Biosynthesis of Erythromycin: Origin of the Methyl Protons

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^{13}C-Nuclear magnetic resonance (^{13}C-NMR) spectroscopy has been used to locate deuterium atoms incorporated biosynthetically into erythromycin from [3-$^{13}CD_3$]- and [3-^{13}C]sodium propionate and L-[$^{13}CD_3$]- and L-[^{13}C]methionine in *Saccharopolyspora erythraea* JCM 4748. The α-shifts of deuterated methyl groups were clearly observed in broad-band deuterium and proton-decoupled ^{13}C-NMR spectra. The seven propionate-derived methyl groups and the four methionine-derived methyl groups were shown to undergo no exchange of methyl protons during the biosynthesis of erythromycin.

Keywords erythromycin; biosynthesis; broad-band deuterium and proton-decoupled ^{13}C-NMR; [3-$^{13}CD_3$]sodium propionate; L-[$^{13}CD_3$]methionine

Erythromycin A (**1**) is an antibiotic substance used in human chemotherapy.[1] It is a macrocyclic (14-membered ring) lactone bearing the sugar moieties desosamine and cladinose (3-*O*-methylmycarose).

Our knowledge of the carbon balance in the pathway leading to the aglycone of erythromycin A (**1**) is essentially complete, due to the application of ^{13}C-nuclear magnetic resonance (^{13}C-NMR) spectroscopy.[2a–e] However, the biochemical inventory of the protons at those positions on the aglycone and sugar moieties that could be involved in oxidation–reduction or prototropic exchange with the medium is still fragmentary.

Separate feeding experiments using [3-^{13}C]- and [3-$^{13}CD_3$]sodium propionate[3] and L-[^{13}C]- and L-[$^{13}CD_3$]-methionine[3b,4] were carried out, and erythromycin was isolated and purified from the culture broths. ^{13}C-NMR spectral analysis was conducted to elucidate the deuterium isotopic effects at the seven propionate-derived methyl groups on the aglycone and the four methionine-derived methyl groups on the sugar moieties.

Results and Discussion

Whole cells of *Saccharopolyspora erythraea* JCM 4748 were incubated with [3-^{13}C]sodium propionate (**6**) (99 atom% ^{13}C; 150 mg), then ^{13}C-labeled erythromycin A (**2**) (4.2 mg) was isolated and subjected to ^{13}C-NMR measurement. The ^{13}C-NMR spectrum of **2** is shown in Fig. 2 (below), and ^{13}C-enriched signals of the seven methyl groups were observed at 9.14, 10.67, 11.98, 15.97, 16.16, 18.23, and 27.09 ppm, corresponding to C15, C21, C18, C14, C19, C17, and C16, respectively.[2b,5] On the other hand, incubation with [3-$^{13}CD_3$]sodium propionate (**7**) (99 atom% ^{13}C, 98 atom% D; 120 mg) gave 3.4 mg of multiply labeled erythromycin A (**3**). Broad-band deuterium and proton-decoupled ^{13}C-NMR (^{13}C-{^{1}H}{D}NMR) spectroscopy[6] is useful in dealing with carbon-13 and deuterium atoms. We observed seven ^{13}C-enriched signals, which were shifted upfield by 77.8—91.0 Hz (−0.77——0.91 ppm) owing to deuterium α-isotope effects, at 8.36 (C15), 9.81 (C21), 11.17 (C18), 15.12 (C14), 15.35 (C19), 17.37 (C17), and 26.18 (C16) ppm (Fig. 2 above and Table I). The results strongly suggest that the deuterium label suffered no exchange during the processes leading from the precursor to **3**. In addition, as regards the two hydroxyl groups at C6 and C12[3b] in erythromycin A, involvement of the epoxide intermediate (**8**) could be ruled out (Fig. 3).

