# BIOSYNTHESIS OF BUTIROSINS. I

## BIOSYNTHETIC PATHWAYS OF BUTIROSINS AND RELATED ANTIBIOTICS

KATSUO TAKEDA, KATSUFUMI AIHARA, TAMOTSU FURUMAI and YUKIO ITO

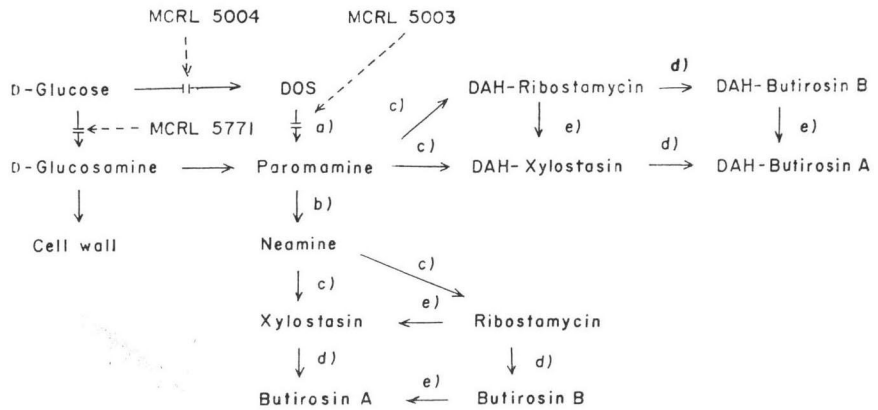
Microbiological Research Laboratory, Tanabe Seiyaku Co., Ltd.
Toda, Saitama, Japan

(Received for publication October 25, 1978)

By using 2 neamine-negative mutants, MCRL 5003 and MCRL 5004, of *Bacillus circulans*, subunit assembly in the biosynthesis of butirosins was investigated. The two mutants were blocked at different steps in the biosynthetic pathway of butirosins, but could produce butirosins when the culture medium was supplemented with neamine. Mutant MCRL 5003 accumulated 2-deoxystreptamine (DOS) in the fermentation broth but could not utilize DOS for the biosynthesis of butirosins, whereas mutant MCRL 5004 could produce butirosins from DOS. Based upon the ability of these 2 mutants to incorporate a series of DOS-containing compounds considered as plausible biosynthetic intermediates of butirosins into butirosins or related antibiotics, the biosynthetic pathways for butirosins and 6′-deamino-6′-hydroxybutirosins were proposed. Feeding experiments with 3′,4′-dideoxy derivatives of neamine, ribostamycin and butirosins supported the pathway proposed for butirosins. A glucosamine auxotroph MCRL 5771 of *B. circulans* afforded some information as to the role of D-glucosamine in the biosynthesis of butirosins.

In the previous papers[1~3], we have reported the preparation of new butirosin analogs by two neamine-negative mutants, MCRL 5003 and MCRL 5004, derived from *Bacillus circulans* MCRL 5001

Fig. 1. Proposed pathways for biosynthesis of butirosins and DAH-butirosins in *B. circulans* MCRL 5001




*Abbreviations*: DOS = 2-Deoxystreptamine. DAH = 6′-Deamino-6′-hydroxy

a) Pseudodisaccharide formation

b) Conversion of a 6′-hydroxyl group to a 6′-amino group

c) Pseudotrisaccharide formation

d) 4-Amino-2-hydroxybutyrylation at a 1-$NH_2$ position

e) Isomerization of ribo-form to xylo-form

⊣⊢ : Possible blocking site of mutants employed (neamine-negative mutants: MCRL 5003 and MCRL 5004; glucosamine auxotroph: MCRL 5771)

which produces butirosins and 6′-deamino-6′-hydroxybutirosins (DAH-butirosins). As an expansion of the above study, the biosynthetic pathways for butirosins and DAH-butirosins have been studied by using the above 2 mutants and these results were communicated briefly[4].

The present paper deals with the details of the study on the biosynthetic pathways elucidated as shown in Fig. 1, in which alternative direct routes from neamine to xylostasin and from paromamine to DAH-xylostasin are added to the pathways reported in the previous communication[4]. This paper also describes the isolation of 2-deoxystreptamine (DOS) from the fermentation broth of mutant MCRL 5003, which provides evidence for the possible involvement of DOS as an intermediate of DOS-containing aminoglycoside antibiotics. It further reports the bioconversion of 3′,4′-dideoxy derivatives of neamine, ribostamycin and butirosins into 3′,4′-dideoxybutirosin A by mutant MCRL 5003, which also supports the above pathway for butirosins. In connection with the role of D-glucosamine in the biosynthesis of butirosins, some characterization of a glucosamine auxotroph MCRL 5771 of *B. circulans* is also described in this paper.

## Materials and Methods

D-Glucosamine(hydrochloride) and N-acetyl-D-glucosamine were obtained from Nakarai Chemicals, Ltd., Japan. The other precursors used in this study were prepared in our laboratory. N-Methyl-N′-nitro-N-nitrosoguanidine and penicillin G were purchased from Aldrich Chemical Co., Inc. and Meiji Seika Kaisha, Ltd., Japan, respectively. Authentic samples of butirosins A and B, and DAH-butirosins A and B were kindly provided by Drs. P. W. K. Woo and H. Kawaguchi, respectively.

