ANTIBIOTICS DERIVED FROM A MUTANT OF *BACILLUS CIRCULANS*

HAROLD D. TAYLOR and HENRY SCHMITZ

Research Division, Bristol Laboratories, Division of Bristol-Myers Company
Syracuse, New York 13201, U.S.A.

(Received for publication January 9, 1976)

A mutant of *Bacillus circulans*, which produces butirosins only when 2-deoxystreptamine is added to the fermentation medium, was employed in the biosynthesis of antibiotics containing modified aminocyclitols. The blocked mutant converted 2,5-dideoxystreptamine into 5-deoxybutirosamine. Streptamine was incorporated into a complex differing from butirosin by an additional hydroxyl at C-2.

Mutant strains of streptomyces have been used in the biosynthesis of new aminoglycoside antibiotics. Deoxystreptamine-negative *Streptomyces fradiae*[1] incorporated either supplemental 2-deoxystreptamine (DOS) to produce neomycins, or streptamine or epi-streptamine to form hybrimycins. Similarly, mutants of *S. kanamyceticus* and *S. ribosidificus*[2] utilized DOS or neamine analogues for the biosynthesis of new antibiotics.

Studies in our laboratories[3] described blocked mutants of *Bacillus circulans* which produced antibiotics only when aminocyclitols (DOS, streptamine, streptidine) were added to the fermentation medium. We now report the structure elucidation of products obtained when exogenous DOS, 2,5-dideoxystreptamine[4] or streptamine was employed in fermentations of the DOS-negative mutant.*

Experimental

The selection of the mutant and the fermentations were carried out as previously reported.[3] The products were isolated and purified by absorption and repeated chromatography on IRC 50 essentially as described.[5] Samples were hydrolyzed with N hydrochloric acid at 60°C for one hour or with 6 N acid at 100°C for 40 hours. The hydrolysates were chromatographed on silica gel thin-layer plates (60F-254, EM Reagents), using the solvent systems chloroform - ammonium hydroxide - methanol (1: 2: 3) and butanol - acetic acid - water (4: 1: 5).

The fragments were trimethylsilylated, separated by gas-liquid chromatography and subjected to mass spectroscopy. Alternatively, the intact antibiotics were N-acetylated and methylated[7] and analysed by mass spectroscopy. The pmr spectra were obtained with a Varian HA 100, 15″; samples were dissolved in DMSO - MeOH. Streptamine and DOS were prepared from streptomycin and kanamycin by published procedures.[8]

The minimum inhibitory concentrations were determined by two-fold serial dilution in MUELLER-HINTON broth.

Results and Discussion

Deoxystreptamine

The antibiotic complex produced by the mutant strain in the presence of added DOS was identical with an equal mixture of butirosins A and B (compounds 1, Fig. 1) as shown by thin-layer chromatography, IR and pmr spectra, the fragments of acid hydrolysis and by mass spectroscopy. The anti-

* Since the completion of this work the use of 2,5-dideoxystreptamine in biotransformations has been reported by TESTA and TILBY, J. Antibiotics 28: 573~579, 1975.

bacterial activities were identical within experimental error (Table 1).

2,5-Dideoxystreptamine

When 2,5-dideoxystreptamine was added to the broth, the major antibiotic produced had the following properties: the pmr spectrum showed six methylene protons in the region 1.2~2.2 ppm, compared to four protons in the butirosins and in butirosamine[6] (compounds 1 and 2, Fig. 1). A single proton (anomeric) appeared at 5.1 ppm (J≈2 Hz). The integral under the curve between 2.3 and 4.5 ppm indicated 13 protons.

On N-acetylation the product gave a tetra-N-acetate $[\alpha]_{589}^{24}$ −4.98 (*c* 0.5, water).

Anal. Calcd. for $C_{24}H_{40}N_5O_{11}$: C, 50.08; H, 7.18; N, 12.17.
Found: C, 50.18; H, 7.20; N, 12.11.

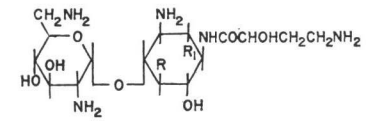
The pmr spectrum of the tetra-acetate showed 12+6 protons in the region 1.2~2.4 ppm and a single anomeric proton at 4.79~4.81 ppm.

