BIOCONVERSION AND BIOSYNTHESIS OF 16-MEMBERED MACROLIDE ANTIBIOTICS. XIII

REGULATION OF SPIRAMYCIN I 3-HYDROXYL ACYLASE FORMATION BY GLUCOSE, BUTYRATE, AND CERULENIN

CHIAKI KITAO, HARUO IKEDA, HIDETAKA HAMADA and SATOSHI ŌMURA*

School of Pharmaceutical Sciences, Kitasato University,
Minato-ku, Tokyo 108, Japan

(Received for publication March 12, 1979)

The effects of glucose, butyrate, and cerulenin on the formation of spiramycin I 3-hydroxyl acylase were investigated by using the cell-free extract prepared from the spiramycin-producing strain of *Streptomyces ambofaciens* KA-1028. Glucose induced the formation of the acylase, and this induction was remarkably repressed by butyrate. Cerulenin, on the other hand, not only canceled the repression by butyrate but also stimulated the formation of the acylase. The unsuccessful trapping of spiramycin I as an intermediate during the bioconversion from neospiramycin I to spiramycin III in the presence of cerulenin was due to the rapid acylation of spiramycin I caused by the acylase induced by cerulenin.

In the previous studies on the biosynthesis of 16-membered macrolide antibiotics using cerulenin, a specific inhibitor of fatty acid and polyketide synthesis[1~4], we have shown that cerulenin can be usefully employed as a tool for examining the biosynthetic pathways of the compounds synthesized *via* polyketide intermediates[5]. In the biosynthetic studies of spiramycins using cerulenin, we recently reported that spiramycin III (SP-III) is derived from forocidin I (FO-I) *via* neospiramycin I (NSP-I) and spiramycin I (SP-I)[6]. The acylation of the aglycone was observed at the various steps in the bioconversion processes such as from FO-I to FO-III, from NSP-I to NSP-III, and from SP-I to SP-III (Fig. 2). Although the accumulation of SP-I was not observed because of its rapid acylation, we concluded that the pathway from NSP-I to SP-III *via* SP-I was reasonable because NSP-III was not converted to any compounds. In order to confirm this result, we prepared the purified enzyme which can acylate the hydroxyl group at C-3 of the aglycone and examined the specificity of the enzyme to several substrates[7]. Of the several substances used SP-I was the most specific for the acylase. This result agreed with that of the previous experiments that the acylation takes place after the formation of SP-I. However it seemed abnormal that the acylation took place so rapidly in several bioconversion steps. So we studied the reason for the rapid acylation by examining not only the effect of glucose and butyrate on the acylation which had already been reported in the biosynthetic studies of leucomycins[8,9], but also the effects of cerulenin on the acylation.

Materials and Methods

Strain

Streptomyces ambofaciens KA-1028 (ISP-5053), a spiramycin-producing organism, was used for the experiments.

* To whom all correspondence should be addressed.

Media

The following media were used for the preparation of the acylating enzyme and for production of spiramycins.

Medium I: 1.0% glucose, 1.0% dried yeast, 0.5% NaCl, 1.0% $CaCO_3$, and 0.1% $NaNO_3$ (adjusted to pH 7.5 with 2 N NaOH prior to autoclaving).

Medium II: the concentration of glucose in medium I was increased to 2.0%.

Medium III: 2.0% glucose, 0.5% meat extract, 0.5% peptone, 0.3% dried yeast, 0.5% NaCl, and 0.3% $CaCO_3$ (adjusted to pH 7.0 with 2 N NaOH prior to autoclaving).

Growth of strain

A loopful of well-grown spores and mycelium on oatmeal agar slant was inoculated to 500-ml Sakaguchi flask containing 100 ml of medium III and incubated at 27°C for 2 days on a reciprocal shaker to give a seed culture. Two ml of the seed culture was then transferred to 100 ml of medium I or II in a 500-ml Sakaguchi flask and cultivated for a desired period under the same conditions. A sodium butyrate solution separately sterilized by a Millipore filter (pore size, 0.45 μm) was added to the culture at the beginning of cultivation. An ethanol solution of cerulenin (0.1 ml, 40 mg/ml) was added to the culture.