### Organisms and fermentation procedure

*Bacillus circulans* MCRL 5003 and MCRL 5004, neamine-negative mutants, were mainly used in this study. These mutants were derived from *B. circulans* MCRL 5001 producing butirosins (80~90% A and 10~20% B) and trace quantities of DAH-butirosins (80~90% A and 10~20% B)[1]. A glucosamine auxotroph MCRL 5771 was also used, which was derived from strain MCRL 5001 as described below.

As reported previously[1], antibiotic production was carried out in a 250-ml flask containing 30 ml of the production medium under shaking at 32°C for 7 days. All of the precursors were added before inoculation.

### Determination of antibiotic activity

The broth to be tested was diluted with 0.1 M potassium phosphate buffer (pH 7.8) and antibiotic activity was determined by a cup plate method using *Escherichia coli* JR 35/C 600, a resistant organism producing aminoglycoside 3′-phosphotransferase I[5], as a test organism. Unless otherwise stated, an amorphous butirosin carbonate salt containing 93% butirosin base (80~90% A and 10~20% B) was used as a reference standard.

### Determination of the ratio of *xylo*- and *ribo*-isomers

Contents of *xylo*- and *ribo*-isomers in butirosins and the related antibiotics were determined by applying a fluorophotometric method of Maeda *et al.*[6] to thin-layer (TL) plates.

Two isomers in the products were separated by thin-layer chromatography (TLC) in system B (cf. Table 1, footnote c), and the TL-plate was sprayed with pyridoxal-zinc solution (pyridoxal hydrochloride, 0.1 g; zinc acetate, 1 g; and 1 liter of 2% pyridine-methanol), heated at 65°~70°C for 10 minutes and then scanned with a fluorophotometer (Hitachi, MPF-2A) set at 365 nm with exitation and at 485 nm with emission.

### Determination of DOS in test solutions

The amount of DOS in the broth or the column chromatographic fraction was determined as follows:

(1) Preparation of standard curve for DOS: Solutions (1.5 ml) each containing 25, 50, 75, 100

and 200 $\mu$g/ml of authentic DOS were individually added to a test tube (diam. 23 mm) containing 1.5 ml of 2-day grown culture of mutant MCRL 5004. These mixtures were incubated at 32°C for 2 days on a reciprocal shaker at 120 c.p.m. and assayed for their butirosin activities. The activities were then plotted against the amounts of DOS added and a linear relationship was observed in the range of the amounts of DOS tested. Thus, standard curve for DOS was prepared.

(2) Determination of DOS in test solutions: The supernatant of the broth obtained by centrifugation, the heat-treated (at 120°C for 20 minutes) whole broth or the column chromatographic fraction (each 1.5 ml) was incubated with 1.5 ml of 2-day grown culture of MCRL 5004 as described above and the resulting butirosin activity was assayed. The amount of DOS in the solution was obtained from the standard curve prepared in every experiment.

Mutagenesis and selection of a glucosamine auxotroph

A glucosamine auxotroph of *B. circulans* MCRL 5001 was obtained by the method of SARVAS[7], in which the penicillin technique[8] was introduced for selection.

Cells growing logarithmically in YE broth (Difco-yeast extract, 10 g; water 1 liter and the pH was adjusted to 7.5 before autoclaving) were collected, suspended in tris-maleic buffer (pH 6.0) and treated with N-methyl-N'-nitro-N-nitrosoguanidine at 300 $\mu$g/ml under shaking at 37°C for 30 minutes (killing rate 92.2%). After being washed with saline, the mutagenized cells were suspended in YE broth supplemented with 200 $\mu$g/ml of N-acetyl-D-glucosamine and grown overnight. The grown cells were then collected, washed, and resuspended in YE broth without N-acetyl-D-glucosamine. After being shaken at 37°C for 1 hour, the culture was added with 2,000 units/ml of penicillin G and further incubated for additional 90 minutes. The survivors were then washed, plated on MM agar (cf. Table 4, footnote b) supplemented with 200 $\mu$g/ml of N-acetyl-D-glucosamine and incubated at 37°C for 3 days. The master plates were replicated on the MM agar plates supplemented with and without 200 $\mu$g/ml of N-acetyl-D-glucosamine. A colony that appeared on an N-acetyl-D-glucosamine supplemented plate but not on an unsupplemented one was picked up and purified. Thus, a glucosamine auxotroph designated as MCRL 5771 was obtained, frequency being approximately $5 \times 10^{-3}$ from the penicillin survivors.

