BIOSYNTHETIC STUDIES ON THE MACROLIDE ANTIBIOTIC TURIMYCIN USING ^{14}C-LABELED PRECURSORS

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Using a strain of *Streptomyces hygroscopicus* JA 6599 several ^{14}C-compounds were investigated as potential precursors of the macrolide antibiotic turimycin followed by partial degradation to localize the radioactivity. L-Methionine-^{14}C-methyl and *n*-butyrate-1-^{14}C were incorporated exclusively and in a specific manner. The incorporation ratios were dependent on the addition time of the precursors. Studies of the incorporation of acyl-mycaroses and demycarosyl turimycin into the antibiotic are also reported.

Turimycin is a basic macrolide antibiotic complex produced by *Streptomyces hygroscopicus* JA 6599[1]. The structure of the individual turimycin components of the used strain is identical to leucomycins consisting of a sixteen-membered lactone ring with a hydroxyl group in the 3-position and the disaccharide mycarosyl-mycaminose bonded to the 5-position of the lactone. The 4''-position of mycarose is acylated with isovaleryl, propionyl, acetyl, *iso*- and *n*-butyryl groups (H. FRICKE, unpublished data) (Fig. 1).

This paper is concerned with the incorporation of several ^{14}C-labeled compounds at different times of fermentation and the biosynthesis of this antibiotic.

Materials and Methods

Organism

Streptomyces hygroscopicus strain No. JA 6599–//R27–158V–2 which is an efficient producer of turimycin was used for the experiments.

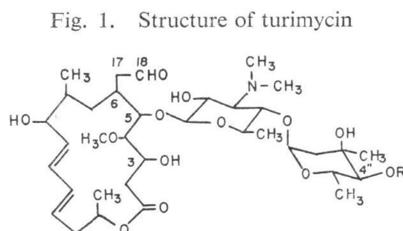
Culture Conditions

The seed culture medium (1.5% glucose, 1% corn steep liquor (dry weight), 0.5% baker's yeast, 0.5% $CaCO_3$, pH 7.2 after sterilization)[2] was inoculated with a spore suspension of *Streptomyces hygroscopicus* grown on a sporulation agar and incubated at 28°C for 44 hours on a rotary shaker. Eight ml of the inoculum were transferred to each series of 500 ml Erlenmeyer flasks containing 100 ml of medium of following composition: 3% potato starch, 0.7% glucose, 0.5% molasses, 2% soybean meal extract, 0.3% yeast extract, 0.07% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 2H_2O$ and 0.3% $CaCO_3$. The fermentation was carried out on a rotary shaker for 72 hours at 25°C by aeration and using a ^{14}C-CO_2-trapping apparatus.

Radioactive Isotopes

Na-Acetate-1-^{14}C (specific radioactivity 57.3 mCi/mmole), Na-propionate-1-^{14}C (40.5 mCi/mmole), *n*-butyric acid-1-^{14}C (230 mCi/mmole), succinic acid-1.4-^{14}C (6 mCi/mmole), ethanol-1-^{14}C (10.7 mCi/mmole), *n*-propanol-1-^{14}C (78 mCi/mmole) were purchased from Isocommerz GmbH, Berlin, GDR; L-valine-U-^{14}C (125 mCi/m-

Fig. 1. Structure of turimycin



mole), L-leucine-U-^{14}C (185 mCi/mmole), L-isoleucine-U-^{14}C (92 mCi/mmole), D-glucose-U-^{14}C (180 mCi/mmole) from the Institute for Research Production and Uses of Radioisotopes, Prague, Czechoslovakia, and methyl-^{14}C-L-methionine (56 mCi/mmole) from The Radiochemical Centre, Amersham, England.

Measurement of Radioactivity

Liquid Scintillation Counter (LKB-Wallac 81 000) was used for the measurement of radioactivity. The scintillation liquid consisted of 5 g PPO and 0.3 g POPOP in 1,000 ml of toluene. The samples were counted in 12 ml of this scintillation cocktail and 3 ml of ethanol.

Radioactivity of thin-layer plates were detected using a thin-layer chromatogram scanner (Berthold/Frieseke GmbH, Karlsruhe-Durlach, GFR).