Bioconversion

For the bioconversion experiment, the final concentration of 40 mcg/ml of cerulenin was added to the culture at the beginning and every 24 hours thereafter. Under this condition, the formation of the aglycone of spiramycin was completely inhibited by cerulenin, but the growth of mycelium was hardly affected. NSP-I dissolved in ethanol (100 μg/ml) was added to the 48-hour culture, and then the culture was incubated for additional 24 hours.

Detection of the bioconversion

The cultured material was centrifuged at 3,000 rpm for 15 minutes and the supernatant fluid adjusted to pH 8 with 2 N NaOH was extracted with ethyl acetate. The extract was concentrated *in vacuo* and applied to a preparative silica gel thin-layer chromatography plate using chloroform - methanol - 1.5 N aqueous ammonia (2: 1: 1, bottom layer) as an eluent. The spiramycin-related substances were detected by a dual wavelength chromatogram scanner (Shimadzu Seisakusho Co., Ltd, Model CS-910) at 232 nm and by the color reaction with 20% H_2SO_4 followed by heating for a few minutes.

Preparation of cell-free extract

The mycelium which was grown in medium I or II for 68 hours were harvested by centrifugation at 10,000 × *g* for 20 minutes, and washed twice with 0.01 M potassium phosphate buffer (pH 7.5). The washed mycelium was suspended in three volumes of 0.05 M potassium phosphate buffer (pH 7.8) containing 5 mM 2-mercaptoethanol (PBS-I). The suspended mycelium was disrupted by sonication (9 KHz, 140 W, Kubota, Model 200 M) for 10 minutes, and the debris was removed by centrifugation at 30,000 × *g* for 30 minutes. The supernatant fluid was collected and recentrifuged at 105,000 × *g* for 150 minutes. The supernatant fluid was dialyzed twice against 50 volumes of PBS-I for 6 hours, and this was used as the crude enzyme.

Enzyme assay

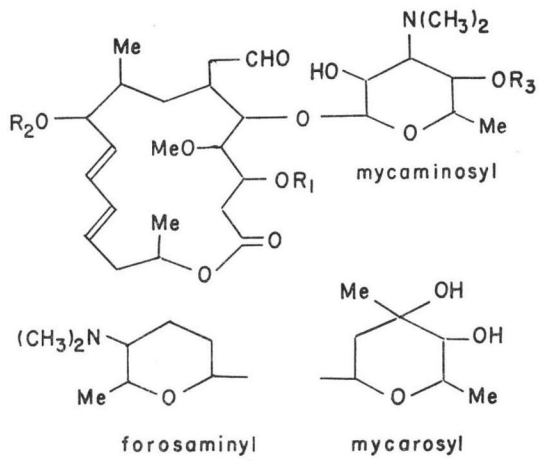
The activity of the crude enzyme was measured by reacting the solution containing 20 nmoles of SP-I, 20 nmoles of [acetyl-^{14}C]-acetyl coenzyme A (0.05 μCi), 4,000 nmoles of potassium phosphate buffer (pH 7.8), 40 nmoles of dithiothreitol, 50 nmoles of $CaCl_2$, and 25 μl of the crude enzyme in a total volume of 50 μl. The reaction was performed at 30°C for 10 minutes, and stopped by adding 10 μl of 1 N KOH containing 1 M potassium acetate and 200 μl of benzene. The benzene layer was separated by centrifugation at 3,000 rpm for 5 minutes. The extraction was repeated twice. Then the combined benzene layers were shaken with 1 M potassium acetate solution to remove the decomposed acetate derived from acetyl coenzyme A, centrifuged and collected in a vial. To this was added 10 ml of scintillation cocktail containing 4 g of 2,5-diphenyloxazole, 0.2 g of *p*-bis(*o*-methylstyryl)benzene in one liter of toluene, and the radioactivity was counted. The specific activity (mU/mg protein) for

the acylating enzyme was defined as follows: one milliunit per one mg of protein is the amount of enzyme catalyzing the formation of one nanomole of SP-II per minute at 30°C per one milligram of protein.