Isolation of DOS from the fermentation broth

Fermentation broths of mutant MCRL 5003, which were incubated for 4 days in 500-ml flasks containing each 100 ml of the production medium, were combined and centrifuged. The supernatant (20 liters) was treated with Amberlite IRC-50 ($Na^+$ form, 2 liters). After being washed with water, the resin was packed into a column and eluted with 1.0 N $NH_4OH$ (*ca.* 7 liters). The eluate was evaporated *in vacuo* to give a concentrate (200 ml), which was adjusted to pH 7.0 with 2 N $H_2SO_4$ and passed through a column of Amberlite CG-50 ($NH_4^+$ form, 100 ml). After being washed with 1 liter of 0.05 N $NH_4OH$, the column was eluted with 0.1 N $NH_4OH$, collecting in portions of 200 ml. Tubes No. 17~22 gave 890 mg of crude DOS. The crude DOS was dissolved in 20 ml of water and the solution was passed through a column of CM-Sephadex C-25 ($NH_4^+$ form, 50 ml). After being washed with water, the column was eluted stepwise with each 250 ml of 0.01 N, 0.02 N and 500 ml of 0.03 N $NH_4OH$. The latter two eluates were combined, concentrated *in vacuo* and lyophilized to give 373 mg of DOS as white amorphous powder: m.p. 197~204°C. Rf value on TLC, and IR and $^1H$-NMR spectra of this compound were identical with those of the authentic DOS.

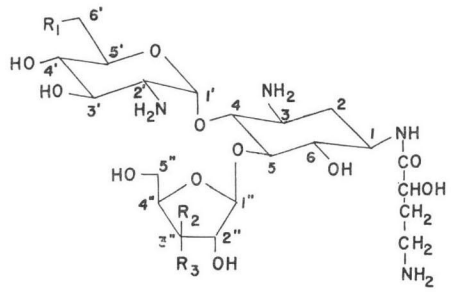
Anal. Calcd. for $C_6H_{14}N_2O_8$: C 44.43, H 8.70, N 17.27
Found: C 44.07, H 8.63, N 16.99

## Results

### Characterization of 2 Neamine-negative Mutants

As previously reported[1], 2 neamine-negative mutants, MCRL 5003 and MCRL 5004, were blocked at different steps in the biosynthetic pathway of butirosins and could not produce the antibiotic. These mutants, however, could produce butirosins when the culture medium was supplemented with neamine. Mutant MCRL 5004 could also produce butirosins from exogeneous DOS,

Fig. 2. Structures of butirosins and DAH-butirosins



| | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Butirosin A | $NH_2$ | OH | H |
| Butirosin B | $NH_2$ | H | OH |
| DAH-butirosin A | OH | OH | H |
| DAH-butirosin B | OH | H | OH |

Fig. 3. Time course of DOS production by mutant MCRL 5003

Mutant MCRL 5003 was cultivated in a 500-ml flask containing 100 ml of the production medium. At given interval of times, the sample of the culture was withdrawn and DOS production was determined by the method described in Materials and Methods.

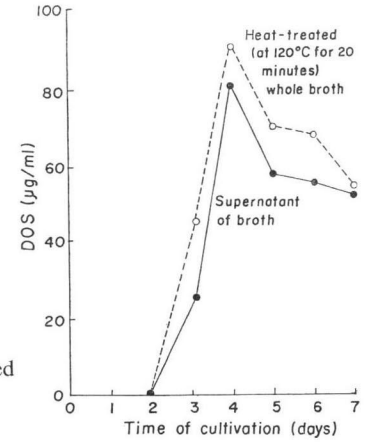
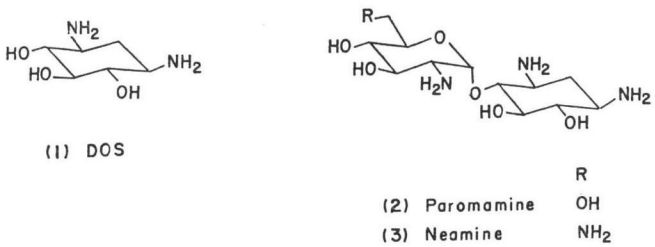


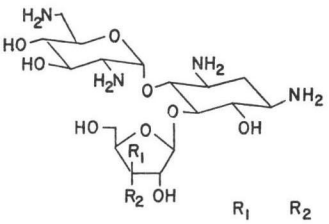

Fig. 4. Structures of DOS-containing compounds tested



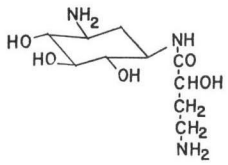
(1) DOS



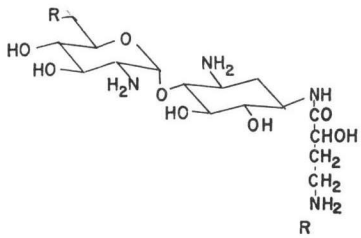
| | R |
|---|---|
| (2) Paromamine | OH |
| (3) Neamine | $NH_2$ |



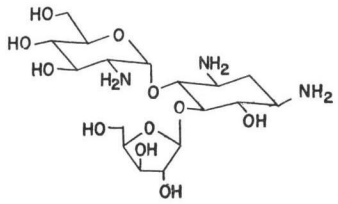
| | $R_1$ | $R_2$ |
|---|---|---|
| (4) Ribostamycin | H | OH |
| (5) Xylostasin | OH | H |



(6) AHB-DOS



| | R |
|---|---|
| (7) AHB-Paromamine | OH |
| (8) AHB-Neamine | $NH_2$ |



(9) DAH-Xylostasin

AHB = 1-N-(4-Amino-2-hydroxybutyryl)

whereas mutant MCRL 5003 could not produce any antibiotic from DOS.

