MUTATIONAL BIOSYNTHESIS OF BUTIROSIN ANALOGS*

I. CONVERSION OF NEAMINE ANALOGS INTO BUTIROSIN ANALOGS BY MUTANTS OF *BACILLUS CIRCULANS*

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By N-methyl-N′-nitro-N-nitrosoguanidine treatment, neamine-negative mutants which required neamine for biosynthesis of butirosins were obtained from a butirosin-producing organism *Bacillus circulans*. These mutants also produced butirosins from paromamine and could be divided into two types I and II. Mutants of type I could not produce butirosins from 2-deoxystreptamine, whereas those of type II could. Two typical mutants MCRL 5003 (type I) and MCRL 5004 (type II) could produce butirosin analogs, 3′,4′-dideoxybutirosins, 6′-N-methylbutirosins, 3′,4′-dideoxy-6′-N-methylbutirosins and 3′,4′-dideoxy-6′-C-methylbutirosins from neamine analogs, gentamine C_{1a}, 6′-N-methylneamine, 6′-N-methylgentamine C_{1a} and gentamine C_2, respectively.

Toxicities and emergence of resistant bacteria have been serious problems in the field of clinically important aminoglycoside antibiotics. As one way to overcome these problems, we took an interest in the modification of butirosins A and B[2], because butirosins are relatively less toxic than other aminoglycoside antibiotics and active against *Pseudomonas* strains together with resistant bacteria having aminoglycoside 3′-phosphotransferase I [APH(3′)-I][3]. Notwithstanding that several derivatives of butirosins active against resistant bacteria having APH(3′)-II[3,4] have been reported[5~10], any butirosin derivative effective against resistant organisms having aminoglycoside 6′-N-acetyltransferase[AAC(6′)][3] has not hitherto been known. We, therefore, undertook to prepare 6′-N-methyl or 6′-C-methyl derivatives and their 3′,4′-dideoxy derivatives of butirosins by a technique of "mutational biosynthesis"[11,12] from neamine analogs. They might conceivably result in widen spectra with improved activity against strains having inactivating enzyme, AAC(6′).

For this purpose, we first isolated neamine-negative mutants (neamine-dependent butirosin-producing mutants) of *Bacillus circulans*. These mutants produced butirosins also from paromamine and could be divided into two types. Mutants of type I could not convert 2-deoxystreptamine (DOS) into butirosins, while those of type II could. Two typical mutants MCRL 5003 (type I) and MCRL 5004 (type II) could further convert neamine analogs, 6′-N-methylneamine, 6′-N-methylgentamine C_{1a} and gentamine C_2 into new butirosin analogs, 6′-N-methylbutirosins, 3′,4′-dideoxy-6′-N-methylbutirosins and 3′,4′-dideoxy-6′-C-methylbutirosins, respectively. As expected, these butirosin analogs were active against butirosin-resistant bacteria having AAC(6′) as described in succeeding papers[13,14]. These two mutants also converted gentamine C_{1a} into 3′,4′-dideoxybutirosins. 3′,4′-Dideoxybutirosins A[6] and B[5] were already prepared by chemical modification of butirosin A and ribostamycin, respectively, however, the present finding is the first example of preparation of 3′,4′-

* A part of this paper was published as a preliminary communication[1].

dideoxybutirosins by biosynthesis.

This paper concerns the isolation and characterization of neamine-negative mutants as well as the production of butirosin analogs by these mutants. The isolation, properties and structures of new butirosin analogs are dealt in accompanying papers[13,14].

Materials and Methods

Organism and culture conditions

Bacillus circulans MCRL 5001 was used as a parent strain for mutation work. The strain was isolated and identified in our laboratory, and produced a mixture of butirosins A and B (A: 80~90%) as major products together with trace quantities of 6′-deamino-6′-hydroxybutirosins[15] (DAH-butirosins) A and B (A: 80~90%).

