BIOSYNTHETIC PRODUCTION OF ^{13}C AND ^{14}C ERYTHRONOLIDE LABELED ERYTHROMYCIN A.

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SUMMARY

(1,3,5,7,9,11,13-^{13}C)-Erythromycin A and [1,3,5,7,9,11,13-^{14}C]-erythromycin A were produced in liquid fermentation broths of *Saccharopolyspora erythraea* CA340 after the administration of (1-^{13}C)-sodium propionate and [1-^{14}C]-sodium propionate, respectively. Fermentations were carried out in shake flasks. Labeled erythromycin A products were isolated by ethyl acetate extraction of the fermentation broth, followed by chromatographic purification on silica gel and Sephadex LH-20. ^{13}C NMR verified (^{13}C) incorporation at the seven possible positions on the erythronolide nucleus. Mass spectral analysis of (^{13}C) labeled erythromycin A revealed a distribution of (^{13}C) label which could be rationalized based on the known biosynthetic pathway for production of the erythronolide nucleus.

Key Words: Erythromycin A, (1-^{13}C)-sodium propionate, [1-^{14}C]-sodium propionate, *Saccharopolyspora erythraea*, erythronolide.

INTRODUCTION

The erythromycins (1) are a group of structurally complex macrolide antibiotics elaborated in the liquid fermentation broths of *Saccharopolyspora erythraea*. The major product is erythromycin A (1, Fig. 1) which has desosamine and cladinose attached to the erythronolide

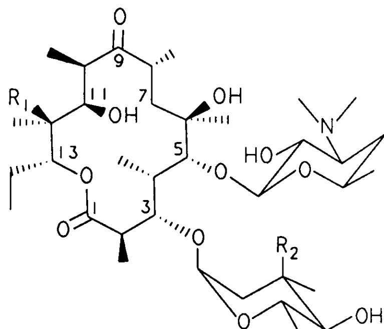

Fig. 1 (1) Erythromycin A, R1 = OH, R2 = OCH3
(2) Erythromycin B, R1 = H, R2 = OCH3
(3) Erythromycin C, R1 = OH, R2 = OH
Numbers indicate the position of the label derived from (1-13C)-sodium propionate and [1-14C]-sodium propionate.

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nucleus at positions 5 and 3, respectively. The organism also produces smaller amounts of erythromycin B (2, Fig. 1), erythromycin C (3, Fig. 1) and several other metabolites.

To monitor the complete degradation of erythromycin A and the erythronolide nucleus to CO_2 requires erythromycin A that is [^{14}C]-labeled on the erythronolide nucleus. Although [^{14}C-N-methyl]-erythromycin A is commercially available, it has only one [^{14}C]-labeled methyl group on the desosamine sugar moiety. Erythromycin A, [^{14}C]-labeled on the erythronolide, was required by the Division of Microbiology to evaluate the environmental impact of erythromycin use in the aquaculture industry. (^{13}C)-Labeled erythromycin A was required by the Division of Chemistry for Liquid Chromatography/Chemical Reaction Ionization-Mass Spectrometry method development.

Erythromycin A is a challenging synthetic target. However, the synthetic methodology developed previously (2) is impractical for the preparation of a labeled compound. Early studies of erythromycin A biosynthesis established that both [^{14}C]-propionate and [^{14}C]-methylmalonate are incorporated into the erythronolide nucleus (3, 4, 5, 6). Based on these results, we proposed that multiple labeled erythromycin A could be prepared biosynthetically by the administration of a suitably labeled precursor to the fermentation broth of *Saccharopolyspora erythraea*. The distribution of the (^{13}C) or [^{14}C] labels should be consistent with derivation of the erythronolide nucleus from seven building blocks, one as propionyl-CoA and six as methylmalonyl-CoA.

RESULTS & DISCUSSION

In preliminary investigations, we optimized the cultural conditions for the erythromycin producing organism, developed analytical methods for determining the most advantageous time to administer the labeled precursor, and developed purification methods for isolating erythromycin A. The *Saccharopolyspora erythraea* CA340 fermentation in shake flasks produced erythromycin A at 95% purity and at levels of approximately 0.5 g/L. HPLC analyses indicated that after an initial lag phase the levels of erythromycin A increased logarithmically to reach maximal levels between eight and eleven days.

Our initial results led us to investigate the incorporation of (1-^{13}C)-sodium propionate into the erythronolide nucleus of erythromycin A. After fermentation for 3 days, (1-^{13}C)-sodium propionate was administered as pulsed daily feedings over the next 3 days. The fermentation was

stopped after 8 days and erythromycin A was isolated. Purification of the (^{13}C) material was achieved using flash silica gel chromatography along with two semi-preparative HPLC steps.