Previously, it was also reported that cofermentation of these 2 mutants gave butirosins[1]. The following facts further clarified the roles of MCRL 5003 and MCRL 5004 as an intermediate-producer (secretor) and a butirosin-producer (converter), respectively, in this system. MCRL 5004 could produce butirosins when the culture was supplemented with the supernatant of the broth of MCRL 5003 or with the heat-treated (at 120°C for 20 minutes) whole broth of MCRL 5003. Under the reversed condition, MCRL 5003 could not produce any antibiotic. These results sug-

gested that MCRL 5003 secreted in the broth at least an active precursor of butirosins which might be DOS or its intermediate. This precursor was isolated from the broth of MCRL 5003 and identified as DOS by direct comparison with the authentic sample. The time course of DOS production by MCRL 5003 is shown in Fig. 3. Maximum production of DOS (about 80 $\mu$g/ml) was obtained at the 4th day after inoculation. Thus, MCRL 5003 and MCRL 5004 must be blocked just after and before the formation of DOS, respectively.

## Bioconversion of Plausible Biosynthetic Intermediates

Bioconversion of a series of DOS-containing compounds, considered as plausible biosynthetic intermediates of butirosins, was investigated to determine the subunit assembly in the biosynthesis of butirosins. The compounds listed in Figs. 2 and 4 were individually added to the culture medium,

Table 1. Production of butirosins[a] and the related antibiotics from DOS and DOS-containing compounds

| Precursor (0.2 mM) | Strain MCRL No. | Butirosin activity in broth ($\mu$g/ml) | | Antibiotics produced[c] | Conversion yield to butirosins (mol. %) |
|---|---|---|---|---|---|
| | | 0 day[b] | 7 days | | |
| DOS | 5003 | 0 | 0 | none | 0 |
| | 5004 | 0 | 93 | Butirosins (A: 80~90%) | 78 |
| Paromamine | 5003 | 0 | 90 | Butirosins (A: 80~90%) | 75 |
| | 5004 | 0 | 91 | Butirosins (A: 80~90%) | 76 |
| Neamine | 5003 | 0 | 88 | Butirosins (A: 80~90%) | 74 |
| | 5004 | 0 | 36 | Butirosins (A: 80~90%) | 30 |
| Ribostamycin | 5003 | 0 | 84 | Butirosins (A: 40~60%) | 70 |
| | 5004 | 0 | 29 | Butirosins (A: 40~60%) | 24 |
| Xylostasin | 5003 | 0 | 80 | Butirosin A | 67 |
| | 5004 | 0 | 29 | Butirosin A | 24 |
| AHB-DOS | 5003 | 0 | 0 | none | 0 |
| | 5004 | 0 | 0 | none | 0 |
| AHB-Paromamine | 5003 | 0 | 3 | AHB-Neamine | 0 |
| | 5004 | 0 | 4 | AHB-Neamine | 0 |
| AHB-Neamine | 5003 | 5 | 11 | Butirosin A (trace) & Neamine (trace) | <9 |
| | 5004 | 4 | 3 | none (unchanged) | 0 |
| DAH-Xylostasin | 5003 | 0 | 2 | DAH-Butirosin A | 0 |
| | 5004 | 0 | 2 | DAH-Butirosin A | 0 |
| DAH-Butirosin B | 5003 | 16 | 14 | DAH-Butirosin A | 0 |
| | 5004 | 14 | 13 | DAH-Butirosin A | 0 |
| DAH-Butirosin A | 5003 | 16 | 15 | none (unchanged) | 0 |
| | 5004 | 15 | 12 | none (unchanged) | 0 |
| Butirosin B | 5003 | 119 | 119 | Butirosin A | (*ca.* 50)[d] |
| | 5004 | 120 | 119 | Butirosin A | (*ca.* 50) |
| Butirosin A | 5003 | 121 | 120 | none (unchanged) | — |
| | 5004 | 120 | 120 | none (unchanged) | — |

a) A mixture of butirosin A and butirosin B.

b) Butirosin activity assayed immediately after the addition of the precursors.

c) All of the products were isolated from the broths (20 ml) by adsorption on Amberlite IRC-50 ($NH_4^+$ form) resin and subsequent elution with 1.0 N ammonia. Each concentrate of the eluates was compared with the reference antibiotics by TLC using 2 systems: System A, silica gel $60F_{254}$ plate (Merck, Art. 5554) using the solvent of $CHCl_3$ – MeOH – 28% $NH_4OH$ – $H_2O$ (1: 4: 2: 1, v/v) and System B, alumina $60F_{254}$ plate (Merck, Art. 5550, type E) using the solvent of the upper phase of $CHCl_3$ – MeOH – 17%$NH_4OH$ (2: 1: 1, v/v). Also, antibiotics were detected by bioautography against *Pseudomonas aeruginosa* No. 12 and *Bacillus subtilis* ATCC 6633.

d) Conversion yield from butirosin B to butirosin A.

and mutants MCRL 5003 and MCRL 5004 were inoculated, respectively. After being incubated for 7 days, the resulting antibiotics were characterized by bioassay and TLC analyses in 2 systems. These results are shown in Table 1.

As already mentioned, MCRL 5004 efficiently produced butirosins from DOS, whereas MCRL 5003 did not. Both strains produced butirosins from paromamine and ribostamycin as well as neamine. These mutants produced butirosin A from xylostasin and butirosin B, but nothing from butirosin A which remained unchanged in the broth.

