RIBOSTAMYCIN, AS AN INTERMEDIATE IN THE BIOSYNTHESIS OF NEOMYCIN

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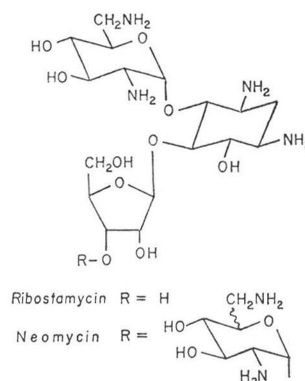
A mutant of a neomycin-producing *Streptomyces fradiae* was found which synthesizes ribostamycin instead of neomycin. After a reverse mutation new colonies were obtained producing neomycin again. Ribostamycin might thus be considered as an intermediate in the biosynthesis of neomycin.

Neomycin is a pseudo-tetrasaccharide antibiotic produced by certain strains of *Streptomyces fradiae* (Fig. 1). Several steps of its biosynthesis have been elucidated by the use of labelled precursors and of mutants of this strain: the monocyclic deoxystreptamine and the pseudo-disaccharide neamine[1] are the main building stones. The further steps in the sequence of assembly are still unknown[2]. In our studies on a neomycin-producing strain of *Streptomyces fradiae* a mutant was found which synthesizes ribostamycin instead of neomycin. After a second and reverse mutation, new colonies were obtained, which were able to produce neomycin again. Ribostamycin, a pseudo-trisaccharide consisting of the first three units of neomycin, and previously obtained from *Streptomyces ribosidificus*[3], might thus be considered as an intermediate in the biosynthesis of neomycin.

Fig. 1. Structure of ribostamycin and neomycin

Neomycins B and C are respectively the α and β isomers at C_5 of the last unit.



Materials and Methods

Strains

Streptomyces fradiae UC75, is an industrial neomycin-producing strain of Roussel-Uclaf Collection.

Streptomyces ribosidificus ATCC 21 294[3], ribostamycin producer.

Sarcina lutea ATCC 15 957, sensitive to most antibiotics of the aminoglycoside family, but not to neamine.

Bacillus pumilus ATCC 14 884, used in the bioautography of paper chromatograms, is sensitive to most antibiotics of the aminoglycoside family.

Staphylococcus aureus ATCC 6538 sensitive to ribostamycin and neomycin.

Staphylococcus aureus B 295[4] and *Escherichia coli* R5/W677[5] sensitive to neomycin and resistant to ribostamycin.

Identification of compounds

The minimum inhibitory concentrations were determined according to SZYBALSKI[6].

The chromatographic comparison of compounds were performed either on Whatman No. 1 paper,

solvent A: methyl ethyl ketone - *tert* butyl alcohol - methanol - 6.5N-ammonia (16: 3: 1: 6), solvent B: 2% *p*-toluene-sulfonic acid in water-saturated 1-butanol, or by thin-layer on SCHLEICHER and SCHÜLL plates, solvent C: chloroform - methanol - ammonia - boric acid 5% in water (1: 4: 2: 1). In all cases mobilities are relative to neomycin B (R rel).

Mutation procedure

A nearly monoconidial preparation of spores of *Streptomyces fradiae* grown on ATCC No. 343 agar was exposed to the mutagenic action of N-methyl-N′-nitro-N-nitrosoguanidine at 0.25 mg per ml in HOPWOOD's medium[7], for 15 hours at 28°C with agitation.

Mutants were detected by the agar block method[8], the culture medium being: potato starch 0.75, soybean flour 0.83, corn steep liquor 0.66, corn protein 0.33, calcium carbonate 0.166, ammonium sulfate 0.16 and agar 2.5 g per 100 ml.

The activity of the colonies was detected by *Sarcina lutea*.

Fermentation procedure

The antibiotic production studies were carried on in shaken flasks, using liquid medium of the following composition: corn starch 9.0, soybean flour 2.0, corn protein 2.0, ammonium sulfate 1.0, calcium carbonate 1.0 g per 100 ml.

