AN APPROACH TO THE BIOSYNTHETIC PATHWAY OF BUTIROSINS AND THE RELATED ANTIBIOTICS

Sir:

In the foregoing communication[1], we have reported the preparation of new butirosin analogs using two neamine-negative mutants derived from *Bacillus circulans* MCRL 5001, a producer of butirosins[2] and 6′-deamino-6′-hydroxybutirosins (DAH-butirosins).[3]

In the present communication, we describe a proposed biosynthetic pathway of butirosins and DAH-butirosins. Previously, CLARIDGE *et al.*[4] attempted to elucidate the pathway of the butirosins biosynthesis using a 2-deoxystreptamine(DOS)-negative mutant of *Bacillus circulans*. However, they could not propose a definite pathway, since their mutant accumulated butirosins from DOS but did not from either pseudodisaccharide or pseudotrisaccharide precursors. In contrast, our results with the neamine-negative mutants suggest that the biosynthesis of butirosins and DAH-butirosins take place according to the pathway in Fig. 1. In these experiments, the method reported by TESTA and TILLEY[5,6] was utilized.

The neamine-negative mutants, MCRL 5003 and MCRL 5004[1], used in this study are blocked in the pathway of butirosins biosynthesis. These mutants, however, are able to produce butirosins when the medium was supplemented with neamine. The biosynthesis was investigated by the addition of DOS and DOS-containing compounds to the culture medium and then characterizing the resulting antibiotics. These results are shown in Table 1 and Table 2.

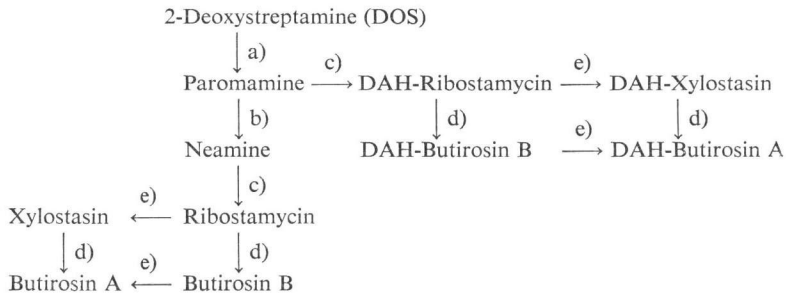
Strain MCRL 5004 produced butirosins from DOS, whereas strain MCRL 5003 did not. Both of these strains efficiently produced butirosins from paromamine and ribostamycin as well as neamine. These mutants also produced butirosin A from xylostasin[7] and butirosin B, but did not convert butirosin A to butirosin B.

The conversion of the compounds tested to butirosins and DAH-butirosins by neamine-negative mutants lead us to the following conclusion about the biosynthesis of butirosins and DAH-butirosins.

(1) Strain MCRL 5004 produced butirosins from DOS, paromamine, neamine and ribostamycin, but not from 1-N-(4-amino-2-hydroxybutyryl) DOS (AHB–DOS), AHB-paromamine and AHB-neamine. Trace amount of butirosin A, however, was produced from AHB-neamine by strain MCRL 5003. The amount was too small, however, to support a route from AHB-neamine to butirosin A as a main pathway (Table 1). It was, therefore, concluded that the 4-amino-2-hydroxybutyryl (AHB) groups of butirosins are probably introduced after the formation of pseudotrisaccharides.

(2) Both strains produced DAH-butirosin A from DAH-xylostasin and DAH-butirosin B. However, these pseudotrisaccharides could not be converted into butirosins (Table 1). This sug-

Fig. 1. A proposed pathway for biosynthesis of butirosins and DAH-butirosins from DOS in *B. circulans* MCRL 5001



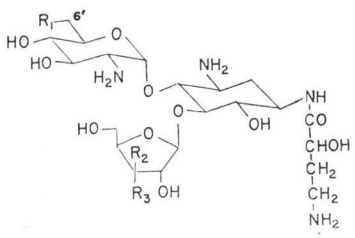
Abbreviation: DAH, 6′-Deamino-6′-hydroxy.

a) Pseudodisaccharide formation
b) Conversion of a 6′-hydroxy 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

gests that the conversion of the 6′-hydroxy groups into the 6′-amino groups of butirosins must take place before pseudotrisaccharides formation. The route from paromamine to ribostamycin *via* neamine was assumed by detection of neamine and ribostamycin among the products derived from paromamine and neamine by strain MCRL 5003, respectively (Table 2).

(3) The mutants, which produced butirosins A and B from ribostamycin, produced only butirosin A from xylostasin. In addition, these mutants produced butirosin A from butirosin B, however, butirosin A was not converted into any other detectable antibiotics (Table 1). These data suggest that the isomerization is involved

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

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

Precursors[b]	Strain MCRL 5003		Strain MCRL 5004	
	Antibiotics produced[c]	Conversion yield to butirosins (mol. %)[e]	Antibiotics produced	Conversion yield to butirosins (mol. %)
DOS	none	0	Butirosins (A: 80~90%)	78
Paromamine	Butirosins (A: 80~90%)[d]	75	Butirosins (A: 80~90%)	76
Neamine	Butirosins (A: 80~90%)	74	Butirosins (A: 80~90%)	30
Ribostamycin	Butirosins (A: 40~60%)	70	Butirosins (A: 40~60%)	24
Xylostasin	Butirosin A	67	Butirosin A	24
AHB–DOS	none	0	none	0
AHB-Paromamine	AHB-Neamine	0	AHB-Neamine	0
AHB-Neamine	Butirosin A (trace) & Neamine (trace)	3	none (unchanged)	0
DAH-Xylostasin	DAH-Butirosin A	0	DAH-Butirosin A	0
DAH-Butirosin B	DAH-Butirosin A	0	DAH-Butirosin A	0
DAH-Butirosin A	none (unchanged)	0	none (unchanged)	0
Butirosin B	Butirosin A	(*ca.* 50)[d]	Butirosin A	(*ca.* 50)
Butirosin A	none (unchanged)	—	none (unchanged)	—