Analytical methods

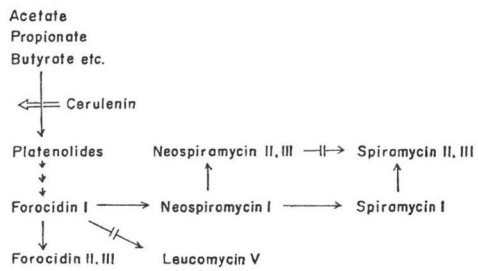
The amount of protein was determined by the protein-dye binding method of BRADFORD[10]. The radioactivity was measured by a liquid scintillation spectrophotometer (Aloka, Model LSC-651). The acylation reaction was detected by the ultraviolet absorption and the radioactivity on a thin-layer chromatogram (silica gel GF_{254}, Merck) developed by chloroform - methanol - 1.5 N aqueous ammonia (2: 1: 1, bottom layer). The ultraviolet absorption was measured by the dual wavelength chromatogram scanner. The radioactivity on a chromatogram was measured by a radio chromatogram scanner (Aloka, Model JTC-202B).

Fig. 1 Structures of spiramycin-related compounds



		R_1	R_2	R_3
Forocidin I	(FO-I)	H	H	H
Forocidin II	(FO-II)	$COCH_3$	H	H
Forocidin III	(FO-III)	COC_2H_5	H	H
Neospiramycin I	(NSP-I)	H	forosaminyl	H
Neospiramycin II	(NSP-II)	$COCH_3$	forosaminyl	H
Neospiramycin III	(NSP-III)	COC_2H_5	forosaminyl	H
Spiramycin I	(SP-I)	H	forosaminyl	mycarosyl
Spiramycin II	(SP-II)	$COCH_3$	forosaminyl	mycarosyl
Spiramycin III	(SP-III)	COC_2H_5	forosaminyl	mycarosyl
Leucomycin V	(LM-V)	H	H	mycarosyl

Fig. 2. Proposed scheme for spiramycin biosynthesis from forocidin I.



Results

Effect of Butyrate on Spiramycin Production

Spiramycin-producing strain, *Streptomyces ambofaciens* KA-1028 accumulates a large amount of spiramycins II and III (SP-II and SP-III), and relatively small amount of spiramycin I (SP-I) when the strain was cultured in medium I. Fig. 3-a shows the quantitative analysis of the 72-hour-cultured products by a dual wavelength chromatogram scanner. The Rf values of both SP-II and SP-III were the same on the TLC which was developed by chloroform - methanol - 1.5 N aqueous ammonia (2: 1: 1, bottom layer). However, it was found that the employed strain produces much more SP-III than SP-II. So this fraction was representatively called as SP-III in the experiments using the growing cell cultures. The ratio of SP-I to SP-III was approximately 1: 15 in this culture. On the other hand, when 0.15% of butyrate was added to the culture, the ratio of SP-I to SP-III was remarkably changed as to approximately 2: 3 caused by the re-

Fig. 3. TLC pattern of spiramycin production at 72 hours. TLC scanned at 232 nm.