Preparation of Radioactive Turimycin

The fermentation was carried out as described under "Culture Conditions". Fifty or 100 μCi quantities of each radioactive compound were added to the fermentation broth 0; 24 and 48 hours, respectively, after inoculation and incubated furthermore until 72nd hour. Normally 10 μCi were used in the case of demycarosyl turimycin and acyl mycaroses. After centrifugation the pH of the fermentation broth filtrate was brought to 8.9 with 1 N NaOH and the filtrate was extracted twice with 80 ml portions of ethyl acetate. The extract was washed with water, dried with Na_2SO_4 and concentrated *in vacuo*, followed by dissolving the residue in 5% citric acid. After filtration the pH was brought back to 8.9 with 1 N NaOH and the resulting alkaline layer was again extracted twice with 80 ml portions of ethyl acetate. After concentration radioactive turimycin was obtained as chromatographic pure product determined on TLC-plates (Polygram, Sil G/UV_{254}, Machery-Nagel & Co., Düren/GFR) in the system benzene - acetone (5: 3).

Degradation of Turimycin

Turimycin obtained from fermentations with several ^{14}C-precursors was diluted with non-radioactive carrier to 0.4 mmole and hydrolyzed in 12 ml of 0.5 N HCl for 8 hours at room temperature. 1 N NaOH was added to the reaction mixture to pH 3 and the solution was extracted three times with 20 ml portions of ethyl acetate. The isolated fraction of acyl mycarose was purified by TLC-chromatography (silica gel PF_{254}, Merck AG, GFR) using the system benzene-acetone (5: 3). The aqueous layer was adjusted to pH 8.9 and extracted three times with ethyl acetate. The extract was washed with water, concentrated and the demycarosyl turimycin was purified by TLC-chromatography as above.

The separated products, acyl mycarose and demycarosyl turimycin, were pure on TLC-chromatograms.

Results

Time-dependent Incorporation of ^{14}C-Substrates

In order to compare the incorporation ratios of various ^{14}C-labeled compounds the precursors were added usually 24 hours after inoculation, when the rate of the antibiotic synthesis increased rapidly. The results of incorporation into turimycin were determined after 72 hours fermentation and summarized in the middle column of Table 1.

On the percentage basis all added substances were incorporated markedly with the exception of succinic acid-1.4-^{14}C, whereas L-methionine-methyl-^{14}C and *n*-butyrate-1-^{14}C were incorporated to the greatest extend. It was of special interest to compare the incorporation of succinate and butyrate as potential precursors of the lactone aldehyde group. *n*-Butyric acid-1-^{14}C was utilized very efficiently, about hundred-fold better than succinate-1.4-^{14}C. All precursors added at zero time showed lower percentage incorporation, only L-methionine-methyl-^{14}C was incorporated reasonably well. Even *n*-butyrate-1-^{14}C was poorly incorporated in a ratio comparable with that of acetate, probably because of butyrate degradation to acetate by ß-oxidation and extensive utilization in metabolic processes other than turimycin synthesis. Not only a significant increase of butyrate-1-^{14}C incorporation but also a

sharp distinction from the ratio of acetate-1-^{14}C incorporation was observed towards the end of growth period about 18 until 20 hours after inoculation. From this time the incorporation ratio of butyrate-1-^{14}C increased rapidly to approximately 40% with variations from 34 to 42%, probably dependent on the actual metabolic situation at this time. In contrast to this finding the incorporation percentages of the most precursors added at 24 hour were relatively constant.

^{14}C-Precursor addition to growing cultures at 48th hour led to a decrease in the incorporation ratio with only L-valine-U-^{14}C and D-glucose-U-^{14}C being better utilized.

Table 1. Incorporation of ^{14}C-labeled substrates into turimycin at different addition times

^{14}C-compound	Percentage incorporation: Addition time (hour) 0	24	48
D-Glucose-U-^{14}C		2.36	3.93
Na-Acetate-1-^{14}C	0.51	2.02	1.14
Na-Propionate-1-^{14}C	1.54	9.4	5.42
Succinic acid-1.4-^{14}C		0.41	
n-Butyric acid-1-^{14}C	0.60	40.5	25.8
L-Leucine-U-^{14}C		4.62	
L-Isoleucine-U-^{14}C		3.17	
L-Valine-U-^{14}C		6.7	9.2
L-Methionine-methyl-^{14}C	21.0	53.2	51.5
Propanol-1-^{14}C	0.51	1.07	
Ethanol-1-^{14}C	1.37	2.29	

Each labeled compound was added as noticed 0, 24 or 48 hours after inoculation and incubated until 72nd hour.