Abbreviation: AHB, 1-N-(4-Amino-2-hydroxybutyryl).

a) A mixture of butirosin A and butirosin B

b) Each precursor (0.2 mM) was added to a 250-ml flask containing 30 ml medium (glycerol, 40 g; 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; $ZnSO_4 \cdot 7H_2O$, 0.5 mg; tap water to 1,000 ml; pH 7.5 adjusted before autoclaving) at the time of inoculation and was incubated at 32°C with shaking (200 rpm) for 7 days.

c) All of the products were isolated from the broths (20 ml) by adsorption on Amberlite IRC-50 (NH_4^+ form) resin followed by elution with 1.0 N ammonia. Each concentrate of the eluate was compared with the reference antibiotics by TLC using two systems: System A, silica gel TLC, $CHCl_3$ - MeOH - NH_4OH - H_2O (1: 4: 2: 1, v/v); System B, alumina TLC, the upper phase of $CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1, v/v). Also, antibiotics were detected by bioautography against *P. aeruginosa* No. 12 and *B. subtilis* ATCC 6633.

d) The ratio of butirosin A to butirosin B was determined by TLC in system B.

e) Conversion yields of added precursors to butirosins were determined by the cup plate method with *E. coli* JR35/C600, a producer of APH(3′)-I. As a reference standard, butirosins carbonate (A: 80~90%) was used.

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

Precursors[a] (100 μg/ml)	Strains (MCRL)	Products isolated (Yield, μg/ml broth)[b]
DOS	5004	Butirosins (114), *DAH-Butirosins (7), Ribostamycin (5) & Xylostasin (2).
Paromamine	5003	Butirosins (49), *DAH-Butirosins (22), Ribostamycin (3), Xylostasin (2) & Neamine (1).
Neamine	5003	Butirosins (64), Ribostamycin (0.4) & Xylostasin (trace).
Ribostamycin	5003	Butirosins (48) & Xylostasin (0.3).
Xylostasin	5003	Butirosin A (42)

* DAH-Butirosins (A: 80~90%)

a) Shaken flask fermentations were carried out by the same conditions as shown in Table 1.

b) The products were isolated from the broth (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 related antibiotics were further purified by column chromatography on CM-Sephadex C-25 (NH_4^+ form) eluted 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 two systems shown in Table 1.

only in the conversion of the *ribo*-isomers to *xylo*-isomers in the biosynthesis of butirosins. The route from ribostamycin to butirosin A *via* xylostasin was assumed by detection of xylostasin among the products derived from ribostamycin by strain MCRL 5003 (Table 2).

(4) DAH-Butirosins were produced from DOS and paromamine, but were not from neamine, ribostamycin and xylostasin (Table 2). This data eliminates the routes from neamine, ribostamycin or xylostasin to paromamine or DOS.

(5) The routes from paromamine to DAH-butirosin A were also assumed by analogy with the routes from neamine to butirosin A, although DAH-ribostamycin was not examined in the present conversion test, because of the difficulty in obtaining the sufficient amount of DAH-ribostamycin.

Based upon these observations, the biosynthetic pathway shown in Fig. 1 was proposed for butirosins and DAH-butirosins formation.

As to the possible blocked sites of the neamine-negative mutants, strain MCRL 5004 appears to be blocked in the formation of DOS while strain 5003 appears to be blocked in the convertion of DOS to paromamine.

Acknowledgements

We are indebted to Dr. H. KAWAGUCHI, Bristol-Banyu Research Institute, Ltd., for supplying a test organism, *Escherichia coli* JR35/C600. We wish to also thank Dr. T. OKUDA in this company for his encouragement and helpful advices throughout this work.

KATSUO TAKEDA
KATSUFUMI AIHARA
TAMOTSU FURUMAI
YUKIO ITO

Microbiological Reseach Laboratory
Tanabe Seiyaku Co. Ltd.
Toda, Saitama, Japan
(Received October 20, 1977)

References

1) TAKEDA, K.; A. KINUMAKI, T. FURUMAI, T. YAMAGUCHI, S. OHSHIMA & Y. ITO: Mutational biosynthesis of butirosin analogs. J. Antibiotics 31: 247~249, 1978
2) WOO, P. W. K.; H. W. DION & Q. R. BARTZ: Butirosins A and B, aminoglycoside antibiotics. III. Structures. Tetrahed. Lett. 1971: 2625~2628, 1971
3) TSUKIURA, H.; K. SAITO, S. KOBARU, M. KONISHI & H. KAWAGUCHI: Aminoglycoside antibiotics. IV. BU-1709 E_1 and E_2, new aminoglycoside antibiotics related to the butirosins. J. Antibiotics 26: 386~388, 1973
4) 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. Microbiol. 15: 101~113, 1974

5) TESTA, R. T. & B. C. TILLEY: Biotransformation, a new approach to aminoglycoside biosynthesis. I. Sisomicin. J. Antibiotics 28: 573~579, 1975
6) TESTA, R. T. & B. C. TILLEY: Biotransformation, a new approach to aminoglycoside biosynthesis. II. Gentamicin. J. Antibiotics 29: 140~146, 1976
7) HORII, S.; I. NOGAMI, N. MIZOKAMI, Y. ARAI & M. YONEDA: New antibiotic produced by bacteria, 5-β-D-xylofranosylneamine. Antimicr. Agents & Chemoth. 5: 578~581, 1974