The product was characterized by ^{13}C NMR spectroscopy. ^{13}C NMR analysis of our isolated erythromycin A showed seven (^{13}C) enriched resonances (7) at carbon atoms 1, 3, 5, 7, 9, 11 and 13 of the erythronolide nucleus (Fig. 1). Interestingly, the enrichment at C13 was approximately twice that at the other carbon atoms. No evidence for (^{13}C) enrichment in the sugar moieties was observed which would be consistent with the observation by Corcoran (8) that D-glucose furnishes the carbon backbone for the desosamine and cladinose sugars.

The product was also characterized by mass spectrometry. Unlabeled erythromycin A and (^{13}C) enriched erythromycin A were analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC/ESIMS). Up to seven (^{13}C) atoms were incorporated into erythromycin A. Fig. 2 represents the isotopic contribution of each (^{13}C) enriched species to the ESI mass spectrum of the (^{13}C) enriched erythromycin A. The figure shows an unusual (^{13}C) distribution. The major species was $^{13}C_1$. The $^{13}C_2$ species is nearly nonexistent. A Gaussian distribution for species enriched from $^{13}C_3$ through $^{13}C_7$ was observed. The relative abundance of the most intense protonated molecule of each species was used to calculate the overall (^{13}C) incorporation into erythromycin A, which is shown in Table 1. Enrichment of the (^{13}C) label was 24.49 mol% (2.47 atom%) for erythromycin A, 4.34 atom% for erythronolide and 13.03 atom% for all of the seven possible erythronolide sites.

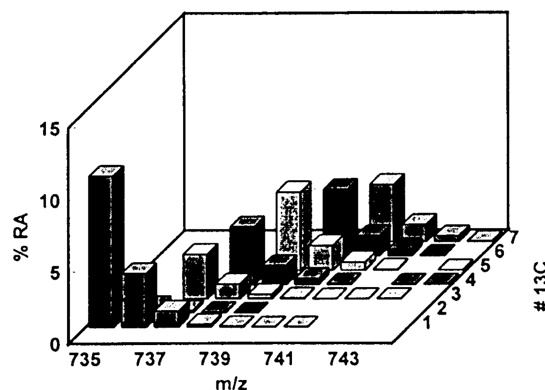

Fig. 2 Isotopic contribution of each (13C) enriched species to the ESI mass spectrum of (13C) labeled erythromycin A.

Table 1 Erythromycin A - (^{13}C) Incorporation

(^{13}C) Label	*m/z*	$[M+H]^+$ % RA	mol %
$^{13}C_0$	734	100	75.51
$^{13}C_1$	735	10.54	7.96
$^{13}C_2$	736	0.51	0.38
$^{13}C_3$	737	3.14	2.37
$^{13}C_4$	738	4.08	3.08
$^{13}C_5$	739	5.49	4.15
$^{13}C_6$	740	4.67	3.53
$^{13}C_7$	741	4.01	3.02
	Total	132.44	100

The distribution of (^{13}C) label can be rationalized because *S. erythraea* uses propionyl-CoA as a starter unit and L-methylmalonyl-CoA for elongation in erythronolide biosynthesis. Propionyl-CoA and L-methylmalonyl-CoA are derived from separate metabolic pools (3, 4). Figure 3 illustrates the metabolic fate of (^{13}C) label derived from (1-^{13}C) sodium propionate (9). Initially, labeled propionyl-CoA is available for incorporation with endogenous unlabeled L-methylmalonyl-CoA, yielding erythronolide with only one (^{13}C) label at C13. As metabolism progresses, labeled propionyl-CoA is eventually converted into labeled L-methylmalonyl-CoA, which is then available for elongation at six possible sites (C1, C3, C5, C7, C9 and C11) as seen in Fig.1. Label that is not incorporated into erythromycin A must be lost as $^{13}CO_2$ via the TCA cycle or by conversion into other metabolic products.