The organism grown on a heart infusion agar (Eiken Chemical Co., Ltd.) slant was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium (per liter); glucose, 4 g; yeast extract (Difco), 8 g; and malt extract (Difco), 20 g; the pH was adjusted to 7.5 before autoclaving, and incubated overnight at 32°C on a rotary shaker. A 3% inoculum thus prepared was transferred to a following production medium (per liter); glycerol, 40g; soybean meal, 20 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 40 mg; $FeSO_4 \cdot 7H_2O$, 5 mg and $ZnSO_4 \cdot 7H_2O$, 0.5 mg and the pH was adjusted to 7.5 before autoclaving. Fermentation was carried out at 32°C on a rotary shaker at 200 r.p.m. for flask culture or on a reciprocal shaker at 120 c.p.m. for test tube (diam. 23 mm) culture.

Detection of converted products

Preliminary detection of antibiotic activity in broth was carried out by paper disc method using two test organisms *Bacillus subtilis* ATCC 6633 and *Pseudomonas aeruginosa* No. 12. The latter might be replaced by an APH(3′)-II producing strain, *Escherichia coli* JR35/C600. Against strains No. 12 and JR35/C600, butirosins were active, whereas DAH-butirosins were slightly active, but neamine, ribostamycin and xylostasin were inactive at 100 μg/ml.

Determination of antibiotic activity

The broth to be tested was diluted with 0.1 M potassium phosphate buffer (pH 7.8). Antibiotic activity was determined by cup-plate method using *E. coli* JR35/C600 as a test organism and butirosins carbonate corresponding to 93% of butirosins base (A: 80~90%) as a reference standard.

Mutagenesis and selection of neamine-negative mutants

Cells growing logarithmically in nutrient broth (Difco) were collected, suspended in tris-maleic buffer (pH 6.0) and treated with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) at 1,000 μg/ml under shaking at 42°C for 30 minutes (killing rate 99.7%). After washing twice with saline, the mutagenized cells were spread on agar plates (per liter); glycerol, 20 g; soybean meal, 10 g and agar, 20 g, and incubated at 32°C for about 1 week. Colonies well separated on these agar plates were cut out with cork borer (diam. 6 mm) and placed on a test plate [(per liter); Polypeptone, 5 g and agar, 13 g; the pH was adjusted to 8.0 before autoclaving] seeded with *P. aeruginosa* No. 12. After incubation at 37°C overnight, colonies showing no inhibitory zone were picked up and purified. Butirosin-nonproducing mutants were then inoculated into two test tubes, each containing 3 ml of the production medium and one of tubes being supplemented with 100 μg/ml of neamine, and incubated for 1 week. Thus, neamine-negative mutants were selected which showed activity against *P. aeruginosa* No. 12 only in the medium supplemented with neamine.

Isolation and identification of converted products

Converted products in broth were adsorbed on Amberlite IRC-50 (NH_4^+) resin and eluted with 1.0 N NH_4OH. The active eluate was concentrated *in vacuo* and lyophilized. The resulting mixture of the products was separated into each component or at least a pair of isomeric antibiotics by repeating column chromatography on Amberlite CG-50 (NH_4^+) or CM-Sephadex C-25 (NH_4^+) using 0.1~0.4 N NH_4OH as eluants. Among the converted products, the known ones were identified by direct com-

parison with the authentic samples by usual methods applying TLC, mass, IR, ^{1}H-NMR, elemental analysis and/or acid hydrolysis.

Materials

All precursors used in this work were prepared in our laboratory. NTG was obtained from Aldrich Chemical Co., Inc. Authentic samples of butirosins A and B, and DAH-butirosins A and B were gifts from Drs. P. W. K. Woo and H. Kawaguchi, respectively.

Results

Isolation and Classification of Neamine-negative Mutants

By NTG treatment and subsequent agar-plug selection, 110 butirosin-nonproducing mutants were obtained from about 3,000 survivors of *B. circulans* MCRL 5001 (frequency, 3.7×10^{-2}). Eight strains of these mutants produced butirosins when cultured in the medium supplemented with neamine (frequency, 2.7×10^{-3}). These mutants are designated as neamine-negative mutants.

As shown in Table 1, these neamine-negative mutants also produced butirosins from paromamine, and could be divided into two types I and II, according to the ability of utilizing DOS for production of butirosins. Mutants belonging to type II could produce butirosins from DOS, suggesting that these mutants are blocked somewhere before DOS formation. In contrast, mutants belonging to type I could not produce any antibiotic from DOS. Mutants of type I failed to produce butirosins when neamine was replaced by an equivalent mixture of its constituents, neosamine C and DOS.