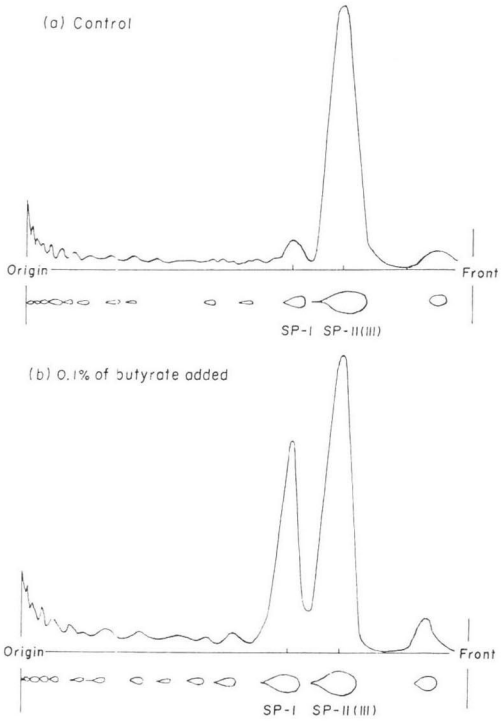
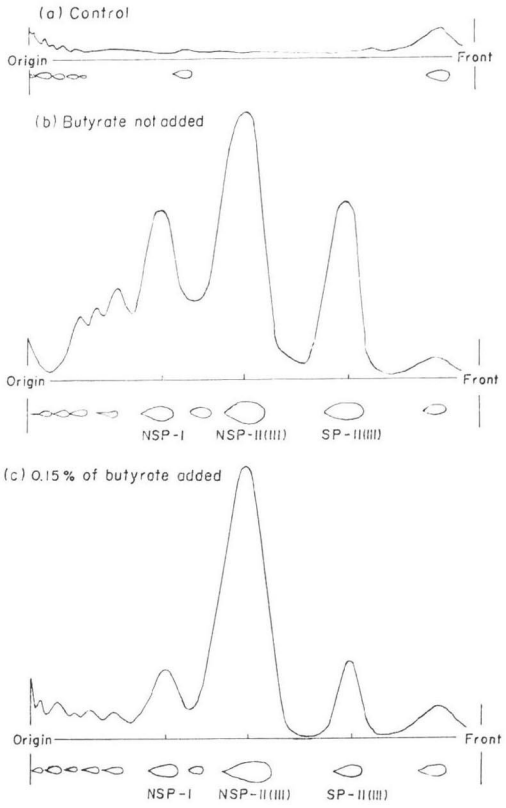


Fig. 4. Bioconversion of neospiramycin I.
The chromatogram developed with $CHCl_3$ - MeOH-1.5 N aqueous ammonia (2: 1: 1, bottom layer) was scanned at 232 nm.



pression of the acylation by butyrate (Fig. 3-b).

Effect of Butyrate on the Bioconversion of Neospiramycin I

The bioconversion of neospiramycin I (NSP-I) was examined under the inhibited condition of the aglycone formation by cerulenin. In the reference culture supplemented with 40 mcg/ml of cerulenin, the antibiotic production was completely inhibited, and under this cultural condition NSP-I was converted to NSP-III and SP-III (Fig. 4-a, b). In order to inhibit the acylation and to trap SP-I as an intermediate, 0.15% of butyrate was added to this bioconversion system. Though the bioconversion of NSP-I to NSP-III and SP-III was clearly observed, the accumulation of SP-I could not be seen in spite of the addition of butyrate (Fig. 4-c). No effect of butyrate on the acylation was observed under the cultural condition supplemented with cerulenin. It was considered that the inhibition of the acylation by butyrate was canceled by another regulatory factor, most probably by cerulenin. So the effects of glucose, butyrate, and cerulenin on the acylation were next examined by using the cell-free system.

Effect of Glucose, Butyrate, and Cerulenin on the Acylation of Spiramycin I

Effects of glucose and cerulenin on the formation of the acylase were first examined in the cell-free system by measuring the conversional activity of SP-I to SP-II. As shown in Table 1, the specific activity of the crude enzyme prepared from the culture grown in medium I containing 1.0% of glucose was 0.496 mU/mg protein, and that prepared from the culture grown in medium II containing 2.0%

Table 1. Effects of glucose and cerulenin on the acylating enzyme formation.

Culture	Enzyme formation (Specific activity; mU/mg protein)
Medium I	0.496
Medium II	0.695
Medium I+cerulenin*	0.758

Fermentations were carried out on a reciprocal shaker at 27°C for 68 hours in a 500-ml Sakaguchi flask containing 100 ml of the medium.

* Cerulenin (40 μg/ml) was added at 0 time, thereafter the same amount of cerulenin was added every 24 hours.

Table 2. Effect of glucose on the acylating enzyme activity.

Addition to cell-free system	Specific activity (mU/mg protein)
None	0.501
Glucose (1.1×10^{-2} M)	0.503
〃 (5.5×10^{-2} M)	0.499
〃 (1.1×10^{-1} M)	0.500

Glucose 10 g/liter = 5.5×10^{-2} M
〃 20 g/liter = 1.1×10^{-1} M

Table 3. Effect of cerulenin on the acylating enzyme activity.