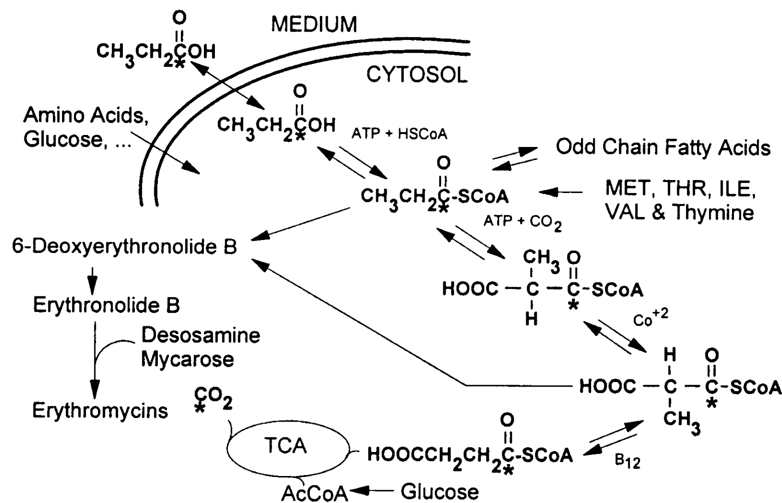

Fig. 3 *Saccharopolyspora erythraea* Metabolism

We became concerned about safety since $^{14}CO_2$ gas would be evolved during the [^{14}C] experiment. Therefore, we tested our fermentation in a system identical to that used in microcosm studies (10, 11). The system was sealed to the atmosphere but had a controlled air inlet and an outlet for trapping volatile organic compounds and evolved $^{14}CO_2$. Several attempts at using this system consistently produced lower yields of erythromycin A than the shaken flasks. Therefore, we decided instead to run our [^{14}C] experiment in shake flasks in an environmental shaker that was housed inside a laboratory fume hood.

[1-^{14}C]-sodium propionate was incorporated into the erythronolide nucleus of erythromycin A. After fermentation for 3 days, [1-^{14}C]-sodium propionate was administered as pulsed daily feedings over the next 5 days. The fermentation was stopped after 8 days and erythromycin A was isolated. The [^{14}C] labeled material was purified by flash silica gel chromatography, which produced material determined to be 96% radiochemically pure. Further purification on Sephadex LH-20 produced 121.7 mg of [^{14}C]-labeled erythromycin A, determined to be 97.5 % radiochemically pure. The specific activity of the product was 327 μCi/mmol and the total activity was 54.2 μCi.

Purification on Sephadex LH-20 after silica gel flash chromatography represented a suitable alternative to time-consuming preparative HPLC procedures and produced material of sufficient purity for microbiological degradation experiments.

The specific activity of erythromycin A could probably be increased by the use of a different labeled precursor. [1,2-^{14}C]-sodium propionate would add an additional seven [^{14}C] atoms to the erythronolide nucleus. However, a better alternative would be [3-^{14}C]-sodium propionate which could be more efficiently incorporated since the labeled carbon would not be involved in any of the enzymatic steps required for biosynthesis of erythronolide.

EXPERIMENTAL

<u>Materials and Methods</u> *Saccharopolyspora erythraea* CA340 was obtained from Abbott Laboratories, Chicago, IL (12). Fermentations were carried out in a New Brunswick Instruments G-25F refrigerated environmental shaker at 32°C and 225 rpm. Fermentation broths were

centrifuged using a Jouan CR 4.22 refrigerated centrifuge. Flash chromatography, TLC and normal phase HPLC used the same mobile phase which was 95% dichloromethane/5% methanol/0.5% ammonium hydroxide. Flash chromatography was performed using EM Science flash silica 40/60 μm. TLC was performed, following the method of Koch (13), using Baker-Flex silica gel plates that were visualized with a 5% anisaldehyde solution in (19:1) ethanol:conc. sulfuric acid by heating. Normal phase HPLC (14) was performed with a Beckman Si (4.6 mm x 250 mm, 5 μm) analytical column or a Si (10 mm x 250 mm, 5 μm) semi-preparative column connected to a Varian 9012 pump, a Varian 9050 UV detector at 220 nm or a Packard Instruments Flo-One Radiomatic HPLC detector. NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. The deuterated methanol resonances at 4.8 ppm and 49.0 ppm were used as a reference for ^{1}H spectra and ^{13}C spectra, respectively. ESIMS analyses were carried out on a Finnigan MAT TSQ7000 triple quadrupole tandem mass spectrometer equipped with an electrospray ion source after HPLC separation of erythromycin A from impurities. The mass spectrometer was operated in the positive ion mode with mass scanning of Q1 from 720 to 750 daltons and with a 0.6 sec cycle time. For accurate ion statistics, mass spectra were summed across the top 50% of the erythromycin A HPLC peak. (1-^{13}C)-Sodium propionate (1-^{13}C, 99%) was purchased from Cambridge Isotope Laboratories, Andover MA. [1-^{14}C]-Sodium propionate (20 mCi at 51 mCi/mmol) was purchased from NEN-Dupont, Boston, MA. For determining specific activity, UV absorbance was recorded on a Varian Cary 219 UV spectrophotometer. Liquid scintillation analyses were recorded on a T M Analytic Instruments Tri-Carb Mark III liquid scintillation counter. All other reagents were used as supplied unless otherwise noted. Water was purified with a Milli-Q system (Millipore, Bedford, MA).

