. erythraea* NRRL 2338 pIG1 was used to inoculate a 300 ml Erlenmeyer flask containing 50 ml of a seed medium containing glucose 0.5%, tryptone 0.5%, yeast extract 0.25% and EDTA 0.0036% in tap water at natural pH. After 36 hours incubation at 28°C on a rotary shaker at 200 rpm, this flask was used to inoculate 3.5 litres of ERY-P medium in a 5 litre minijar (Electrolab, Gloucester, U.K. GL20 7LR). ERY-P medium contains glucose 5.0%, Nutrisoy® flour 3.0%, ammonium sulphate 0.3%, sodium chloride 0.5% and calcium carbonate 0.6% maintained at a natural pH of 7.0. Thiostrepton (105 mg in 5 ml methanol) was filter sterilised into the autoclaved medium. The broth was incubated at 28°C with an aeration rate of 1.75 litres/

minute. Cyclopentane carboxylic acid, 1.4 ml, was added after 24 hours and the fermentation continued for 168 hours. The fermentation was repeated using a range of other short chained carboxylic acids.

Wild Type Culture:

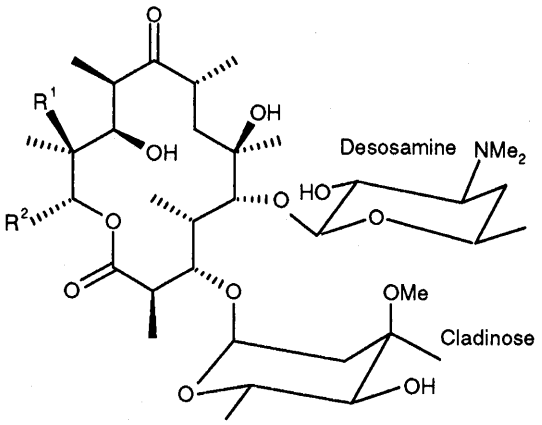
A slant culture of the wild type *S. erythrae* NRRL 2338 was inoculated into a 300 ml Erlenmeyer flask containing 50 ml of the same seed medium. After a 36 hour incubation under the same conditions above 2 ml of seed was inoculated into two 300 ml Erlenmeyer flasks containing 50 ml ERY-P medium. These were incubated at 28°C on a rotary shaker at 200 rpm and at 24 hours, 20 μl of cyclopropane carboxylic acid was added to one flask and 20 μl cyclobutane carboxylic acid was added to the other. The flasks were incubated for a further 6 days.

Isolation

The pink culture broth from the minijar, fermentation of *S. erythraea* NRRL 2338 pIG1, which was at pH 8.5 at the point of harvest, was extracted with ethyl acetate (10 litres). The organic solubles were then concentrated to give a gum (4.2 g). One gram of this extract was dissolved in ethyl acetate (5 ml) and added to a prepacked silica cartridge (10 g; International Sorbent Technology) previously conditioned with ethyl acetate (10 ml). The column was sequentially eluted with ethyl acetate (4 × 10 ml), dichloromethane : methanol (50 : 50) (2 × 10 ml), dichloromethane : methanol : ammonia (80 : 19 : 1) (1 × 10 ml) and methanol (2 × 10 ml). The last four 10 ml fractions were combined and evaporated to dryness. This fractionation was repeated on the remaining 3.2 g of material to yield 920 mg of a gum solid containing the desired product. The solid was further purified by preparative reversed phase HPLC using a Zorbax 7 μ ODS column (21.2 mm × 25 cm) with an isocratic mobile phase of acetonitrile : 0.05 M ammonium acetate (50 : 50) over 18 minutes with a flow rate of 4 ml/minute. Fractions shown by LC-APCI-MS to contain the product of interest from five separate injections were combined and evaporated to dryness to afford a white solid (7 mg). The structure of the solid was determined as 13-cyclopentyl-13-desethyl erythromycin-B (**2b**) as shown in Fig. 2. APCI-MS data gave a molecular ion *m/z* 758 ([M + H$^+$]). High resolution FAB-MS established the molecular formula as $C_{40}H_{11}NO_{12}$. A full structural assignment is shown in the following paper.

The wild type organism fed with cyclopropane carboxylic acid and cyclobutane carboxylic acid was extracted from the Erlenmeyer flask with ethyl acetate (150 ml). The ethyl acetate solubles were taken to dryness and the gum redissolved in methanol (1 ml). They were then assayed for novel erythromycin production using the chromatographic method described under Table 1.

Fig. 2. Structure of compound **2b** compared with erythromycin A and B.



