^{13}C-NMR EVIDENCE FOR THE BIOSYNTHETIC INCORPORATION OF ACETATE INTO MINIMYCIN AND COMPOUNDS RELATED TO KREBS CYCLE

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1. Introduction

In recent years, application of carbon 13 NMR (CMR) for biosynthetic study utilizing ^{13}C-labeled acetate has been extensively developed for polyketide and isoprenoid compounds. However, its application to the biosynthesis of the compounds derived from the Krebs cycle has been limited because of the high isotope dilution and extensive randomization through the cycle. To our knowledge, the maleimide ring of showdomycin [2] and the α-aminoadipoyl group of penicillin N and cephalosporin C [3] are the only examples of this class of compounds utilizing [1- or 2-^{13}C]acetate for their biosynthetic study. We considered that this difficulty may be overcome by utilizing doubly-labeled [1,2-^{13}C]acetate from the following reasons. First, CMR analysis depends not on enrichment of signals, but on ^{13}C–^{13}C coupling which appears as independent satellite bands. Therefore, high incorporation may not be required for analysis. Second, one cleavage of the C–C bond originating from the same acetate unit occurs, there would be no coupling observed. Thus, the extensive recycling through the Krebs cycle or its shunt pathways would not result in the complex coupling pattern.

As an example, we report herein the utilization of [1,2-^{13}C]acetate for the biosynthetic study of the oxazine ring of the C-nucleoside antibiotic, minimycin [4,5], which has recently been found to be biosynthesized from α-ketoglutarate [6]. Succinic acid and fumaric acid from the culture filtrate as well as glutamic acid from the cell protein were also analyzed to confirm the coupling pattern.

2. Materials and methods

[1,2-^{13}C]Sodium acetate was purchased from MSD Canada Ltd. and [1-^{14}C]sodium acetate was purchased from Radiochemical Centre, Amersham. CMR spectra were recorded on a JEOL FX-100 spectrometer at 15°C. Precision of J values is ± 0.36 Hz. Radioactive measurements were made in a Packard 3330 liquid scintillation spectrometer using Bray's scintillation solution. Maintenance and fermentation of *Streptomyces hygroscopicus* were same as described before [7] except that the glucose content was reduced to 2% and 500 ml fermentation flasks containing each 70 ml of the medium were used.

2.1. *Biosynthesis of labeled minimycin*

[1,2-^{13}C; 1-^{14}C]Acetate (1 g, 90 atom% of ^{13}C; spec. act. of ^{14}C, 2.09 × 10^{-2} Ci/mol) was added into 20 flasks of the shaking culture in two portions, 22 and 45 hr after inoculation. After the additional 72 h incubation, the fermentation broth was acidified (pH 3) with 10% HCl and centrifuged. Minimycin was isolated from the supernatant as described before [7]. Yield: 8.0 mg, mp 164–166°C (dec).

2.2. *Isolation of succinic acid from the culture broth*

Succinic acid was eluted from the silicic acid column with $CHCl_3$–EtOH (8:2) in advance to minimycin. The fractions were concentrated to dryness. The residue was purified by sublimation in vacuo (160–180°C) followed by preparative thin-layer chromatography (Avicel cellulose, butanol–acetic acid–water, 4:1:2), affording 2.5 mg of crystalline

residue, mp 175–185°C. It was shown to be contaminated with a small amount of fumaric acid by CMR analysis (fig.1b).

2.3. *Isolation of cell protein glutamic acid*

The centrifuged cells (wet wt., 75 g) were washed twice with water and homogenized with a French press. Protein was fractionated and hydrolyzed with 6 N HCl (110–120°C, 20 h). Glutamic acid was isolated as described [8]. Yield 2.2 mg, mp 214°C.