The percentage incorporation was determined as (Total dpm of turimycin-^{14}C)/(Total dpm of added ^{14}C-compound) $\times$ 100

Degradation Studies

In order to determine the distribution of radioactivity, turimycin labeled by several ^{14}C-compounds was subjected to a degradation by acid hydrolysis as described under "material and methods". In this way the antibiotic was split into demycarosyl turimycin (I) and the acylated mycarose (II). Because the turimycin isolated from the strain of *Streptomyces hygroscopicus* used was a complex mixture of compounds with different 4″-acyl residues, an approximate molecular weight of the antibiotic (M=760) and of the acyl mycaroses (M=238) were used for calculating the specific radioactivities.

The results given in Table 2 showed that most of the radioactivity derived from *n*-buty-

Table 2. Distribution of radioactivity among the degradation products of turimycin-^{14}C labeled from different ^{14}C-precursors

^{14}C-Precursor	Addition time (hour)	Specific radioactivity: Turimycin-^{14}C	Degradation products: ^{14}C-acyl mycarose		^{14}C-demycarosyl turimycin	
		μCi/mmole	μCi/mmole	%	μCi/mmole	%
n-Butyric acid-1-^{14}C	24	27.86	3.36	12	22.85	82
n-Butyric acid-1-^{14}C	48	32.37	4.38	14	25.31	78
Na-Propionate-1-^{14}C	24	16.1	3.96	24	11.0	68
Na-Propionate-1-^{14}C	48	6.78	2.71	40	3.6	53
L-Valine-U-^{14}C	24	8.39	4.67	56	3.16	38
L-Valine-U-^{14}C	48	11.52	3.4	30	7.3	63
L-Leucine-U-^{14}C	24	11.54	9.49	82	1.45	12
L-Isoleucine-U-^{14}C	24	7.94	4.58	58	2.73	34
L-Methionine-methyl-^{14}C	0	10.5	2.27	22	7.5	71
L-Methionine-methyl-^{14}C	24	28.1	7.34	26	19.36	69
L-Methionine-methyl-^{14}C	48	49.20	18.5	38	29.1	59

A middle value of molar weights in the case of turimycin (M=760) and acyl mycarose (M=238) was used for calculating the specific radioactivity.

rate-1-^{14}C was found in the demycarosyl turimycin.

The specific radioactivity of L-methionine-methyl-^{14}C-labeled turimycin was disproportionally divided between the demycarosyl fragment (I) (71 %) and the acyl mycarose fraction (II) (22 %). If the methionine precursor had been added in a later fermentation stage, the percentage of radioactivity of I decreased, whereas that of II increased. In the case of propionate-1-^{14}C as a precursor of the aglycone as well as the acyl part most of the radioactivity was found in I (68 %). Dependent on increasing addition time, this ratio shifted in favour of the acyl residue. The reverse result was obtained with L-valine-U-^{14}C.

Incorporation Experiments of ^{14}C-Demycarosyl Turimycin and ^{14}C-Acyl Mycarose into Turimycin

^{14}C-Demycarosyl turimycin (I) and ^{14}C-acyl mycarose (II) prepared and purified as described above were added to growing cultures 24 hours after inoculation and the turimycin produced was isolated from 72-hour cultures. The added molecules were labeled either from L-methionine-methyl-^{14}C or from *n*-butyric acid-1-^{14}C.

Both labeled ^{14}C-demycarosyl turimycins (I-met, I-but) gave comparable maximal incorporation ratios (I-met 21 % and I-but 25 %). The results of several experiments varied in a range from 14 to 25 %. The unused demycarosyl turimycin substrate could be recovered only in part.

The labeling patterns of turimycin components, as determined by radioactivity TLC-scanning, were different depending on the labeled substrate employed. They showed higher 4″-butyryl and 4″-acetyl labeling in the case where I-but was the substrate indicating degradation of the added demycarosyl turimycin.

The radioactivity of ^{14}C-acyl mycarose obtained from L-methionine-methyl-^{14}C-labeled turimycin (II-met) was incorporated only to a negligible extent (1 %). In contrast to this result, 18~20 % of the radioactivity of the corresponding *n*-butyric acid-1-^{14}C-derived fragment (II-but) was found in the turimycin with especially high amounts in the 4″-butyryl and 4″-acetyl moieties.

Discussion

Summarizing, the results of incorporation of ^{14}C-labeled substrates into turimycin indicate that several carbon compounds can serve as precursors of this macrolide antibiotic. The addition of the substrates 24 hours after inoculation gave the best results.