The mass spectrum of the N-acetylated and permethylated product contained peaks for the molecular ion M (*m/e* 701), M−15 (loss of methyl, *m/e* 686) and M−32 (loss of methanol, *m/e* 669). The

Table 1. The antimicrobial spectra of butirosin and derivatives

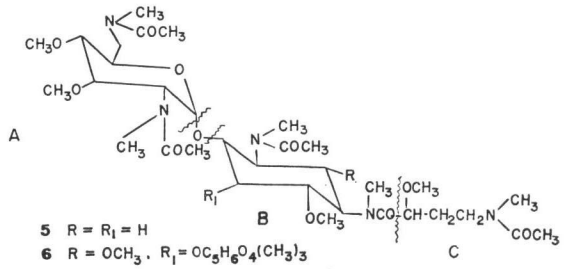
Test organisms	Bristol A No.	Minimum inhibitory concentrations (mcg/ml)				
		Butirosin A + B	Mutant + DOS	Butiros-amine	5-Deoxybuti-rosamine	2-Hydroxy butirosin
Streptococcus pneumoniae	9585	63	63	>125	125	>63
S. pyogenes	9604	63	63	>125	125	>63
Staphylococcus aureus	9537	0.5	0.5	4	2~4	—
S. aureus	9606	1.0	1.0	4	1~2	—
S. aureus	20240	32	32	>1,000	>125	>63
Escherichia coli	9632	0.5	1.0	8	2~8	—
E. coli	21218	16	32	>1,000	>125	>63
E. coli	20895	1	1	8	4~8	—
E. coli	20732	0.5~4	1	8	8	—
E. coli	20665	2	1	63	16~32	16
E. coli	20683	>63	63	>1,000	>125	>125
Enterobacter cloacae	9656	0.5~2	1	8	4~8	—
E. cloacae	20364	0.5~2	0.5	16	8	—
E. cloacae	21006	>63	16	>1,000	>125	>63
Klebsiella pneumoniae	9977	0.06	0.13	2	0.5~1	0.5
Proteus mirabilis	9900	2~4	4	32	4~8	16
P. rettgeri	9637	4	1	32	16~32	16
P. rettgeri	21207	>63	>63	1,000	125	>63
Providentia stuartii	21210	16	4	32	16	>63
P. stuartii	20894	>63	>63	>1,000	125	>63
Serratia marcescens	20019	8	4	16	16	8
S. marcescens	20460	8	4	16	32~63	8
Pseudomonas aeruginosa	9843A	8	8	8	4	63
P. aeruginosa	20653	>63	>63	>1,000	>125	>63
P. aeruginosa	20741	8	8	32	63	>63
P. aeruginosa	20717	8	8	8	2~4	63
P. aeruginosa	20601	2	2	8	2	2

Fig. 1



1. Butirosin, R = Pentosyl, R_1 = H
2. Butirosamine, R = OH, R_1 = H
3. 5-Deoxybutirosamine, R = R_1 = H
4. 2-Hydroxybutirosin, R = Pentosyl, R_1 = OH

Fig. 2. Fragmentation in mass spectroscopy



5 R = R_1 = H
6 R = OCH_3, R_1 = $OC_5H_6O_4(CH_3)_3$

peaks *m/e* 301 and 144 corresponded to fragments A and C of compound **5** (Fig. 2) also obtained from butirosin. The peak 384 is compatible with N-acetyl-permethyl N-1-γ-amino-α-hydroxybutyramido-2,5-dideoxystreptamine (fragment B).

The products of mild acid hydrolysis were γ-amino-α-hydroxybutyric acid and 2,5-dideoxystreptamine, as evidenced by TLC and GLC comparisons and a third product presumed to be the 5-deoxy analog of neamine. Vigorous acid hydrolysis gave 2,6-diaminoglucose which is also obtained from butirosin and from neamine under similar conditions. These data are consistent with the assignment of structure **3** to the fermentation product.