1a Erythromycin A $R^1 = OH$ $R^2 = -CH_2CH_3$
1b Erythromycin B $R^1 = H$ $R^2 = -CH_2CH_3$
2b $R^1 = H$ $R^2 =$ 

Antibacterial Assays

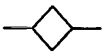
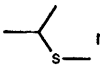
Minimum inhibitory concentrations (MICs) against *Pasteurella multocida*, *Escherichia coli*, and *Staphylococcus aureus* were analyzed by a microdilution method similar to that described in the NCCLS document M7-A3[15], with the exception that the total volume of the well was 200 μl instead of 100 μl, and BHI (brain-heart infusion) broth was used in place of CAMH broth in the case of *P. multocida* and *E. coli* assays.

Results and Discussion

Table 1 describes a range of short-chained fatty acid substrates that were incorporated by the recombinant organism into the erythromycin molecule. The novel erythromycin products were characterised by APCI mass spectrometry and/or NMR assignment. The fed substrates listed in Table 1 all produced the corresponding erythromycin B analogues. To facilitate the uptake of substrate, the *N*-acetyl cysteamine ester of thiophene-3-carboxylic acid was prepared and fed to the organism. This ester incorporated, whereas the thiophene-3-carboxylic acid did not.

In addition, the cyclopropane carboxylic acid and

Table 1. Erythromycin analogues from feeding experiments with *S. erythrae* NRRL2338pIG1.

Erythromycin	R^1	R^2	Substrate	Novel peak HPLC retention time * (mins)	Mass of observed peak $(M+H)^+$	Expected mass for novel erythromycin analogue with appropriate C-13 substituent $(M+H)^+$
2b	H		cyclopentane carboxylic acid	26	758	758
3b	H		1-cyclopentene carboxylic acid	24.2	756	756
4a	OH		cyclopropane carboxylic acid	13.3	746	746
4b	H		cyclopropane carboxylic acid	17.9	730	730
5a	OH		cyclobutane carboxylic acid	17.6	760	760
5b	H		cyclobutane carboxylic acid	21.9	744	744
6b	H		3-methyl cyclobutane carboxylic acid	25	758	758
7b	H		3-furoic acid	17	756	756
8b	H		thiophene 3-N-acetyl cysteamine ester	20.9	772	772
9b	H		methyl thiolactic acid	20	764	764
10b	H		isobutyric acid **	22	732	732
11b	H		2-methylbutyric acid **	24	746	746

* HPLC conditions: Column: Beckman Ultrasphere 5μ ODS column 25 × 0.4 cm, mobile phase: acetonitrile/0.05 M ammonium acetate 28 : 72 to 50 : 50 over 22 minutes and maintaining at 50 : 50 from 22 ~ 25 minutes; APCI mass detection 200 ~ 1500 mu, flow: 0.85 ml/minute.

** These acids are naturally occurring metabolites, and hence were 'unfed', endogenous substrates.

cyclobutane carboxylic acids produced the corresponding erythromycin A analogues. Thus, enzymes involved in the post polyketide modifications of the 6-DEB ring can be active even in the presence of these novel, bulky C-13 substituents. Unexpectedly, a similar profile was observed when these two particular substrates were fed to the wild type *S. erythraea* NRRL 2338 organism. This observation suggests that the specificity of the *S. erythraea* loading domain is not limited to the starter fatty acids propionate and acetate, as previously believed. However all the other substrates listed in Table 1 did not incorporate into the wild strain demonstrating that the recombinant avermectin loading domain is required for the production of the other erythromycins.

As reported previously, the 'unfed' NRRL 2338 pIG1 organism produced the isopropyl B and *sec*-butyl B analogues from endogenous fatty acids[14]. To encourage the production of additional novel erythromycins, secondary and tertiary feeds of substrate were often made to suppress the production of these two analogues. Erythromycin A (*i.e.*, C-13 ethyl) production from the recombinant organism was also suppressed by the addition of the antibiotic thiostrepton, which is used as a resistance marker in preparation of the recombinant organism.

Table 2 lists the *in vitro* antibacterial activity of the isolated novel erythromycins as compared with erythromycin A and B. Most of the novel erythromycins were less active than the parent antibiotics, with the exception of C-13 cyclopentyl analog (**2b**), which showed

Table 2. *In vitro* antibacterial activity of erythromycin analogues.

Compound	*P. multocida* (μg/ml)	*E. coli* (μg/ml)	*S. aureus* (μg/ml)
1a	0.78	12.5	<0.1
1b	0.39	25	0.2
2b	0.025	0.1	1.56
4a	1.56	25	0.39
4b	1.56	25	0.39
5a	6.25	100	0.39
5b	6.25	100	0.2
7b	25	>200	0.78
9b	3.13	100	0.2
10b	0.78	12.5	0.2
11b	6.25	>200	0.39

impressive activity against *P. multocida* and *E. coli.*

In summary, we report that biologically active, novel C-13 erythromycins were produced by the feeding of exogenous fatty acids to a recombinant *S. erythraea* strain. This work suggests the potential to produce a wide-range of novel polyketides by the precursor-directed biosynthetic technology together with PKS genetic manipulation.

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