Table 1. Production of butirosins from neamine, paromamine and DOS by neamine-negative mutants of *B. circulans* MCRL 5001.

Mutant strain		Production of butirosins[a)]		
Type	MCRL No.	Neamine	Paromamine	DOS
I	5003	27.0[b)]	20.5	—
	5083	18.5	15.0	—
	5615	18.0	14.0	—
	5621	22.0	21.0	—
II	5004	20.5	17.0	26.5
	5602	12.0	16.0	15.5
	5605	16.5	24.5	23.0
	5607	16.0	15.0	21.5

Abbreviation: DOS, 2-deoxystreptamine.

a) Precursors (100 μg/ml) were added individually to the test tubes each containing 3 ml of the production medium before inoculation. Fermentation was carried out for 7 days.

b) Diameter (mm) of inhibitory zone obtained by paper disc (8 mm) assay using *P. aeruginosa* No. 12 (−showed no activity).

Mixed culture of two strains of these neamine-negative mutants was further studied in every combination. The results are shown in Table 2. The complementation pattern for production of butirosins suggested that these mutants are probably blocked in different steps in the formation of neamine. Two typical mutants of each type, MCRL 5003 (type I) and MCRL 5004 (type II), were used for preparation of butirosin analogs. MCRL 5004 could produce butirosins in the mixed culture with MCRL 5003 (Table 2), suggesting that MCRL 5003 accumulates DOS or its related compounds in the cultured broth. DOS was really isolated from the normal fermentation broth of MCRL 5003 as will be dealt elsewhere.

Neamine- or DOS-dependent Butirosin Production

Fig. 1 shows the time courses of typical fermentations by mutants MCRL 5003 and MCRL 5004, when the medium was supplemented with 100 μg/ml of neamine before inoculation. Antibiotic production by both strains began to increase 3 days after inoculation and reached a maximum (MCRL 5003:

Table 2. Production of butirosins by mixed culture[a] of two neamine-negative mutants.

Mutant strain		Type I				Type II			
Type	MCRL No.	5003	5083	5615	5621	5004	5602	5605	5607
I	5003	—							
	5083	—	—						
	5615	—	11.0	—					
	5621	20.5[b]	15.0	—	—				
II	5004	13.5	—	19.5	20.0	—			
	5602	19.5	—	—	17.0	—	—		
	5605	21.5	—	—	11.5	—	18.5	—	
	5607	16.0	—	14.0	11.5	—	13.5	—	—

a) Strains were individually cultivated for 3 days in a 250-ml flask containing 30 ml of the production medium. The culture broths (each 1.5 ml) of two strains were then mixed in a test tube and incubated for additional 2 days.

b) See footnote b) in Table 1.

Fig. 1. Production of butirosins from neamine by mutants MCRL 5003 and MCRL 5004

Neamine (100 μg/ml) was added to 250-ml flasks each containing 30 ml of the production medium before inoculation.

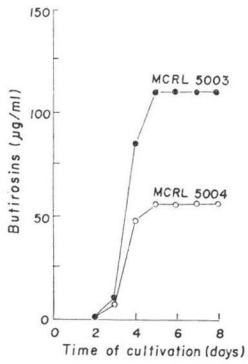


Fig. 2. Production of butirosins from DOS by mutant MCRL 5004

DOS (100 μg/ml) was added to a 250-ml flask containing 30 ml of the production medium before inoculation.

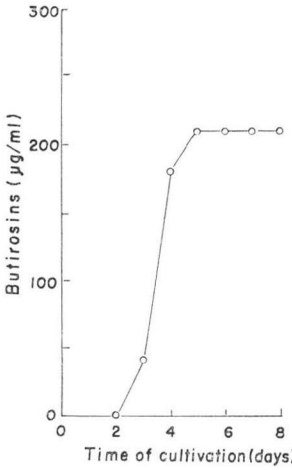
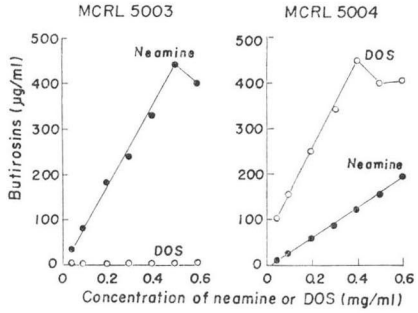


Fig. 3. Effect of the concentration of neamine or DOS on production of butirosins.