Both mutants could not produce butirosins from 1-N-(4-amino-2-hydroxybutyryl)-DOS(AHB-DOS), AHB-paromamine, DAH-xylostasin and DAH-butirosins. Among these, AHB-DOS was not converted to any other antibiotic. Both strains produced AHB-neamine from AHB-paromamine. MCRL 5003 produced trace quantities of butirosin A and neamine from AHB-neamine, but the larger part of the added AHB-neamine remained unchanged in the broth of this strain. Two mutants produced DAH-butirosin A from DAH-xylostasin and DAH-butirosin B, however, they could not convert DAH-butirosin A to any other antibiotic and the added DAH-butirosin A remained unchanged in the broths.

These results suggested the following possibility about the biosynthesis of butirosins and DAH-butirosins.

(1) DOS, paromamine, neamine, ribostamycin, xylostasin and butirosin B are possible intermediates of butirosin A, because these compounds were efficiently converted to butirosin A by MCRL 5003 (except for DOS) or MCRL 5004. However, DAH-butirosins, which are produced as minor products by the parent strain, are probably shunt metabolites in the biosynthesis of butirosins.

(2) Insertion of the 6′-amino group of butirosins must take place before pseudotrisaccharide formation, since DAH-xylostasin and DAH-butirosins were not utilized in the biosynthesis of butirosins by both mutants.

(3) The 4-amino-2-hydroxybutyryl (AHB) group is probably introduced after the formation of pseudotrisaccharides, because AHB-DOS, AHB-paromamine and AHB-neamine were not or not significantly converted to butirosins by MCRL 5004 or MCRL 5003.

Table 2. Characterization and yield of the products under supplement of DOS, paromamine, neamine, ribostamycin and xylostasin

| Strain | MCRL 5004 | MCRL 5003 | | | |
|---|---|---|---|---|---|
| Precursor (100 $\mu$g/ml) | DOS | Paromamine | Neamine | Ribostamycin | Xylostasin |
| Products isolated[a] (Yield, $\mu$g/ml broth) | Butirosins (114), DAH-Butirosins (7),[b] Ribostamycin (5) & Xylostasin (2) | Butirosins (49), DAH-Butirosins(22),[b] Ribostamycin (3), Xylostasin (2) & Neamine (1) | Butirosins (64), Ribostamycin (0.4) & Xylostasin (trace) | Butirosins (48) & Xylostasin (0.3) | Butirosin A (42) |

a) The products were isolated from the broths (1~3 liters) by adsorption on Amberlite IRC-50 ($NH_4^+$ form) resin. The crude products were then separated by column chromatography on Amberlite CG-50 ($NH_4^+$ form) eluted with dilute ammonia (0.05~0.3 N). The butirosins and the related antibiotics were further purified by column chromatography on CM-Sephadex C-25 ($NH_4^+$ form) eluted with dilute ammonia. The structures of butirosins and DAH-butirosins were confirmed by mass spectrometric comparison with reference antibiotics and by their degradation studies. The other minor products were identified by TLC in 2 systems shown in Table 1.

b) A mixture of DAH-butirosin A (80~90%) and DAH-butirosin B (10~20%).

(4) Isomerization step is probably involved only in the conversion from *ribo*-isomer to *xylo*-isomer in the biosynthesis of butirosins, because ribostamycin and butirosin B were converted to butirosin A, but xylostasin and butirosin A were not converted to butirosin B by these 2 mutants.

The products converted from DOS, paromamine, ribostamycin and xylostasin were then characterized in detail and these results shown in Table 2 afforded further information as to the biosynthetic sequence of butirosins and DAH-butirosins.

(5) Pathways for butirosins and DAH-butirosins are derived from paromamine, because DAH-butirosins were produced only from DOS and paromamine, but not from neamine, ribostamycin and xylostasin.

(6) The route from paromamine to neamine, involving amination of paromamine at the C-6′ position, was deduced, because neamine was isolated as one of the minor products converted from paromamine.

(7) The following 3 routes are probably involved in the formation of butirosin A from neamine: (i) neamine→xylostasin→butirosin A, (ii) neamine→ribostamycin→butirosin B→butirosin A, and (iii) neamine→ribostamycin→xylostasin→butirosin A. The presence of these routes was strongly suggested by identification of the products converted from neamine, ribostamycin, xylostasin and butirosin B (Tables 1 and 2).

(8) The routes from paromamine to DAH-butirosin A are also assumed by analogy with the routes from neamine to butirosin A.

Based upon these observations, the biosynthetic pathways for butirosins and DAH-butirosins are summarized as shown in Fig. 1. Blocking sites of the above 2 mutants MCRL 5003 and MCRL 5004 would be as marked.

## Bioconversion of 3′,4′-Dideoxy Derivatives of Neamine, Ribostamycin and Butirosins

Among the 3 routes proposed for the pathway from neamine to butirosin A, the route (ii) was supported by the feeding experiments with 3′,4′-dideoxy derivatives of neamine, ribostamycin and butirosins as follows:

As reported previously[1], mutants MCRL 5003 and MCRL 5004 could convert 3′,4′-dideoxyneamine to 3′,4′-dideoxybutirosins as major products and 3′,4′-dideoxyribostamycin as a minor one.