The ^{13}C-NMR spectrum of ^{13}C-enriched erythromycin A (**4**) (5.6 mg) derived from L-[^{13}C]methionine (**9**) (99 atom% ^{13}C; 124 mg) is shown in Fig. 4 (below). Three

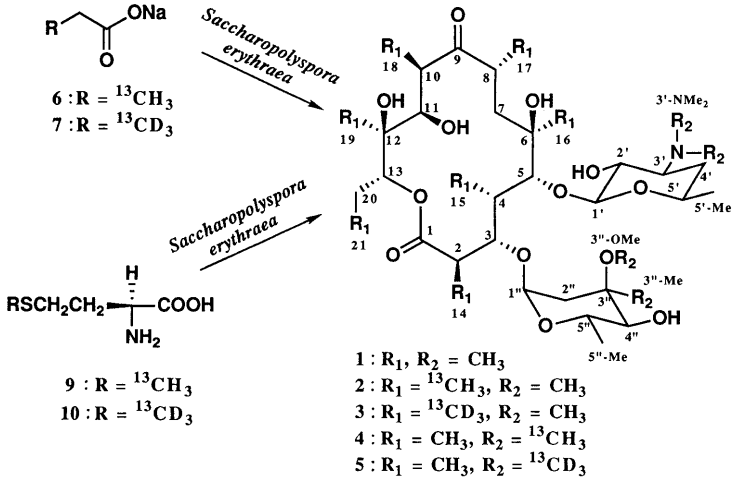

Fig. 1. Chemical Structures of Labeled Erythromycins (**2**, **3**, **4**, and **5**) and Their Labeled Precursors (**6**, **7**, **9**, and **10**)

signals appeared at 21.51, 40.30, and 49.51 ppm, corresponding to C3″-Me, C3′-NMe_2, and C3″-OMe. Furthermore, L-[$^{13}CD_3$]methionine (**10**) (99 atom% ^{13}C, 98 atom% D; 124 mg) provided multiply labeled erythromycin A (**5**) (5.3 mg), which was isolated and examined by the above ^{13}C-NMR technique. [$^{13}CD_3$]Enriched signals of **5** were seen at 20.67 (C3″-Me), 39.39 (C3′-NMe_2), and 48.69 ppm (C3″-OMe) with α-shifts of 82.1—91.0 Hz upfield (Fig. 4 above and Table II). This shows that the methyl group of **10** was incorporated into the sugar moieties of erythromycin A (**5**) without exchange of the methyl deuteriums. These results are consistent with the hypothesis that the biosynthesis of the sugar moieties on erythromycin A is similar to the known pathways from D-glucose to L-cladinose and D-desosamine.[7]

TABLE I. α-Shifts of Labeled Erythromycins. Comparison of **2** with **3**

Carbon No.	Chemical shifts (ppm)		α-Shifts	
	[3-^{13}C]	[3-$^{13}CD_3$]	ppm	Hz
C15	9.137	8.363	−0.774	−77.8
C21	10.669	9.808	−0.861	−86.5
C18	11.983	11.165	−0.818	−82.2
C14	15.967	15.121	−0.846	−85.1
C19	16.157	15.354	−0.803	−80.7
C17	18.229	17.368	−0.861	−86.5
C16	27.089	26.184	−0.905	−91.0

Conclusion

The α-shifts associated with the seven propionate-derived [$^{13}CD_3$]methyl groups on the aglycone and the four methionine-derived [$^{13}CD_3$]methyl groups on the sugar moieties of erythromycin, observed by broad-band deuterium and proton-decoupled ^{13}C-NMR spectroscopy, indicated that these deuteriums suffered no exchange during the biosynthetic processes of erythromycin A.

Experimental

Instruments ^{13}C-NMR and ^{13}C-{^1H}{D}NMR spectra were recorded on a JEOL GSX-400 spectrometer (100 MHz) in $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard. The conditions were: spectral width 24,038.5 Hz; acquisition time 0.682 s; repetition delay 2.5 s; and 30° pulse.