Addition to cell-free system	Specific activity (mU/mg protein)
None	0.501
Cerulenin (10^{-3} M)	0.547
〃 (10^{-4} M)	0.484
〃 (10^{-5} M)	0.551

Cerulenin 40 μg/ml = 1.8×10^{-4} M

Table 4. Effect of butyrate on the acylating enzyme formation.

Addition to medium II	Enzyme formation (Specific activity; mU/mg protein)
None	0.695
Sodium butyrate (0.3%)	0.467
〃 (0.4%)	0.271
〃 (0.5%)	0.161

Fermentations were carried out on a reciprocal shaker at 27°C for 68 hours in a 500-ml Sakaguchi flask containing 100 ml of the medium.

Table 5. Effect of butyrate on the acylating enzyme activity.

Addition to cell-free system	Specific activity (mU/mg protein)
None	0.501
Sodium butyrate (10^{-1} M)	0.464
〃 (5×10^{-2} M)	0.517
〃 (10^{-2} M)	0.503

Sodium butyrate 0.3% = 2.72×10^{-2} M
〃 0.4% = 3.64×10^{-2} M
〃 0.5% = 4.54×10^{-2} M

Table 6. Repression of acylating enzyme by butyrate and inhibition of its repression by cerulenin.

Addition to medium II	Enzyme formation (specific activity; mU/mg protein)
Sodium butyrate (0.4%)	0.354
Sodium butyrate (0.4%) +cerulenin*	1.13
Sodium butyrate (0.5%)	0.254
Sodium butyrate (0.5%) +cerulenin*	0.914

Fermentations were carried out on a reciprocal shaker at 27°C for 68 hours in a 500-ml Sakaguchi flask containing 100 ml of the medium.

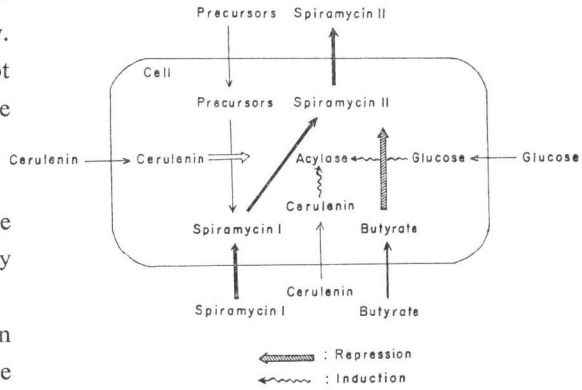
* Cerulenin (40 μg/ml) was added at 0 time, thereafter the same amount of cerulenin was added every 24 hours.

of glucose increased to 0.695 mU/mg protein. Furthermore, the specific activity of the crude enzyme prepared from the culture grown in medium I supplemented with 40 mcg/ml of cerulenin markedly increased to 0.758 mU/mg protein. The effects of glucose and cerulenin in the cell-free system were not observed under these experimental conditions (Tables 2 and 3). These results obviously show that the formation of the acylase was stimulated by both glucose and cerulenin.

The effect of butyrate on the formation of the acylase was next examined in the same cell-free system. The specific activities of the crude enzymes prepared from the mycelium grown in medium

II with or without sodium butyrate (0.3~0.5%) are shown in Table 4. It is obvious from the table that the increased concentration of butyrate remarkably decreased the specific activity. Butyrate added to the cell-free system did not affect the enzyme activity (Table 5). Therefore the drastic decrease of the specific activity of the crude enzyme prepared from the butyrate-supplemented culture was concluded to be due to the repression of the acylase formation by butyrate.

Fig. 5. Proposed action mechanism of glucose, butyrate, and cerulenin on the bioconversion from spiramycin I to spiramycin II.