Streptamine

A medium supplemented with streptamine produced a mixture of two antibiotics which were separated on Brinkmann alumina plates (FT 22) using the system chloroform - methanol - 17% ammonium hydroxide (2: 1: 1, upper phase). Acid hydrolysis yielded streptamine, aminohydroxybutyric acid and ribose or xylose, respectively. Pmr data for the mixture showed two protons at 1.8~2.1 ppm, two anomeric protons at 5.3~5.7 and 19 protons from 2.5~4.4 ppm.

High resolution mass spectroscopy of the N-acetylated-permethylated mixture resulted in exact masses for fragments A and C of compound 6 (Fig. 2) (*m/e* 301.1752 and 144.1010 *vs.* the required values 301.1763 and 144.1025). Low resolution mass spectral analysis gave *m/e* 604 compatible with fragment B. These data are consistent with compound **4** (Fig. 1).

Antibiotic Activity

The minimum inhibitory concentrations (Table 1) show little difference between **3** and butirosamine, *i.e.*, the lack of the 5-hydroxyl does not change the effect on the microorganisms tested. This is to be expected, since, to our knowledge, no inactivating enzymes act on that site.

The antibacterial spectrum of **4** shows a reduction of activity compared to that of butirosin. The aminohydroxybutyric acid side chain, however, increases the activity of **3** over that of neamine, and that of **4** over that of ribostamycin. The effects are noted in the case of strains which phosphorylate the 3′-hydroxyl or acetylate the 3-amino group of aminoglycoside antibiotics.

Acknowledgements

We are indebted to Dr. R. D. BROWN for the mass spectra, to Mr. K. L. DEN BLEYKER for MIC's, to Mr. R. GRULICH for the pmr spectra, and to Miss M. D. DEFURIA for the fermentations and bio-autographs. The authors would like to thank Dr. G. KAVADIAS for the 2,5-dideoxystreptamine used in this work.

References

1) SHIER, W. T.; K. L. RINEHART, Jr. & D. GOTTLIEB: Preparation of four new antibiotics from a mutant

of *Streptomyces fradiae*, Proc. Nat. Acad. Sci. U.S. 63: 198~204, 1969

2) KOJIMA, M. & A. SATOH: Microbial semi-synthesis of aminoglycosidic antibiotics by mutants of *S. ribosidificus* and *S. kanamyceticus*. J. Antibiotics 26: 784~786, 1973
3) CLARIDGE, C. A.; J. A. BUSH, M. D. DEFURIA & K. E. PRICE: Fermentation and mutational studies with a butirosin-producing strain of *Bacillus circulans*. Devel. Indust. Microb. 15: 101~113, 1974
4) KAVADIAS, G.: unpublished results.
5) WOO, P. W. K.; H. W. DION & Q. R. BARTZ: Butirosins A and B. I. Structural units. Tetrahedron Lett. 1971-26: 2617~2620, 1971
   DION, H. W.; P. W. K. WOO, N. E. WILLMER, D. L. KERN, J. ONAGA & S. A. FUSARI: Butirosin, a new aminoglycosidic antibiotic complex: Isolation and characterization. Antimicr. Agents & Chemoth. 2: 84~88, 1972
6) TSUKIURA, H.; K. FUJISAWA, M. KONISHI, K. SAITO, K. NUMATA, H. ISHIKAWA, T. MIYAKI, K. TOMITA & H. KAWAGUCHI: Aminoglycoside antibiotics. III. J. Antibiotics 26: 351~357, 1973
7) DEJONGH, D. C.; E. B. WILLS, J. D. HRIBAR, S. HANESSIANS & T. CHANG: A mass spectral investigation of derivatives of kanamycin A. Tetrahedron 29: 2707~2713, 1973
8) PECK, R. L.; C. E. HOFFINE, Jr., E. W. PEEL, R. P. GRABER, F. W. HOLLY, R. MOZINGO & C. FOLKERS: The structure of streptidine. J. Am. Chem. Soc. 68: 776~778, 1946
   CRON, M. J.; D. L. JOHNSON, F. M. PALERMITTI, Y. PERRON, H. D. TAYLOR, D. W. WHITEHEAD & I. R. HOOPER: Kanamycin. I. Characterization and acid hydrolysis studies. J. Am. Chem. Soc. 80: 752~753, 1958