Materials and Organism [3-^{13}C]Sodium propionate (**6**)[8] and

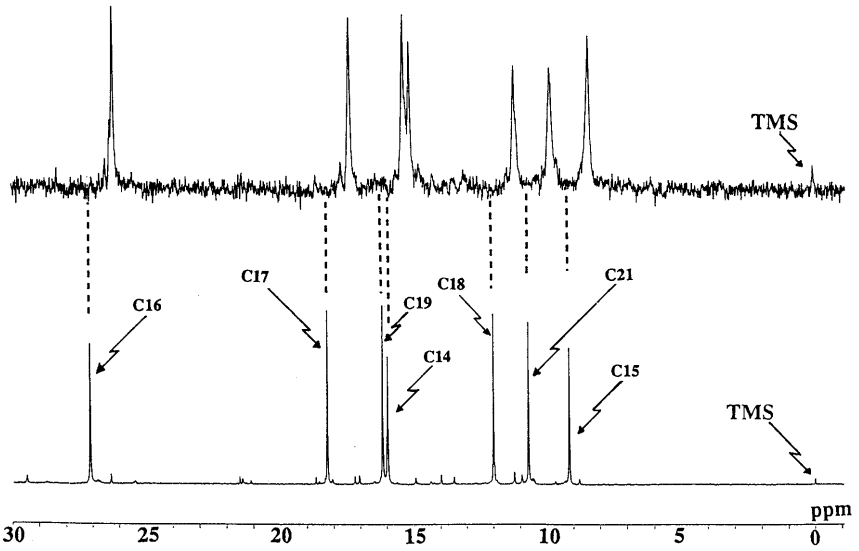

Fig. 2. ^{13}C-NMR Spectra ($CDCl_3$, 100 MHz) of Labeled Erythromycins (**2** and **3**)
Below: ^{13}C-NMR Spectrum of **2**. Above: ^{13}C-{^1H} {D} NMR Spectrum of **3**.

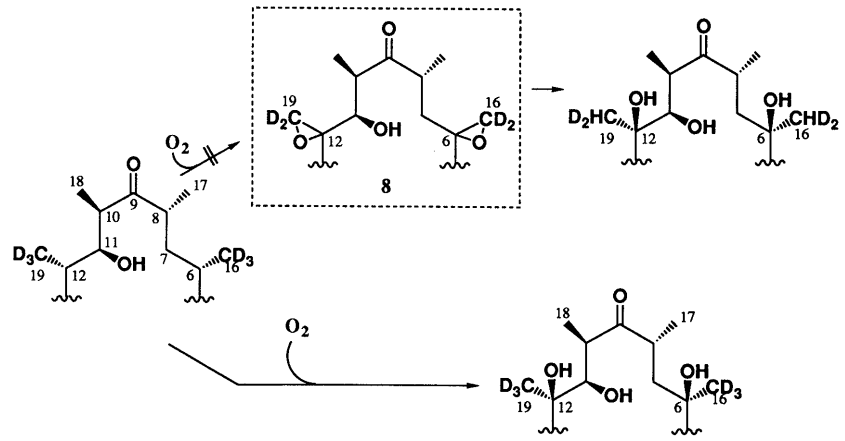

Fig. 3. Generation of the Hydroxyl Groups at C6 and C12 in the Biosynthesis of Erythromycin A