The acylase formation by the culture grown in medium II supplemented with both butyrate and cerulenin was then examined by using the same cell-free system. As shown in Table 6, the specific activities were reduced to 0.354 and 0.254 mU/mg protein by adding 0.4 and 0.5% of sodium butyrate, respectively. However, the addition of cerulenin to those cultures remarkably increased the enzyme activities to 1.13 and 0.914 mU/mg protein, respectively. These results indicated that the inhibitory effect on the acylase formation by butyrate was completely neglected by the induction with cerulenin. From these experiments, the hypothetical action mechanisms of glucose, butyrate, and cerulenin on the acylation of SP-I to SP-II were proposed as shown in Fig. 5.

Discussion

In the biosynthetic studies of leucomycins produced by *Streptomyces kitasatoensis*, we have reported that the formation of the acylase which catalyzes the bioconversion of leucomycin A_1 (C-3, hydroxyl) to leucomycin A_3 (C-3, acetyl) is induced by glucose and this induction process is inhibited by butyrate[8,9]. In the present experiments on *Streptomyces ambofaciens*, we again showed the inductive effect on the formation of the acylase by glucose and the inhibition of the induction by butyrate. According to the classification of 16-membered macrolide antibiotics based on the skeleton of the lactone ring, spiramycin as well as leucomycin belongs to the magnamycin-group antibiotics[11]. Therefore it seemed that the similar effects of glucose and butyrate on the acylation of the lactone ring might be observed in other microorganisms which produce the antibiotics belonging to magnamycin-group.

Cerulenin possessed the inducible ability of the acylase formation like the effect of glucose. However, the action mechanism of cerulenin seemed to be different from that of glucose. The induction by glucose was easily inhibited by butyrate, while the induction by cerulenin was not negated by butyrate. This inductive effect of the acylase formation by cerulenin is the first report of the action of cerulenin. The unsuccessful trapping of SP-I as the intermediate in the bioconversion experiment of NSP-I by the growing cell systems is therefore due to the unusual inductive effect of the acylase formation by cerulenin.

References

1) ŌMURA, S.: The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bact. Rev. 40: 681~697, 1976
2) OHNO, H.; T. OHNO, J. AWAYA & S. ŌMURA: Inhibition of 6-methylsalicylic acid synthesis by the antibiotic cerulenin. J. Biochem. 78: 1149~1152, 1975

3) MARTIN, J. R. & E. MCDANIEL: Specific inhibition of candicidin biosynthesis by the lipogenic inhibitor cerulenin. Biochim. Biophys. Acta 411: 186~194, 1975
4) TAKESHIMA, H.; C. KITAO & S. ŌMURA: Inhibition of the biosynthesis of leucomycin, a macrolide antibiotic, by cerulenin. J. Biochem. 81: 1127~1132, 1977
5) ŌMURA, S.; C. KITAO, J. MIYAZAWA, H. IMAI & H. TAKESHIMA: Bioconversion and biosynthesis of 16-membered macrolide antibiotic, tylosin, using enzyme inhibitor: Cerulenin. J. Antibiotics 31: 254~256, 1978
6) ŌMURA, S.; C. KITAO, H. HAMADA & H. IKEDA: Bioconversion and biosynthesis of 16-membered macrolide antibiotics. X. Final steps in the biosynthesis of spiramycin, using enzyme inhibitor, cerulenin. Chem. Pharm. Bull. 27: 176~182, 1979
7) ŌMURA, S.; H. IKEDA, C. KITAO & H. HAMADA: Bioconversion and biosynthesis of 16-membered macrolide antibiotics. XII. Isolation and property of spiramycin I 3-hydroxyl acylase. J. Biochim. submitted.
8) ŌMURA, S.; J. MIYAZAWA, H. TAKESHIMA, C. KITAO, K. ATSUMI & M. AIZAWA: Bioconversion of leucomycins and its regulation by butyrate in a producing strain. J. Antibiotics 29: 1131~1133, 1976
9) ŌMURA, S.; J. MIYAZAWA, H. TAKESHIMA & C. KITAO: Induction of the bioconversion of leucomycins by glucose in a producing strain. J. Antibiotics 30: 192~193, 1977
10) BRADFORD, M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248~254, 1976
11) ŌMURA, S. & H. TAKESHIMA: Biosynthesis of macrolide antibiotics. Kagaku to Seibutsu 15: 381~386, 1977 (In Japanese)