Optimization of streptomycete strains producing polyether and macrolide antibiotics

ZDENKO VANĚK, STANISLAV POSPÍŠIL, PETR SEDMERA, PAVEL TICHÝ, TOMÁŠ ŘEZANKA and VLADIMÍR KRUMPHANZL

Department of Biogenesis of Natural Substances, Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Prague 4, Czechoslovakia

Polyether and macrolide antibiotics represent numerous groups of compounds formed by condensation of acetate, propionate and, in some cases, butyrate units. However, their biogenesis has not yet been completely solved and theoretical discrepancies exist already at the level of the formation of basic building units.

This holds primarily for the formation of the butyrate building unit. Aglycones of some 16-membered macrolides (e.g. magnamycin, tylosin, turimycin, platenolids, leukomycins, spiramycins) contain this unit. It was assumed that in case of magnamycin this unit is formed from succinic acid (Achenbach & Grisebach, 1964). The views of Woodward *et al.* (1965) were similar. Grisebach & Weber-Schilling (1968) later withdrew the assumption about the

incorporation of succinic acid. On the basis of incorporation experiments with ^{14}C-labelled substrates Srinivasan & Srinivasan (1967) assumed that this unit is formed from acetate.

Studies in which the biosynthesis of 16-membered macrolides was investigated by means of ^{13}C-labelled precursors and nuclear magnetic resonance led Ōmura *et al.* (1977) to the conclusion that the significant randomization of isotopes in the aglycone moiety can be, at least partially, explained by oxidation of butyric acid to succinic acid which is further isomerized to methylmalonic acid (at the level of corresponding enzymes). After decarboxylation methylmalonic acid yields the propionate unit. Carboxylation of butyric acid gives rise to ethylmalonic acid which is isomerized, oxidized and decarboxylated yielding again methylmalonic acid, but, in this case, with an altered isotopic labelling (Ōmura & Nakagawa, 1981). Ethylmalonyl-CoA can only hardly be considered as a direct precursor of the butyrate unit as its absolute configuration is not known; in addition, the existence of both butyryl-CoA carboxylase and ethylmalonyl-CoA epimerase has not yet been demonstrated (Hutchinson *et al.*, 1981). Gersch *et al.* (1977) studying the biosynthesis of turimycin faced apparently different problems. Position 4' of the sugar mycarose of this antibiotic can be acylated by isovaleryl, propionyl, acetyl and iso- and n-butyryl. They degraded turimycin to mycarose and demycarosylturimycin but were not brave enough to conclude that L-[U-^{14}C]valine might be a precursor of the butyrate unit of the lactone moiety. Reuter & Huttner (1976) reported that both theoretical considerations and experimental data give evidence against the direct synthesis of n-butyric acid from valine.

A similar situation with the biogenesis of the butyrate unit was observed when studying oligoketides of the polyether type (Dorman *et al.*, 1976). In studying the basic building units of narasin they came to the conclusion that enzymes of the producer, i.e. *Streptomyces aureofaciens*, are capable of the interconversion of propionate and butyrate. The mechanism of these interconversions is not clear. It cannot be explained by simple schemes of α- and β-oxidation of basic building units. These authors were the first to demonstrate the conversion of propionate to butyrate.

Monensins A and B (differing only in a single butyrate and propionate unit) appear to be a highly valuable model which makes it possible to solve the origin of the butyrate unit. We found that *Streptomyces cinnamonensis* culture in a synthetic medium with L-valine produces predominantly monensin A, whereas in the presence of DL-leucine the synthesis of monensin B increases (Pospíšil *et al.*, 1982).

When using radioactive substrates it is not necessary to degrade chemically the compounds under investigation but the differences in specific radioactivity between monensin A and monensin B can be directly related with the butyric acid unit of monensin A. On the basis of these experiments it could be concluded that L-valine and isobutyric acid are transformed to n-butyric acid which is a direct precursor of monensin A (Vaněk *et al.*, 1982).

In studies using precursors labelled with the stable ^{13}C it was clearly demonstrated that the butyrate unit of monensin A originates via a new metabolic pathway including isomerization of isobutyrate to n-butyrate (Pospíšil *et al.*, 1983).

The monensins A and B from *S. cinnamonensis* C-100-5 (non-pigmenting strain) were isolated and separated by preparative thin-layer chromatography. Labelled compounds were administered in three portions (1/2, 1/4, 1/4) at 24th, 48th and 72nd hour of the fermentation. The doses per 50ml flask were 17, 25, and 50mg of sodium [1-^{13}C]butyrate, [1-^{13}C]isobutyrate, and [1,2-$^{13}C_2$]acetate respectively.