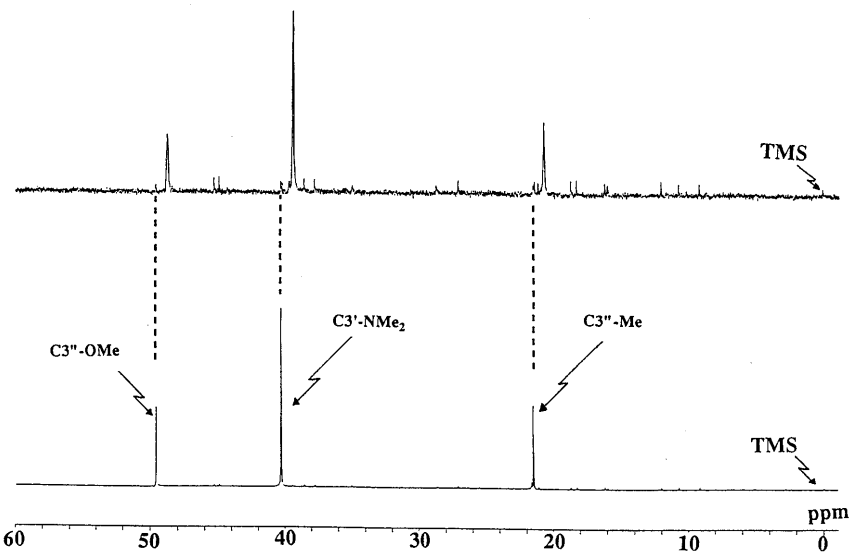

Fig. 4. ^{13}C-NMR Spectra ($CDCl_3$, 100 MHz) of Labeled Erythromycins (**4** and **5**)
Below: ^{13}C-NMR Spectrum of **4**. Above: ^{13}C-{^{1}H} {D} NMR Spectrum of **5**.

TABLE II. α-Shifts of Labeled Erythromycins. Comparison of **4** with **5**

Carbon No.	Chemical shifts (ppm)		α-Shifts	
	^{13}C	$^{13}CD_3$	ppm	Hz
C3″-Me	21.513	20.667	−0.846	−85.1
C3′-NMe_2	40.297	39.393	−0.904	−91.0
C3″-OMe	49.507	48.690	−0.817	−82.1

L-[^{13}C]methionine (**9**)[9] were synthesized from [^{13}C]iodomethane (99 atm% ^{13}C), which was supplied by CIL (U.S.A.). [3-$^{13}CD_3$]Sodium propionate (**7**)[8] and L-[$^{13}CD_3$]methionine (**10**)[9] were synthesized from [$^{13}CD_3$]iodomethane (99 atom% ^{13}C, 98 atom% D), which was supplied by MDS (Canada). The strain used was *Saccharopolyspora erythraea* JCM 4748.

Media and Growth Conditions Seed culture medium and fermentation culture medium were CSL medium, which consisted of 1.5% glucose, 0.5% corn steep liquor powder, 1.5% bacto-soytone, and 1.5% sodium chloride adjusted to pH 7.4 with 0.1 N sodium hydroxide before autoclaving. Seed culture was done in 20 ml of the medium at 28 °C for 3—5 d in a test tube. Then, 1 ml was transferred to erythromycin fermentation culture in 50 ml of the medium at 28 °C for 4 d in a 500 ml baffled Erlenmeyer flask on a rotary incubator (200 rpm).

Incorporation of Labeled Precursor Amounts of labeled precursor used were as follows: [3-^{13}C]sodium propionate (**6**) (150 mg), [3-$^{13}CD_3$]sodium propionate (**7**) (120 mg), L-[^{13}C]methionine (**8**) (124 mg), and L-[$^{13}CD_3$]methionine (**9**) (124 mg). Their aqueous solutions were filtered through a membrane filter (Nalgen) and added to the fermentation culture medium after 24 h.

Extraction from Culture Broth and Purification of Erythromycin Culture broth was centrifuged for 30 min at 12300 × *g* (8000 rpm). The supernatant was filtered through cotton, and adjusted to pH 9.0 with 1 N sodium hydroxide. Products were extracted with chloroform, and were purified by silica gel chromatography (dichloromethane : methyl alcohol : ammonium hydroxide = 200 : 10 : 1) to give erythromycin A. (**2**, 4.2 mg; **3**, 3.4 mg; **4**, 5.3 mg; **5**, 5.6 mg).

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