3. Results and discussion

Chemical shift (δ_c in ppm, calculated from internal dioxane, 67.4 ppm, solvent: D_2O-H_2O, 1:1) and assignment of minimycin is as follows: 150.8 (2), 163.9 (4), 114.9 (5), 155.5 (6), 78.8 (1′), 74.3 (2′), 71.4 (3′), 83.9 (4′), 62.2 (5′). Assignment of the ribose moiety follows the reported data of C-nucleosides such as formycin [9], showdomycin [10], and pseudouridine [10]. C5 and C6 can easily be assigned by the chemical shifts and a gated decoupling spectrum [11]. Assignment for two carbonyl signals (C2 and C4) made from the three bond couplings (${}^3J_{C4\text{-}H1'} = 3.7$, ${}^3J_{C2\text{-}H6} = 9.5$, ${}^3J_{C4\text{-}H6} = 8.8$ Hz), which were confirmed by a selective long-range proton decoupling spectrum [12].

A spectrum of minimycin biosynthesized from [1,2-^{13}C; 1-^{14}C]acetate is shown in fig.1a. Small but distinct satellites are observed for both C4 and C5. The coupling constant (J_{cc} = 64.5 Hz) is a reasonable value for a sp^2–sp^2 interaction [13]. This is an unambiguous proof for the incorporation of an intact acetate unit. As granted, acetyl coenzyme A is involved in the Krebs cycle to give α-ketoglutarate, in which the primary incorporation site is C4–C5 (shown by a box in scheme 2). Therefore, this data support the finding that the carbons 3, 4, and 5 of glutamate were incorporated asymmetrically into the carbons 6, 5, and 4 of minimycin (scheme 1) [6]. Indeed, the chemical degradation has shown that 65% of ^{14}C incorporated into minimycin resided on C4. The rest of ^{14}C was found to be on C2 (table 1). The incorporation into C4 determined from radioactive assay was 0.26% in good agreement with the CMR data (centre singlet and satellite signal ratio, 1:0.24). This coupling is also to

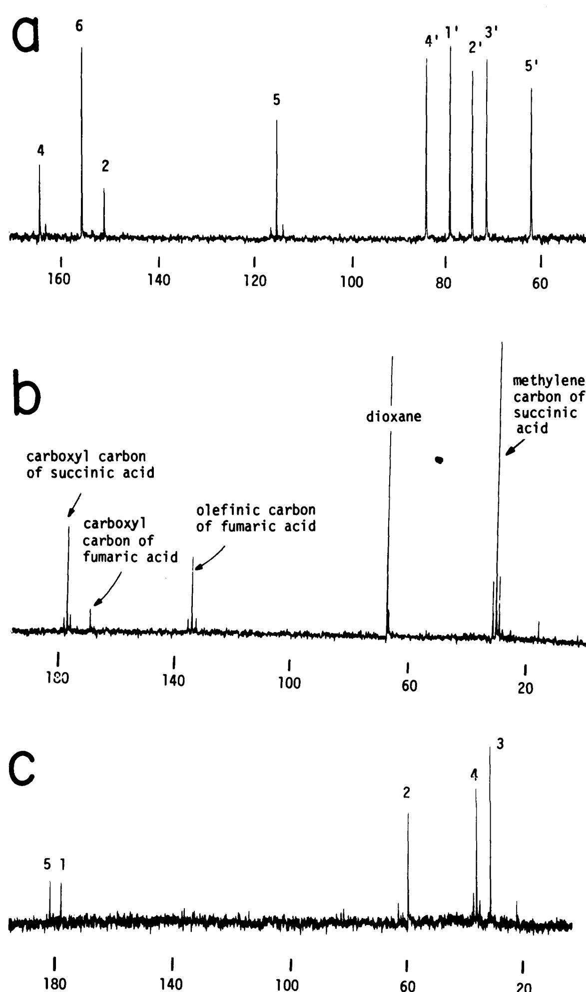
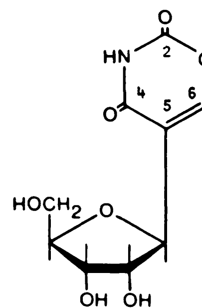


Fig.1. CMR spectra of (a) minimycin, (b) succinic acid (plus fumaric acid), and (c) glutamic acid labeled with [1,2-^{13}C] acetate. Spectra were recorded at 25.2 MHz in D_2O–H_2O (1:1).