As expected, C-15 of monensin A is predominantly labelled by sodium[1-^{13}C]butyrate; a lower level of enrichment was observed at C-1, -3, -5, -11, -17, -21, and -23, i.e. at carbons arising from C-1 of propionate. In monensin B only C-1 propionate sites (now also C-15) were labelled. Sodium-[1-^{13}C]isobutyrate produced the same incorporation pattern. Using double-labelled acetate, we found that five intact acetate units are incorporated into monensin B but seven into monensin A. The additional labelled carbons are those of butyrate origin.

The successful incorporation of isobutyrate into monensin A demonstrates a new metabolic pathway involving its isomerization to n-butyrate. Both isobutyrate and n-butyrate can be converted to propionate by *S. cinnamonensis* with the carboxyl label retained. Such an effect has already been observed in lysocellin, tylosin, and probably also in lasalocid A. Butyrate synthase participates in the biosynthesis of monensin A. This finding explains a higher than theoretical radioactivity found by Day *et al.* (1973) in [1-^{14}C]acetate-derived monensin A.

Our results show that in spite of simple building units, the biosynthesis of monensins is complex due mainly to multiple relationships among them.

Similar results were also reached by Ōmura *et al.* (1983) studying the formation of the butyrate unit in *Streptomyces fradiae*, a tylosin producer, and in *Streptoverticillium kitasatoensis*, producing leukomycin A_5, and thus confirmed the general validity of this new metabolic pathway.

Analyses of fatty acids in *S. cinnamonensis* showed (Řezanka *et al.*, 1984) that iso- and anteiso-acids have a dominant representation. Iso-branching occurs most frequently in acids with an even number of carbon atoms while anteiso-branching is found in those acids having an odd number of carbons. The present results indicate that the amino acids participating in the biosynthesis of these fatty acids are mainly valine and isoleucine, while leucine does so to a much lesser extent (Table 1).

The strain *Streptomyces cinnamonensis* C-100-5 produced roughly identical amounts of monensins A and B. In further studies we attempted to investigate the origin of the butyrate unit in strains producing predominantly monensin A. We used amino acid analogues of the group of aspartic acid and derived from pyruvic acid, i.e. 2-aminobutyrate, DL-norvaline, L-norleucine, 2-amino-3-chlorobutyrate, and, in addition, DL-ethionine, an inhibitor of transmethylation reactions (Pospíšil *et al.*, 1984).

The usual procedures were applied. We verified the sensitivity of *S. cinnamonensis* to the antimetabolites in the presence of different carbon source and tested the effect of amino acids on the increased inhibitory role of DL-norvaline and L-norleucine. After treatment with mutagens (u.v. light, $NH_2OH \cdot HCl$; survival 0.1–0.5%) the spore suspension was transferred to a liquid medium. The required concentrated

Table 1. *Fatty acids of S. cinnamonensis*

Fatty acid	Normal acids (%)	Iso-acids (%)	Anteiso-acids (%)
6:0	0.21	—	—
7:0	0.16	—	—
10:0	0.17	0.13	0.14
12:0	0.17	0.39	—
13:0	0.13	1.17	1.29
14:0	2.24	8.06	0.10
15:0	0.10	4.64	10.90
16:0	9.88	11.44	—
17:0	1.36	—	9.50
18:0	0.50	—	—
19:0	0.13	—	—

Table 2. *Mutant strains resistant to antimetabolites of amino acids*

*Mon, content of monensin A.

Antimetabolite	No. of obtained strains	(+) strains Mon* %	(+) strains No.	(+) strains %	(−) strains Mon %	(−) strains No.	(−) strains %
2-Aminobutyrate	31	70–81	18	58	50–70	13	42
DL-Norvaline	14	—	0	0	50	14	100
L-Norleucine	8	70–80	2	25	50–70	6	75
DL-Ethionine	20	—	0	0	30	20	100
2-Amino-3-chlorobutyrate	9	85–90	3	33	50	6	67

solutions of the antimetabolites were added after a 42h incubation which was continued for a further 72h. The results of these experiments are summarized in Table 2. In the strains resistant to 2-amino-3-chlorobutyrate the production of monensin A varied from 85 to 90%.

When investigating the excretion of amino acids into the synthetic culture medium valine (0.415 μmol/ml) was detected in the strains resistant to 2-aminobutyrate and only traces of this amino acid were found in the 2-amino-3-chlorobutyrate-resistant strains. The strains also differed in the spectrum of other amino acids excreted into the culture medium, indicating that the regulation of the biosynthesis of the amino acids investigated in these strains is also different.

Therefore, in further experiments, we tried to prepare mutant strains of *S. cinnamonensis* resistant to 2-amino-3-chlorobutyrate and even to some other antimetabolites. These experiments were successful and stable mutant strains producing 95–97% of monensin A were obtained.

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