Neamine or DOS was individually added at 2 days to 500-ml flasks each containing 40 ml of the production medium and the culture was further incubated for additional 5 days.



110 μg/ml and MCRL 5004: 54 μg/ml) at the 5th day and the activities remained unchanged up to the 8th day. The products were extracted from the broths by a procedure using Amberlite IRC-50 resin. Bioautographic analyses of the crude extracts showed that these substances produced by both strains were ascribed to a mixture of butirosins A and B (A: 80~90%), as in the fermentation of the parent strain. In the case of MCRL 5003, butirosins (192 mg, A: 80~90%) and trace quantities of ribostamycin (1.2 mg) and xylostasin (less than 1 mg) were isolated from the broth (3 liters).

Fig. 2 shows the time course of a typical fermentation by mutant MCRL 5004 when the medium was supplemented with 100 μg/ml of DOS before inoculation. Antibiotic production began to increase

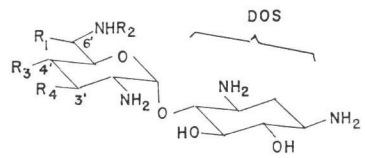
3 days after inoculation and reached a maximum (210 μg/ml) at the 5th day, and the activity remained unchanged up to the 8th day. Butirosins (228 mg, A: 80~90%) and trace quantities of DAH-butirosins (14 mg, A: 80~90%), ribostamycin (10 mg) and xylostasin (4 mg) were isolated from the broth (2 liters).

Concentration-dependency of neamine or DOS for production of butirosins was shown in Fig. 3. Addition of higher concentration of neamine (more than 600 μg/ml) resulted in lysis of both strains.

Conversion of Neamine Analogs into Butirosin Analogs

By using above neamine-negative mutants, five neamine analogs, gentamine C_{1a} (**I**), 6′-N-methylneamine (**II**), 6′-N-methylgentamine C_{1a} (**III**), gentamine C_2 (**IV**) and gentamine C_1 (**V**), were tested for the conversion into butirosin analogs. Structures of these neamine analogs are shown in Fig. 4.

Fig. 4. Structures of neamine analogs tested.



	R_1	R_2	R_3	R_4
I	H	H	H	H
II	H	CH_3	OH	OH
III	H	CH_3	H	H
IV	CH_3	H	H	H
V	CH_3	CH_3	H	H
Neamine	H	H	OH	OH

Table 3 shows antibiotic production by mutants MCRL 5003 and MCRL 5004 when the medium was supplemented with 100 μg/ml of neamine analogs 24 hours after inoculation. In the conversion of **IV** and **V**, Polypeptone (1.5%) must be added to the medium to avoid marked growth inhibition of both strains caused by **IV** and **V**. Under these conditions, strains MCRL 5003 and

Table 3. Production of butirosin analogs by mutants MCRL 5003 and MCRL 5004

Precursor[a] (100 μg/ml)	Strain MCRL No.	Antibiotic activity[b] (μg/ml)	Major antibiotics produced[c] (ratio of main isomer)	Conversion yields[d] (mol. %)
Neamine	5003	100	Butirosins A & B (A: 80~90%)	58
	5004	64	Butirosins A & B (A: 80~90%)	37
Gentamine C_{1a} (**I**)	5003	80	DDB-A & DDB-B (B: 50~60%)	53
	5004	55	DDB-A & DDB-B (B: 60~70%)	36
6′-N-Methyl-neamine (**II**)	5003	24	NMB-A & NMB-B (A: 90~95%)	21
	5004	11	NMB-A & NMB-B (A: 90~95%)	10
6′-N-Methyl-gentamine C_{1a} (**III**)	5003	45	DMB-A & DMB-B (B: 70~80%)	41
	5004	28	DMB-A & DMB-B (B: 70~80%)	26
Gentamine C_2 (**IV**)	5003	23	DCB-A & DCB-B (B: 80~90%)	21
	5004	25	DCB-A & DCB-B (B: 80~90%)	22
Gentamine C_1 (**V**)	5003	1.4	none	0
	5004	1.5	none	0