Scheme 1. The structure of minimycin.

Table 1
Incorporation and distribution of ^{14}C from [1,2-^{13}C; 1-^{14}C]acetate into minimycin

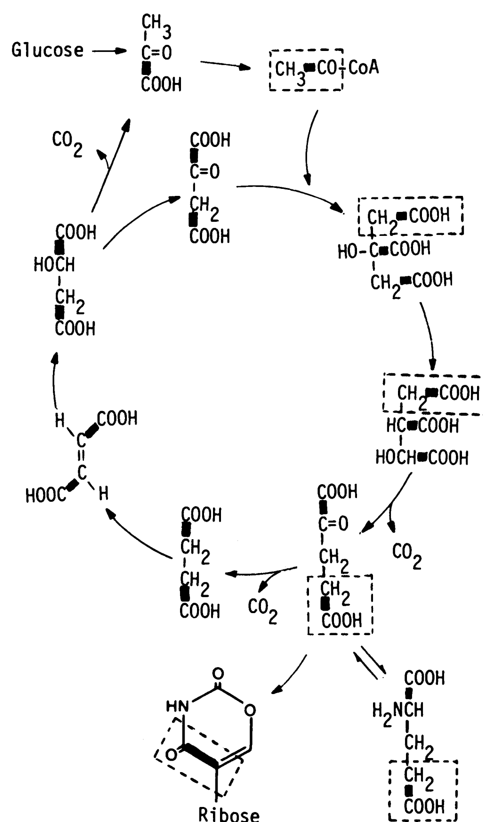
Acetate added	20.9 µCi/mol
Minimycin isolated	0.017 µCi/mol
Dilution[a]	228
^{14}C on carbon 2[b]	33.2%
^{14}C on carbon 4[c]	64.5%

[a]Spec. act. of acetate added ÷ spec. act. of minimycin isolated
[b]Obtained from CO_2 liberated by alkaline hydrolysis [7]
[c]Obtained by acid hydrolysis followed by ceric sulfate decarboxylation of crystalline 2,3-*O*-isopropylidene-β-D-ribofuranosylglyoxylamide, which is a $KMnO_4$ oxidation product of minimycin acetonide [6,7]

confirm the C4 signal assignment, because C4 has the adjacent C5, however, C2 is isolated.

As shown in this case, the use of [1,2-^{13}C]acetate offers a sensitive and reliable method for studying the biosynthesis of the compounds which are derived from the Krebs cycle (e. g. amino acids, nucleic acids and the related compounds). The extensive recycling through the Krebs cycle and the shunt pathways would not result in randomization of the label seen in the case of [2-^{13}C]acetate [2,3]. Scheme 2 illustrates that the C–C coupling pattern of the Krebs cycle member acids remains unchanged after multiple recycling. To support this argument and to show further the efficiency of this method, succinic acid acid was isolated from the culture filtrate and glutamic acid was isolated from the cell protein. CMR spectra of these compounds are shown in fig.1 (b and c). It is to be noted that only a few mg of samples were enough for the analysis. Even fumaric acid, a small contaminant in a succinic acid sample (J_{cc} = 54.9 Hz) was able to demonstrate satellites (J_{cc} = 70.2 Hz) on its olefinic carbon (fig.1b). Satellites were observed on C4 and C5 (J_{cc} = 53.7 Hz) of a glutamic acid sample (fig.1c). However, no apparent coupling was observed between C1 and C2, indicating that the contribution of recycling is less than one-forth.

In summary, we have been able to analyze by CMR the selective incorporation of an intact acetate unit into the oxazine ring of minimycin using [1,2-^{13}C]acetate. This method may be regarded as a useful tool to study the biosynthesis of compounds which are derived from the Krebs cycle.



Scheme 2. The role of the Krebs cycle and malic enzyme on the distribution of acetate unit (C▬C) and the biosynthesis of minimycin. Note that this coupling pattern would remain unchanged after the multiple recycling. The boxes show the primary site for an incorporated acetate unit.

Acknowledgement

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