FEMS Microbiology Letters 84 (1991) 33–36

Published by Elsevier

FEMSLE 04659

Isobutyrate as a precursor of *n*-butyrate in the biosynthesis of tylosine and fatty acids

T. Řezanka [1], J. Reichelová [2] and J. Kopecký [2]

[1] *Institute of Microbiology, Prague, and* [2] *Institute of Chemical Technology, Prague, Czechoslovakia*

Received 26 July 1991
Accepted 12 August 1991

Key words: Tylosine; Fatty acid; Isobutyrate; *Streptomyces fradiae*

1. SUMMARY

Labelled sodium isobutyrate [$(CD_3)_2$-CHCOONa] was added to the culture medium of *Streptomyces fradiae* and up to 14 atoms of deuterium were found to be incorporated into a molecule of tylosin aglycone (tylactone). This observation is in accordance with the data in the literature. When fatty acids were analyzed, as much as 34% of the isobutyrate incorporated into the cell was formed to be transformed into butyrate that was used for the synthesis of even, straight-chain fatty acids; 57% of the labelled isobutyrate was incorporated into the even iso-acids, whereas 9% was degraded to propionate and further used for the synthesis of the odd acids.

2. INTRODUCTION

Tylosin is a 16-membered macrolide antibiotic produced by *Streptomyces fradiae*. It is commercially important both as a foodstuff supplement and as a veterinary antibiotic against Gram-positive bacteria and *Mycoplasma* sp.

The synthesis of protylonolide was explained [1] by using precursors labelled with ^{13}C. By subsequent analysis of the labelled tylosin (^{13}C-NMR), the carbon atoms of the aglycon part of the molecule were found to originate from one butyrate, five propionate and two acetate units. These lower fatty acids can be assumed to be supplied by primary metabolism and serve for both the formation of macrolide lactone and the synthesis of long-chain fatty acids.

Similar features of the biosynthesis of macrolide antibiotics, as compared to that of higher fatty acids, were confirmed by observation of the inhibition of the biosynthesis of tylosin, leucomycin and other oligoketide antibiotics (formed by the same route of condensation) by cerulenin [2], an inhibitor of the biosynthesis of fatty acids.

Recently, two papers have appeared [3,4] described both the incorporation of a branched acid (*N*-acetylcysteamine ester of 2-methyl-3-hydroxypentanoic acid) into tylactone and the production of branched-chain fatty acids (e.g., 7-hydroxy-4,6-dimethyl-nona-2,4-dienoic acid) by mutants of *S. fradiae*.

Correspondence to: T. Řezanka, Institute of Microbiology, Vídeňská 1083, 14220 Prague, Czechoslovakia.

The transformation of butyric into isobutyric acid is not known to be carried out directly [5,6]. Experiments conducted with oligoketide antibiotics in the first half of 1980, however, suggested that a direct rearrangement of isobutyric to butyric acid can occur [5,6]. In addition, the former acid is also decomposed to produce propionic acid.

On the basis of the above observations, the enzymes synthesizing oligoketide (tylactone) and the fatty acid synthase could be considered very similar or, maybe, identical. Therefore, we attempted to provide evidence for the similarity of primary and secondary metabolisms. The biosynthesis of fatty acids and oligoketides was studied in *S. fradiae*, a producer of tylosin.

3. MATERIALS AND METHODS

Isobutyric-d_6-acid, $(CD_3)_2CHCOOH$, was synthesized [7,8] from diethylmalonate and methyl-d_3-iodide, CD_3I (Sigma, USA).

Streptomyces fradiae strain 13/1 from the collection of the Research Institute of Biofactors and Veterinary Drugs in Koufim, Czechoslovakia was used. The procedures for culture maintenance and fermentation are described in literature [9,10]. A concentration of 1 g of isobutyrate-d_6 (added at 24 h) per 1 l of the culture medium was found to be the most suitable. The concentrations of isobutyrate (unlabelled) were tested in the range of 0.04–4 g/l.

Methylesters of corresponding fatty acids (FAME) were prepared by alkaline hydrolysis and reaction of free acids (FA) with BF_3-methanol [11]. Methylesters were identified by GC-MS in a Shimadzu QP-1000 apparatus (Shimadzu, Kyoto, Japan) equipped with a fused silica capillary column (60 m × 0.32 mm ID/SPB-1; Supelco, U.S.A.); splitless injection, carrier gas He. Oven temperature was programmed from 100 to 320 °C at a rate of 4 °C/min. Ionization energy was 70 eV and electron multiplier voltage 2.5 kV.

For desorption chemical ionization-mass spectrometry (DCI-MS) of tylosine, a Finnigan MAT 90 (Finnigan, F.R.G.) mass spectrometer was used. Ion-source temperature was 200 °C, heating rate 1 °C/s; reaction gas ammonia (3×10^{-4} bar) was the most suitable of three gases tested (methane, isobutane, ammonia).