Methionine is well known as a methyl donor in biological transmethylation reactions[3,4,5]. The effective utilization of L-methionine-methyl-^{14}C corresponds with the data given by other authors[6,7]. The distribution of radioactivity among demycarosyl turimycin (71 %) and the acylated mycarose (22 %) after zero time addition of the precursor agrees with the findings by ACHENBACH *et al*[6] and ONO *et al.*[7] They reported that in magnamycin and maridomycin, respectively, the radioactivity of the methyl group of methionine is incorporated into two methyl groups of mycaminose, one methyl residue of mycarose and one of the lactone ring.

Our observation that the incorporation ratio of the substrates is changing during the course of fermentation may be explained by the assumption that mycaminose synthesis occurs in an earlier period of growth, whereas the methylation of a glucose derivative in mycarose biosynthesis may take place in a later stage.

Other experiments with different addition times also reflect the influence of the metabolic situation on the incorporation ratio. *n*-Butyric acid-1-^{14}C incorporation increases rapidly to a very high level towards the end of the rapid growth period (about 40 %). This finding together with the results of degradation studies showing a distribution ratio among demycarosyl turimycin and acyl mycarose near

7: 1 may be explained by the butyrate origin[11] of the 5-6-17-18-carbons of the aglycone. Because normally all 2n-fatty acids were degradated to acetic acid units, this rapid increase may be understood as an indicator of a metabolic change to the so-called secondary metabolism.

Succinate is not directly involved as a precursor[8,9] of this part of turimycin as shown by poor utilization (0.4%) of the added succinic acid-1.4-^{14}C. An alternative way in which the butyric acid can be incorporated is by β-oxidation followed by hydrolysis to acetate units prior to incorporation[10]. This possibility is unlikely based on the poor utilization of acetate-1-^{14}C (2.02%). Our conclusion concerning the butyrate metabolism is in accordance with the results by ŌMURA and coworkers[11] using ^{13}C-precursors as well as by those of FURUMAI and SUZUKI[12,13,14] who have isolated intermediates containing a C-18-methyl group instead of the aldehyde function from the fermentation broth of blocked mutants of *Streptomyces platensis* subsp. *malvinus*.

In contrast to results reported by ONO *et al.*[7] we could not detect such a high and corresponding incorporation of ^{14}C-propionate and -propanol. Our observation that lower alcohols (especially propanol) stimulate the antibiotic production in this strain (unpublished results) as also described in the case of erythromycin synthesis[18,19] may not be explained as a precursor effect.

Most of the radioactivity of L-valine-U-^{14}C, L-isoleucine-U-^{14}C and L-leucine-U-^{14}C was present in the acyl mycarose fraction, particularly that of leucine as an excellent precursor of the isovaleryl group[14,15]. Valine and isoleucine are normally metabolized to α-methyl-malonyl-CoA *via* isobutyryl-CoA and α-methyl-butyryl-CoA, respectively, indicating an incorporation similar to that of propionate. L-Valine is suggested by REUTER *et al*[16] as a precursor of the isobutyryl group.

The experiments with differently labeled degradation products of turimycin led to some conclusions on the biosynthesis of this antibiotic. The radioactivity of the acyl mycarose fraction obtained from methionine-methyl-^{14}C labeled turimycin (therefore only mycarosyl-labeled) was incorporated to a negligible extend in contrast to the butyric acid-1-^{14}C derived substrate (acyl-labeled). This difference may suggest a preliminary deacylation prior to utilization of the acyl-mycarose. Additionally, it is evident that only the acyl residue can be incorporated suggesting that free mycarose as such may not be activated to a nucleotide derivative (possibly a necessary intermediate for binding to the mycaminose moiety of demycarosyl turimycin). PAPE and BRILLINGER[17] have found that thymidine-diphosphate-L-mycarose, derived from thymidine-diphosphate-D-glucose accumulates in a tylosin-producing strain of *Streptomyces rimosus*.

No more than 1/4th (14~25%) of the added radioactivity of demycarosyl turimycin labeled from L-methionine-methyl-^{14}C or *n*-butyric acid-1-^{14}C could be found in the turimycin after fermentation was stopped. The estimation of the labeled patterns showed remarkable differences dependent on the ^{14}C-precursor. In the case of the butyrate-labeled molecule the butyryl and also the acetyl component were labeled to a greater extend than usual. These observations may reflect a partial degradation of the added demycarosyl turimycin. On the other hand unchanged demycarosyl turimycin was also recovered. In this field it is interesting to remember the hypothesis by LYNEN and TADA[20] involving principles in the biosynthesis of macrolides similar to that of fatty acids.[21]

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