Table 3. Production of 3′,4′-dideoxybutirosin A from 3′,4′-dideoxy derivatives of neamine by MCRL 5003

| Precursor[a] (100 μg/ml) | Antibiotic activity[b] in broth (μg/ml) | | Antibiotics produced[d] | Conversion yield[e] (mol. %) |
|---|---|---|---|---|
| | 0 day[c] | 7 days | | |
| none | 0 | 0 | none | 0 |
| 3′,4′-Dideoxyneamine | 0 | 63 | 3′,4′-Dideoxybutirosins (A: 50~60%) | 35 |
| 3′,4′-Dideoxyribostamycin | 0 | 89 | 3′,4′-Dideoxybutirosins (A: 50~60%) | 72 |
| 3′,4′-Dideoxybutirosin B | 100 | 100 | 3′,4′-Dideoxybutirosin A | (*ca.* 50) |
| 3′,4′-Dideoxybutirosin A | 110 | 110 | none (unchanged) | — |

a) Precursors were added to the medium supplemented with 1.5% Polypeptone to avoid the growth inhibition.

b) As a reference standard, 3′,4′-dideoxybutirosin base (50~60% A and 40~50% B) was used.

c) Antibiotic activity assayed immediately after the addition of the precursor.

d) See foot note c) in Table 1.

e) Conversion yield from precursor to 3′,4′-dideoxybutirosins (or that from 3′,4′-dideoxybutirosin B to 3′,4′-dideoxybutirosin A).

As shown in Table 3, MCRL 5003 could efficiently convert 3′,4′-dideoxyribostamycin to the 3′,4′-dideoxybutirosins. Mutant MCRL 5003 also converted 3′,4′-dideoxybutirosin B to 3′,4′-dideoxybutirosin A, but not *vice versa*. These results suggested the following pathways in MCRL 5003: 3′,4′-dideoxyneamine → 3′,4′-dideoxyribostamycin → 3′,4′-dideoxybutirosin B → 3′,4′-dideoxybutirosin A. Support for the above route (i) may similarly be provided by the feeding experiment with 3′,4′-dideoxyxylostasin, however, this compound was regretfully unavailable.

## Production of Butirosins by a Glucosamine Auxotroph

As shown in Table 4, a glucosamine auxotroph MCRL 5771 could utilize as a sole carbon source for growth either D-glucosamine or N-acetyl-D-glucosamine, but not D-glucose, suggesting that MCRL 5771 is blocked at some point in the conversion from D-glucose to D-glucosamine. The blocking site was not yet clarified in detail, but it may be the same as that of glucosamine auxotrophes of *Bacillus subtilis* which, as reported by FREESE *et al.*[9], lacked the L-glutamine-D-fructose-6-phosphate aminotransferase. Neosamine C (2,6-diamino-2,6-dideoxy-D-glucose) as a sole carbon source could not support the growth of this mutant as well as the parent strain (Table 4).

Butirosin production by MCRL 5771 was dependent upon the amounts of exogeneous D-glucosamine or N-acetyl-D-glucosamine in the range of 50~500 μg/ml (Table 5). Under the presence of limiting amounts (less than 100 μg/ml) of D-glucosamine or N-acetyl-D-glucosamine, formation of abnormal cells (round, protuberant or filamentous form) was observed. Similar morphological change was reported on the glucosamine auxotrophes of *B. subtilis* in limiting D-glucosamine[9]. It is interesting that in even such insufficient conditions, MCRL 5771 could produce butirosins. D-Glucosamine appeared to be more preferable than N-acetyl-D-glucosamine for butirosin production and these glucosamines could be replaced by N-acetyl-D-glucosaminides such as methyl N-acetyl-α(or β)-D-glucosaminide(s) and benzyl N-acetyl-α-D-glucosaminide as shown in Table 5.

Mutant MCRL 5771 was further examined as to whether or not glucosamine analogs such as 3-deoxy-D-glucosamine, 3,4-dideoxy-D-glucosamine, purpurosamine C (3,4-dideoxyneosamine C), 6-N-methylpurpurosamine C[10], 6-N-methylneosamine C as well as their 2-N-acetyl derivatives and/or their methyl or benzyl glucosaminides might be competitively utilized for the formation of butirosin analogs under the presence of limiting amounts of D-glucosamine or N-acetyl-D-glucosamine (50~200 μg/ml) in the media. This auxotroph, however, failed to produce any butirosin analogs from these glucosamine analogs when supplemented with these analogs in the range of the concentrations of 200 ~1,000 μg/ml. Furthermore, even if neosamine

Table 4. Growth response of glucosamine auxotroph MCRL 5771 and the parent strain MCRL 5001 on agar plates

| Addition (1 mg/ml) | Growth on agar plates[a] | | | |
|---|---|---|---|---|
| | MCRL 5771 | | MCRL 5001 | |
| | MM[b] | YE[c] | MM | YE |
| none | − | − | − | + |
| D-Glucose | − | − | + | + |
| D-Glucose+L-asparagine | − | − | + | + |
| D-Glucose+L-glutamine | − | − | + | + |
| D-Glucose+D-glucosamine | + | + | + | + |
| D-Glucose+N-acetyl-D-glucosamine | + | + | + | + |
| D-Glucose+Neosamine C | − | − | + | + |
| D-Glucosamine | + | + | + | + |
| N-Acetyl-D-glucosamine | + | + | + | + |
| Neosamine C | − | − | − | + |

a) Plates were inspected after 3 days of growth at 37°C. +: good growth and −: no growth.

b) MM agar plate: $K_2HPO_4$, 14 g; $KH_2PO_4$, 6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; $(NH_4)_2SO_4$, 2 g; agar, 15 g and water to 1 liter.

c) YE agar plate: yeast extract (Difco), 10 g; agar, 20 g and water to 1 liter (pH 7.5).