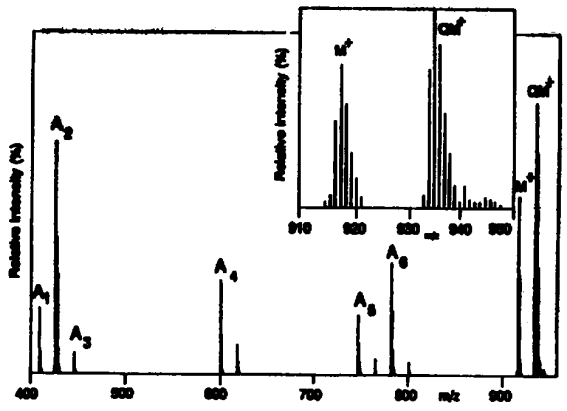

Fig. 1. DCI-MS of labelled (deuterated) tylosine. Range of QM^+ is shown in the extended scale (top, right).

4. RESULTS AND DISCUSSION

The content of fatty acids in *Streptomyces fradiae* [10] was very similar to other *Streptomyces* species [12]. The ionization by ammonia [13,14] enabled us to identify the pseudomolecular ion (QM^+) as well as the split ions. By using ^{13}C-NMR [1], the incorporation of as many as 19 atoms of D was detected. From our mass spectrum we assume that isobutyrate was transformed to butyrate and partially degraded to propionate. The shifted values of the m/z ions (see Fig. 1 and

Table 1

Structures and values of some important ions from tylosine

Abbreviation	Structure of ions	m/z
A_1	$(Agl+H)^+$	409
A_2	$(Agl+NH_4)^+$	426
A_3	$(Agl+OH)H^+$	427
A_4	$(Agl+Mycam)^+$	600
A_5	$(Agl+Mycam+Mycar)^+$	744
A_6	$(Agl+Mycam+Mycin)^+$	774
M^+	M^+	917
QM^+	$(M+NH_4)^+$	935

Agl = tylactone; Mycam = mycaminose; Mycar = mycarose; Mycin = mycinose.

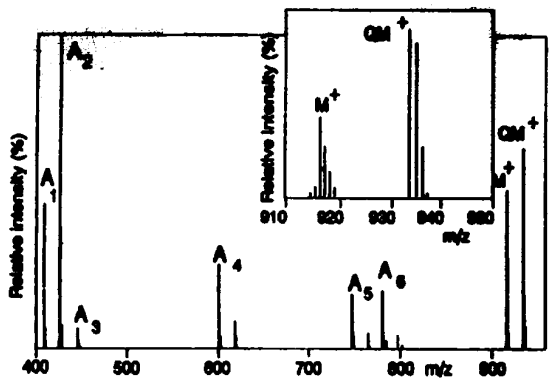

Fig. 2. DCI-MS of unlabelled tylosine. Range of QM^+ is shown in the extended scale (top, right).

Table 1) which originated from the splitting of the tylosin molecule confirm this conclusion. The mass spectrum of unlabelled tylosine is shown in Fig. 2.

In the case of the transformation of isobutyrate to butyrate, especially iso-even FA and straight-chain FA would be enriched in deuterium. As shown in Fig. 3, during the chromatographic determination of the total FA in the form of methylesters, mostly the maxima of i-16:0 acids

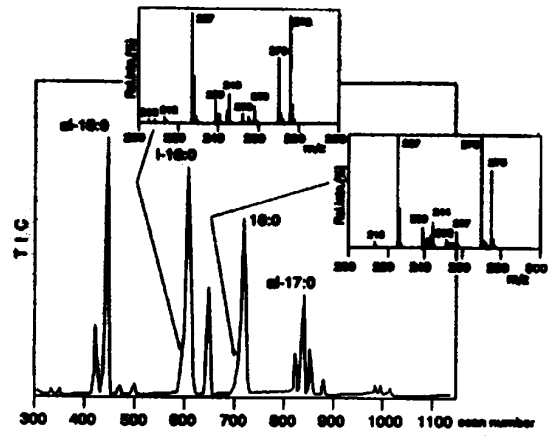

Fig. 3. Partial GC-MS chromatogram of methylesters from *S. fradiae*. Mass spectrum of labelled and unlabelled FAME (i-16:0 and 16:0) is shown. Exact conditions, see MATERIALS AND METHODS. The assumed structures of ions from mass spectra are shown in Table 1. i, isoacid; ai, ante-isoacid; first number, number of carbon atoms in the chain; second number, number of double bond(s). TIC, total ion current.