Preparation of (1,3,5,7,9,11,13-^{13}C)-Erythromycin A One loopful of mature spores from a slant of *Saccharopolyspora erythraea* CA340 was used to inoculate 100 mL of seed culture (15), composed of soy flour (15 g/L), glucose monohydrate (15 g/L) and calcium carbonate (1 g/L) in water. The inoculated medium was incubated for 3 days at 32°C with shaking at 225 rpm in an environmental shaker. The mature seed culture was used to inoculate six 500 mL

fermentation flasks, each containing 50 mL production medium (16) composed of soy flour (20 g/L), corn starch (15 g/L), corn steep liquor (5 g/L) and calcium carbonate (1 g/L) in water. The inoculated fermentation flasks were incubated at 32°C with shaking at 225 rpm and monitored for erythromycin A production by HPLC. The fermentation was allowed to proceed for 3 days. (1-^{13}C)-Sodium propionate was dissolved in water and aseptically administered through a 0.45 μm nylon filter to each fermentation flask. A total of 90 mg of precursor was administered as daily pulsed feedings over the next three days. The fermentation was stopped after it had proceeded for 8 days. The broth was centrifuged at 4500 rpm at 5°C for 30 min to remove the organism. The supernatant was filtered through glass wool and the pH adjusted to 10 by the careful addition of NaOH (1.0 M). The broth was extracted three times with one half volumes of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate, filtered and concentrated *in vacuo*. This gave 1.45 g as a yellow oil. Analysis of the extract by TLC indicated that the major product was erythromycin A. The crude extract was partitioned in a 1:1 mixture of hexane and methanol (50 mL), the lower methanol layer was collected and concentrated *in vacuo*. The residue was further purified by flash chromatography on a silica gel column eluted with 95% dichloromethane/5% methanol/0.5% ammonium hydroxide. Ten mL fractions were collected and analysed for erythromycin A by TLC. Concentration of the fractions containing erythromycin A yielded 90 mg of crude erythromycin A as an off-white powder. HPLC analysis of this material showed a major impurity in addition to erythromycin A. The solid was further purified by semi-preparative HPLC (Si, 10 mm x 250 mm, 5 μm column eluted with 95% dichloromethane/5% methanol/0.5% ammonium hydroxide). This gave 15 mg of erythromycin A as a white powder, shown to be 95% pure by HPLC. Analysis of the sample by ^{13}C NMR showed enrichments for the resonances due to C1, C3, C5, C7, C9, C11 and C13 of the erythronolide nucleus. The assignments of the ^{13}C resonances were based on previous work by Ager and Sood (7).

<u>Preparation of [1,3,5,7,9,11,13-^{14}C]-Erythromycin A.</u> Culture conditions were the same as those used to prepare (1,3,5,7,11,13-^{13}C)-erythromycin A. After 3 days of fermentation, a

total of 20 mCi (37.6 mg) [1-^{14}C]-sodium propionate was administered in five daily doses to twenty fermentation flasks. The fermentation was stopped after 8 days. Crude erythromycin, 0.960 g as a yellow oil, was isolated and TLC analysis indicated that erythromycin A was the major product. The crude product was purified by flash chromatography yielding 165.8 mg crude erythromycin A as a white solid. HPLC analysis indicated a major peak for erythromycin A with some earlier eluting impurities. Radio-HPLC analysis showed the product to be only 96% radiochemically pure. To avoid time-consuming purification by preparative HPLC, the material was loaded onto a Sephadex LH-20 column and eluted with 47.6% chloroform/47.6% hexane/4.8% methanol. Five mL fractions were collected and analysed for erythromycin A by TLC. The fractions containing erythromycin A were pooled and concentrated *in vacuo*. This gave 157 mg of erythromycin A as a white powder. HPLC analysis indicated that the sample was free of the impurities encountered in the commercial sample of erythromycin A and showed a major peak which co-eluted with an authentic standard. Radio-HPLC analysis indicated that the product was 97% radiochemically pure. The specific activity of the product, measured by the methods of Koch (13), was 0.327 mCi/mmol and the total activity was 54.2 μCi. Based on the distribution of the (^{13}C) label in (1,3,5,7,9,11,13-^{13}C)-erythromycin A, the same carbon atoms were assumed to be labeled with [^{14}C] to give [1,3,5,7,9,11,13-^{14}C]-erythromycin A. However, with the lower dose to culture ratio, it would not be surprising if the bulk of the label was at the C13 position of the erythronolide nucleus.

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