Table 5. Production of butirosins from D-glucosamine and its derivatives by glucosamine auxotroph MCRL 5771

| Precursor ($\mu$g/ml)[a] | | Butirosin activity in 7-day broth ($\mu$g/ml) | Morphological characteristic of cells[b] | Conversion yield to butirosins (mol. %) |
|---|---|---|---|---|
| D-Glucosamine | ( 50) | 15 | abnormal | 11 |
| | (200) | 168 | normal | 30 |
| | (500) | 260 | normal | 19 |
| N-Acetyl-D-glucosamine | ( 50) | 13 | abnormal | 10 |
| | (200) | 28 | normal | 5 |
| | (500) | 105 | normal | 8 |
| Methyl N-acetyl-$\alpha$-D-glucosaminide | (200) | 115 | — | 23 |
| Methyl N-acetyl-$\beta$-D-glucosaminide | (200) | 132 | — | 26 |
| Benzyl N-acetyl-$\alpha$-D-glucosaminide | (200) | 265 | — | 69 |
| none | | 0 | abnormal | 0 |

[a] Strain MCRL 5771 was cultivated in YE broth supplemented with 200 $\mu$g/ml of N-acetyl-D-glucosamine at 32°C over night, and the culture (3 ml) was inoculated into the production medium (30 ml) added with each precursor.

[b] The shape of the cells in 3-day broth was inspected in the phase contrast microscope: normal cells were observed as short rod form and abnormal cells were observed as round (protoplasting), protuberant or filamentous form. —: not observed.

C was added to the medium, no significant effect on the production of butirosins was observed under the above condition. These results suggested that the hydroxyl groups of D-glucosamine at the C-3 and C-6 positions might play an important role in the incorporation of D-glucosamine into butirosins.

## Discussion

Although the biosynthesis of some of the aminoglycoside antibiotics have been extensively studied[11], little is known about the biosynthesis of butirosins[12]. As to the antibiotics such as ribostamycin and neomycin which are structurally related to butirosins, the following biosynthetic sequence has been postulated by using DOS-negative mutants of *Streptomyces ribosidificus* (ribostamycin producer)[11,13] and *Streptomyces rimosus* forma *paromomycinus* (paromomycin producer)[14,15] or a ribostamycin-accumulating mutant of *Streptomyces fradiae* (neomycin producer)[16]: neosamine C+DOS→neamine→ribostamycin or further to neomycin. However, the first step for the neamine formation has not been experimentally demonstrated.

The present study performed with 2 neamine-negative mutants of *B. circulans* showed that, in butirosin biosynthesis, DOS was first attached by D-glucosamine (probably, UDP-N-acetyl-D-glucosamine) to form paromamine which was then converted to butirosins or DAH-butirosins (Fig. 1). This finding might give some suggestion to the biosynthetic pathway for ribostamycin and neomycin, especially to the formation of neamine.

DOS undoubtedly participates in the biosynthesis of butirosins, since it was isolated from the fermentation broth of mutant MCRL 5003, and converted to butirosins by mutant MCRL 5004. Involvement of neamine, ribostamycin and xylostasin as the intermediates were supported by positive conversion experiments to butirosins and also isolation of these compounds in the conversion tests (Table 2).

Additional evidence for the participation of xylostasin and ribostamycin as the intermediates was obtained by the study with the other type of blocked mutants of *B. circulans* MCRL 5001 which could not produce butirosins but could accumulate both xylostasin (80~90%) and ribostamycin (10~

20%) in the broths, as will be reported in future. The cofermentation of these mutants with MCRL 5003 or MCRL 5004 gave butirosins.

Among the 2 routes involving xylosylation and ribosylation of neamine at the C-5 position (Fig. 1), the route from neamine to xylostasin appears to be predominant when compared the A: B ratios of butirosins converted from ribostamycin (A: 40~60%), xylostasin (A: 100%) and butirosin B (A: *ca.* 50%) with those of butirosins from DOS, paromamine and neamine (A: 80~90%). If the route from neamine to ribostamycin is predominant, butirosins converted from neamine would give butirosin mixture in which the A: B ratio is similar to that from ribostamycin.

The following consideration and facts eliminated the possibility that pseudodisaccharides or pseudotrisaccharides tested were once broken down to DOS (for MCRL 5004) or paromamine (for MCRL 5003) and then reincorporated into butirosins. First, if these routes are present, the A: B ratio in the biosynthesized butirosins would be similar, irrespectively of the precursors added. Secondly, DAH-butirosins were derived only from DOS and paromamine, but not from neamine, ribostamycin and xylostasin (Table 2). Thirdly, as previously reported[1], neamine analogs were directly converted to the corresponding analogs of butirosins by mutants MCRL 5003 and MCRL 5004.