Table 2

Structure and values (m/z) of some important ions, from C_{16} acids

Structure of ions	Type of acids, which are labelled by			
	n(H)	i(H)	n(D)	i(D)
M^+	270	270	275	276
$M\text{-}CH_3$	255	255	–	–
$M\text{-}H_2O$	252	252	257	258
$M\text{-}CD_3$	–	–	257	258
$M\text{-}CH_3O$	239	239	244	245
$M\text{-}C_2H_5$	241	241	–	–
$M\text{-}C_2H_2D_3$	–	–	243	244
$M\text{-}C_3H_7$	227	227	–	–
$M\text{-}C_3H_2D_5$	–	–	227	–
$M\text{-}C_3HD_6$	–	–	–	227
$M\text{-}H_2O\text{-}CH_3OH\text{-}CH_3$	–	205	–	–
$M\text{-}H_2O\text{-}CH_3OH\text{-}CD_3$	–	–	–	208

n = straight chain acid; i = iso acid; (H) = unlabelled; (D) = labelled by deuterium.

were broadened. This broadening was caused by the shorter retention time of the deuterated compounds [15,16]. We were not able to separate non- and partially-deuterated FAME, which is in accordance with other studies. The full confirmation of the hypothesis concerning the transformation of isobutyrate to butyrate came from the mass spectra of i-16:0 and 16:0. The ions formed belong to both non-deuterated and partially-deuterated FAME (Table 2, Fig. 3). The ions represent mainly molecular ions (M^+) giving clear evidence of the presence of two compounds in one peak. If a simple extension of the labelled isobutyrate molecule takes place, the i-16:0 FAME formed has an M^+ value higher by 6 amu. In the case of the transition, an M^+ value is increased by only 5 amu. The carboxyl group replaces one of six D atoms in one of the two CD_3 groups of isobutyrate.

On the basis of the intensity of M^+ deuterium-labelled and unlabelled FAME, we were able to quantify the intensity of metabolism (rearrangement or degradation of isobutyrate). For quantification of the rearrangement of isobutyrate to butyrate the intensity of M^+ was measured (by means of single-ion monitoring, e.g., m/z 270, respectively m/z 276). On the basis of these intensities, we found that 34% of the Na

isobutyrate incorporated into the cell was transformed to butyrate that was further used for the synthesis of even straight-chain FA (mostly 16:0). Fifty-seven percent of the isobutyrate labelled was incorporated into even iso-acids (mainly i-16:0) measured on the basis of ion intensities (m/z 270 and m/z 275), while 9% was degraded to propionate and further used for the synthesis of odd acids (minor 15:0 and 17:0). This result was obtained by the sum of ion intensities: unlabelled and labelled M^+ with 3 atoms of deuterium.

Our findings concerning the transition of isobutyrate to butyrate give important evidence of the similarity between the biosynthesis of oligoketide antibiotics and of fatty acids.

REFERENCES

[1] Omura, S., Nakagawa, A., Takeshima, H., Miyazava, J., and Kitao, C., (1975) Tetrahedr. Lett. 50, 4503–4506.
[2] Omura, S. (1986) Microbiol. Rev. 50, 259–279.
[3] Huber, M.L.B., Paschal, J.W., Leeds, J.P., Kirst, H.A., Wind, J.A., Miller, F.D., and Turner, J.R. (1990) Antimicrobiol. Agents Chem. 34, 1535–1541.
[4] Yue, S., Duncan, J.S., Zamamoto, Z., and Hutchinson, C.R. (1987) J. Am. Chem. Soc. 109, 1253–1255.
[5] Omura, S., Tsuzuki, K., Tanaka, Y., Sakakibara, H., Aizawa, M., and Lukacs, G. (1983) J. Antib. 36, 614–616.
[6] Pospíšil, S., Sedmera, P., Havránek, M., Krumphanzl, V., and Vaněk, Z., (1983) J. Antib. 36, 617–619.
[7] Singh, R.K. (1985) Synthesis 54–55.
[8] Sherman, M.M., Yue, S., Hutchinson, C.R., (1976) J. Antib. 39, 1135–1143.
[9] Marišálek, J., (1987) Ph.D. Thesis, Faculty of Science, Charles University, Prague.
[10] Vančura, A., Řezanka, T., Marišálek, J., Křišťan, J., Basařová, G. (1987) J. Basic Microbiol. 27, 167–171.
[11] Řezanka, T., Sokolov, M.Yu., and Víden, I. (1990) FEMS Microbiol. Ecol. 73, 231–238.
[12] Ballio, A., and Barcelona, S. (1968) Ann. Inst Pasteur 114, 121–137.
[13] Suzuki, M., Harada, K., Takeda, N. and Tatematsu, A. (1981) Heterocycles 15, 1123–1130.
[14] Suzuki, M., Harada, K., Takeda, N. and Tatematsu, A. (1977) Heterocycles 8, 199–205.
[15] Patton, G.M., and Lowenstein, J.M. (1986) In: CRC Handbook of Chromatography, Lipids, (Mangold, H.K., Zweig, G. and Sherma, J., Eds.) Vol. II, pp. 225–231. CRC Press, Boca Raton, FL.
[16] Rohwedder, W.K., Duval, S.M., Wolf, D.J. and Emken, E.A. (1990) Lipids 25, 401–405.