Abbreviations: DDB-A, 3′,4′-dideoxybutirosin A; DDB-B, 3′,4′-dideoxybutirosin B; NMB-A, 6′-N-methylbutirosin A; NMB-B, 6′-N-methylbutirosin B; DMB-A, 3′,4′-dideoxy-6′-N-methylbutirosin A; DMB-B, 3′,4′-dideoxy-6′-N-methylbutirosin B; DCB-A, 3′,4′-dideoxy-6′-C-methylbutirosin A, and DCB-B, 3′,4′-dideoxy-6′-C-methylbutirosin B.

a) Precursor was added at 24 hours to a 250-ml flask containing 30 ml of the production medium and the culture was further incubated for additional 6 days.

b) The broth at the 7th day was assayed against *E. coli* JR35/C600 using butirosins as the reference standard. When assayed immediately after the addition of the precursor (100 μg/ml), the broth supplemented with neamine or **II** showed no activity against this test organism, whereas that for **I**, **III**, **IV** or **V** showed activity as follows; **I** (5.0 μg/ml calculated as butirosins), **III** (1.6 μg/ml), **IV** (1.7 μg/ml) and **V** (1.9 μg/ml).

c) A (*xylo*-isomer) and B (*ribo*-isomer)

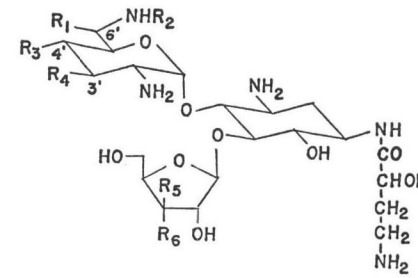
d) Conversion yields from neamine or its analogs into butirosins and their analogs were obtained by using the major products as the reference standard.

Table 4. TLC of butirosins and their analogs

Compounds	Rf values	
	System A	System B
DDB-A	0.20	0.49
DDB-B	0.20	0.03
NMB-A	0.15	0.29
NMB-B	0.15	0.03
DMB-A	0.22	0.57
DMB-B	0.22	0.03
DCB-A	0.24	0.57
DCB-B	0.24	0.03
Butirosin A	0.13	0.21
Butirosin B	0.13	0.03

System A: silica gel $60F_{254}$ plate (Merck, Art. 5554) using the solvent of $CHCl_3$ - MeOH - 28% NH_4OH (1 : 3 : 2, v/v). 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).

Fig. 5. Structures of butirosin analogs produced.



	R_1	R_2	R_3	R_4	R_5	R_6
DDB-A	H	H	H	H	OH	H
DDB-B	H	H	H	H	H	OH
NMB-A	H	CH_3	OH	OH	OH	H
NMB-B	H	CH_3	OH	OH	H	OH
DMB-A	H	CH_3	H	H	OH	H
DMB-B	H	CH_3	H	H	H	OH
DCB-A	CH_3	H	H	H	OH	H
DCB-B	CH_3	H	H	H	H	OH
Butirosin A	H	H	OH	OH	OH	H
Butirosin B	H	H	OH	OH	H	OH

MCRL 5004 could convert neamine analogs such as **I**, **II**, **III** and **IV** into the corresponding analogs of butirosins. In contrast, **V** was not converted into any other detectable antibiotics by these mutants.

A complex of 3′,4′-dideoxybutirosins A and B (DDB-A & -B, 235 mg, B: 50~60%) and trace quantities of 3′,4′-dideoxyribostamycin[16] (28 mg) and 1-N-(4-amino-2-hydroxybutyryl)gentamine C_{1a} (14 mg) were isolated from the broth (3.6 liters) of MCRL 5003 supplemented with 100 μg/ml of **I**. Similar result was obtained in the case of MCRL 5004, although the yields of DDB-A and DDB-B were less than that of MCRL 5003. The complex of DDB-A and DDB-B was separated into each component and characterized as follows:

DDB-A: m.p. 195~200 °C (dec.); $[\alpha]_D^{25}$ +25.0° (*c* 0.4, H_2O), IR (KBr), 1635 and 1570 cm^{-1} (amide); Anal. Calcd. for $C_{21}H_{41}N_5O_{10}\cdot H_2CO_3\cdot H_2O$: C 43.78, H 7.51, N 11.60. Found: C 43.69, H 7.43, N 11.61.