It was well known that some of the subunits in aminoglycoside antibiotics are biosynthesized from D-glucosamine[11], branching from the pathway for cell wall synthesis. This branched route may be regulated by a certain mechanism. In connection with this mechanism, it is interesting to note that the glucosamine auxotroph MCRL 5771 could utilize D-glucosamine or N-acetyl-D-glucosamine for the biosynthesis of butirosins, even when this mutant was harvested in the medium supplemented with limiting amounts (less than 100 $\mu$g/ml) of these compounds insufficient for the normal growth.

Acknowledgements

We are indebted to Dr. H. KAWAGUCHI of Bristol-Banyu Research Institute, Ltd., for supplying the strain of *E. coli* JR35/C600 and samples of DAH-butirosins A and B, and Dr. P. W. K. WOO of Parke, Davis & Company for supplying samples of butirosins A and B used for our comparison studies. Special thanks go to Dr. T. OKUDA of this company for his encouragements and helpful discussions throughout this work.

## References

1) TAKEDA, K.; S. OKUNO, Y. OHASHI & T. FURUMAI: Mutational biosynthesis of butirosin analogs. I. Conversion of neamine analogs into butirosin analogs by mutants of *Bacillus circulans*. J. Antibiotics 31: 1023~1030, 1978
2) TAKEDA, K.; A. KINUMAKI, H. HAYASAKA, T. YAMAGUCHI & Y. ITO: Mutational biosynthesis of butirosin analogs. II. 3′,4′-Dideoxy-6′-N-methylbutirosins, new semisynthetic aminoglycosides. J. Antibiotics 31: 1031~1038, 1978
3) TAKEDA, K.; A. KINUMAKI, S. OKUNO, T. MATSUSHITA & Y. ITO: Mutational biosynthesis of butirosin analogs. III. 6′-N-Methylbutirosins and 3′,4′-dideoxy-6′-C-methylbutirosins, new semisynthetic aminoglycosides. J. Antibiotics 31: 1039~1045, 1978
4) TAKEDA, K.; K. AIHARA, T. FURUMAI & Y. ITO: An approach to the biosynthetic pathway of butirosins and DAH-butirosins. J. Antibiotics 31: 250~253, 1978
5) MITSUHASHI, S.; L. ROSIVAL & V. KRCMERY: Drug inactivating enzymes and antibiotic resistance. pp. 115~119, Springer-Verlag, Berlin, 1975
6) MAEDA, M.; A. TSUJI, S. GANNO & Y. ONISHI: Fluorophotometric assay of amino acids by using automated ligand-exchange chromatography and pyridoxal-zinc (II) reagent. J. Chromatogr. 77: 434~438, 1973
7) SARVAS, M.: Mutant of *Escherichia coli* K-12 defective in D-glucosamine biosynthesis. J. Bacteriol. 105: 467~471, 1971
8) GORINI, L. & H. KAUFMAN: Selecting bacterial mutants by the penicillin method. Science 131: 604~605, 1961
9) FREESE, E. B.; R. M. COLE, W. KLOFAT & E. FREESE: Growth, sporulation, and enzyme defects of glucosamine mutants of *Bacillus subtilis*. J. Bacteriol. 101: 1046~1062, 1970
10) OHASHI, Y.; S. OKUNO, K. TAKEDA & Y. ITO: A convenient synthesis of N-methylpurpurosamine C. Carbohyd. Res. 67: 503~508, 1978

11) RINEHART, Jr., K. L. & R. M. STROSHANE: Biosynthesis of aminocyclitol antibiotics. J. Antibiotics 29: 319~353, 1976
12) CLARIDGE, C. A.; J. A. BUSH, M. D. DEFURIA & K. E. PRICE: Fermentation and mutation studies with a butirosin-producing strain of *Bacillus circulans*. Devel. Industr. Microbial. 15: 101~113, 1974
13) KOJIMA, M. & A. SATOH: Microbial semi-synthesis of aminoglycosidic antibiotics by mutants of *S. ribosidificus* and *S. kanamyceticus*. J. Antibiotics 26: 784~786, 1973
14) PEARCE, C. J.; J. E. G. BARNETT, C. ANTHONY, M. AKHTAR & S. D. GERO: The role of the pseudo-disaccharide neamine as an intermediate in the biosynthesis of neomycin. Biochem. J. 159: 601~606, 1976
15) PEARCE, C. J.; M. AKHTAR, J. E. G. BARNETT, D. MERCIER, A.-M. SEPULCHRE & S. D. GERO: Sub-unit assembly in the biosynthesis of neomycin. The synthesis of 5-O-$\beta$-D-ribofuranosyl and 4-O-$\beta$-D-ribofuranosyl-2,6-dideoxystreptamines. J. Antibiotics 31: 74~81, 1978
16) BAUD, H.; A. BETENCOURT, M. PEYRE & L. PENASSE: Ribostamycin, as an intermediate in the biosynthesis of neomycin. J. Antibiotics 30: 720~723, 1977