Table 1. Paper chromatographic comparison of the antibiotic produced in liquid cultures of strain N_4C_2 to related antibiotics. (see Methods)

	R rel	
	Solvent A	Solvent B
Neomycin B	1.00	1.00
Neomycin C	0.55	1.00
Ribostamycin	1.35	0.85
N_4C_2 Strain broth	1.35	0.85
Neamine	1.65	1.00

Table 2. Identification of the antibiotic produced by strain N_4C_2 (see Methods)

		Ribostamycin standard	Antibiotic from strain N_4C_2
Purified product (base)		R rel	R rel
	TLC Solvent C	2.8	2.8
	Solvent D	1.65	1.65
	$[\alpha]_D$ (1%, H_2O)	+58.5°	+59.0°
N-Acetylated derivative	$[\alpha]_D$ (1%, H_2O)	+43.0°	+44.8°
	NMR (proton and ^{13}C)	in agreement	
	Microanalysis for $C_{25}H_{42}N_4O_{14}$		
	calcd.: C 48.23	found: 48.2	found: 48.3
	H 6.8	6.9	6.8
	N 9.0	8.8	8.9

Table 3. Bacteriostatic activity of the antibiotic from strain N_4C_2 determined by the SZYBALSKI method[6] (see Materials and Methods)

	Minimum inhibitory concentration (μg/ml)		
	Ribostamycin from strain N_4C_2	Ribostamycin standard	Neomycin
Staphylococcus aureus ATCC 6538	1	0.8	0.1
Staphylococcus aureus B295 (10)	100	100	6
Sarcina lutea ATCC 15 957	30	32	0.1
Escherichia coli R5/W677 (11)	100	100	1

Results and Discussion

The mutagenic treatment of *Streptomyces fradiae* killed about 99.9% of the spores and, among the survivors, gave non-antibiotic-producing mutants with a frequency of 0.1%. One colony out of 11,000 showed a zone of inhibition much smaller than those of the control. This colony, designated as strain N_4C_2, was picked out and checked for homogeneity; its morphology and sporulation capacity are not different from those of the parent strain.

The fermentation broth of this mutant strain had a high antibiotic potency but the antibiotic spectrum did not correspond to neomycin. Analysis by paper chromatography (Table 1) suggested that the sole antibiotic spot detected by bioautography on *Bacillus pumilus* was ribostamycin instead of neomycin. In order to confirm this result the active product was isolated and purified by the procedure used for most aminoglycosides[9]: 1) alkaline lysis of the microorganism, 2) absorption on carboxylic ion-exchange resin and, 3) elution by ammonium hydroxide using a concentration gradient. The purified antibiotic was obtained with a yield of about 80% (from the fermented broth). This compound and its N-acetylated derivative were analysed in comparison to a sample of authentic ribostamycin. The data collected (Table 2) showed identity and the information was in agreement with the literature[10,11]. Moreover, the authentic ribostamycin and the product from strain N_4C_2 showed the same bacteriostatic activity towards bacterial strains which distinguished them from neomycin (Table 3).

Thus, the strain N_4C_2, blocked in one of the last steps of neomycin biosynthesis accumulates ribostamycin. We also noted that the antibiotic production is much higher in the conditions of neomycin production than in those[3] of ribostamycin (respectively 1.8 and 0.5 mg/ml), while *Streptomyces ribosidificus* produces under the same conditions 0.05 mg and 0.75 mg/ml respectively.

Furthermore, a second mutagenic treatment was performed on strain N_4C_2 using the same procedure as mentioned above. The spores were plated on the agar medium. We found 5 colonies out of 45,000 with a fairly large zone of inhibition using the overlayer method of DULANEY and DULANEY[12]. Checked for antibiotic biosynthesis, these clones turned out to present revertant phenotypes, that is to say that they produced neomycin and not ribostamycin. We wish to propose the following scheme for the main steps in the neomycin biosynthetic pathway: the four building blocks are formed exclusively from glucose, and ammonia for three of them,[13] neosamine C and deoxystreptamine are linked to give neamine. In the next step ribose is linked with a glycoside bond to neamine to give ribostamycin. Finally, the second molecule of neosamine is linked to ribostamycin and neomycin is obtained.

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