DDB-B: m.p. 190~195°C (dec.); $[\alpha]_D^{25}$ +32.0° (*c* 0.4, H_2O); IR (KBr), 1635 and 1570 cm^{-1} (amide); Anal. Calcd. for $C_{21}H_{41}N_5O_{10}\cdot H_2CO_3\cdot H_2O$: C 43.78, H 7.51, N 11.60. Found: C 43.62, H 7.64, N 11.67.

Furthermore, new butirosin analogs, 6′-N-methylbutirosins (NMB-A & -B), 3′,4′-dideoxy-6′-N-methylbutirosins (DMB-A & -B) and 3′,4′-dideoxy-6′-C-methylbutirosins (DCB-A & -B) were isolated as the major products from the cultured broths supplemented with **II**, **III** and **IV**, respectively, as will be described in the succeeding papers[13,14].

Rf values for differentiation purpose and the structures of these butirosin analogs are shown in Table 4 and Fig. 5, respectively.

Discussion

Since SHIER *et al.*[11] first demonstrated the application of the technique of "mutational biosynthesis" for production of semisynthetic aminoglycoside antibiotics, several DOS-negative mutants of aminoglycoside-producing organisms have been isolated and utilized for preparation of new antibiotics modified in DOS moiety of the parent antibiotics[17,18]. These mutants were proved to be useful for production of new antibiotics from DOS analogs. However, before initiation of our present work, incorporation of pseudodisaccharide precursors by such mutants was successful only in one case.* Namely, KOJIMA and SATOH[19] reported that a DOS-negative mutant of *Streptomyces ribosidificus* incorporated neamine and gentamine C_{1a} (**I**) into ribostamycin and 3′,4′-dideoxyribostamycin, respectively. However, CLARIDGE *et al.*[24] reported that neamine was not incorporated into butirosins by their DOS-negative mutants of *Bacillus circulans*. In contrast, we have succeeded in isolating neamine-negative mutants of *B. circulans*. Two typical strains of them, MCRL 5003 (type I) and MCRL 5004 (type II), could produce butirosin analogs from neamine analogs (Table 3).

In order to analyze structural specificity in the incorporation of neamine analogs, conversion yields of the precursors into butirosin analogs were compared. As evident in Table 3, the conversion of neamine analogs took place readily by both mutants in the following order: neamine > **I** > **III** > **IV** ≧ **II** > **V**. The order was unchanged even when all the precursors were examined in the medium added with Polypeptone. Similar conversion yields shown by **I** and neamine suggested that the presence of the hydroxyl groups of neamine at C-3′ and C-4′ positions might bring no serious effect on the biosynthesis of butirosins from neamine. In contrast, modification of neamine at C-6′ position generally resulted in the reduction of the conversion yields. From these results, the conversion rate of **II** was expected to be similar to that of **III**, however, the incorporation of **II** was less than that of **III** as shown in Table 3. Although the origin of this reduced rate for **II** is not clear now, it may connect with the presence of a minor route toward a shunt metabolite in the conversion of **II**. We do not now deal with the minor products from **II** and **III**, however, we noticed that the accumulation of by-product from **II** was more significant than in the case of **III**. In the conversion of **II**, the by-product was isolated and identified as 1-N-(4-amino-2-hydroxybutyryl)-6′-N-methylneamine, as described in a succeeding paper[14]. This by-product was not converted into the corresponding analogs of the butirosins and remained unchanged in the cultured broth of both mutants. These results suggested the presence of a minor route from **II** into the shunt metabolite in the conversion of **II**.

It is also interesting to note here that the ratio of *xylo*-isomer to *ribo*-isomer in a pair of butirosin analogs was greatly dependent upon the structures of the added precursors (Fig. 4). In the case of **I**, **III** and **IV**, the *ribo*-isomer was predominant, whereas **II** gave mainly the *xylo*-isomer as did neamine (Table 3).

Acknowledgements

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* Up to the present time, the following three DOS-negative mutants have been reported which can incorporate pseudodisaccharide precursors: *Micromonospora inyoensis* (sisomicin)[20], *Bacillus vitellinus* (butirosin A)[21] and *Streptomyces rimosus* forma *paromomycinus* (paromomycin)[22,23].

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