6'''-DEAMINO-6'''-HYDROXY DERIVATIVES, AS INTERMEDIATES IN THE BIOSYNTHESIS OF NEOMYCIN AND PAROMOMYCIN

D. AUTISSIER, P. BARTHELEMY, N. MAZIERES
M. PEYRE and L. PENASSE

Centre de Recherches Roussel Uclaf, 93, Romainville, France

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From broths of a neomycin producing *Streptomyces fradiae* and of a mutant of *Streptomyces rimosus* forma *paromomycinus* respectively, 6'''-deamino-6'''-hydroxyneomycin and 6'''-deamino-6'''-hydroxyparomomycin were obtained and their structures established by mass and ^{13}C-NMR spectroscopy and by the study of hydrolytic fragments. These new compounds, which are both present as two epimers at C-5''', are suggested as intermediates in the biosynthesis of the parent antibiotics. The place and the mechanism of the 5'''-epimerisation and of the 6'''-amination are discussed.

Until recently the last steps in the biosynthesis of the pseudotetrasaccharide antibiotics neomycin and paromomycin were the least well known.

The extensive work of RINEHART and his group[1] had shown that the individual subunits were first synthesized and then subsequently joined. In the case of neomycin, it was then demonstrated[2] that neamine, which consists of 2,6-diamino-2,6-dideoxy-D-glucose and deoxystreptamine, is an obligatory step in the pathway, with paromamine, consisting of D-glucosamine and deoxystreptamine, playing a similar role in the case of paromomycin. Moreover, it was suggested that paromamine could be an intermediate in the neamine pathway, and it is known also that small quantities of paromomycin are detectable in broths of most neomycin producing strains[3,4].

In 1977, it was shown in this laboratory[5], by the use of blocked mutants, that in a further step ribose is joined to neamine, leading to ribostamycin, which was thus suggested as an intermediate in neomycin biosynthesis. A similar view has been independently adopted by TAKEDA *et al.*[6] for the biosynthesis of butirosin. In the paromomycin pathway, 6'-deamino-6'-hydroxyribostamycin or BM 408 α[7] could play a similar role.

In this communication, we wish to describe the isolation, in small quantities, from broths of a normal neomycin producing strain and of a blocked mutant of a paromomycin producing strain, of 6'''-deamino-6'''-hydroxyneomycin (compound Y) and 6'''-deamino-6'''-hydroxyparomomycin (compound X), respectively, both as two stereoisomers, presumably epimers at the 5''' carbon. The use of the mutant in the case of paromomycin does not mean that 6'''-deamino-6'''-hydroxyparomomycin is not obtainable from the parent strain, as in the case of its neomycin analogue. This mutant was, however at hand, and its inability to produce paromomycin made it particularly convenient for detecting and isolating the new, almost inactive, compounds.

Materials and Methods

Strains

Streptomyces fradiae UC 75 is an industrial neomycin-producing strain of the Roussel Uclaf collection.

Streptomyces rimosus forma *paromomycinus* UC 57 is an industrial paromomycin-producing strain of the Roussel Uclaf collection.

Bacillus pumilus ATCC 14884, used in the bioautography of paper chromatograms and in the agar block method, is highly sensitive to most antibiotics of the aminoglycoside family.

Mutation Procedure

Stock cultures of *S. rimosus* f. *paromomycinus* UC 57 were allowed to grow for 7 days at 30°C on slopes of a medium of the following composition: potatoes 10 g, NZ amine B (Sheffield chemical) 1 g, agar 2.5 g per 100 ml. The strain sporulates poorly and gives an aerial mycelium easily fragmented in small pieces. The cultures were kept at 4°C for 3 months.

A well fragmented preparation of mycelium grown on agar was exposed to the mutagenic action of N-methyl-N-nitro-N'-nitrosoguanidine (NTG) at 0.50 mg/ml in HOPWOOD's medium[8] for 7 hours at 30°C with agitation.

The mutagenic treatment killed about 99.9% of the colony forming units and, among the survivors, auxotrophic mutants were obtained with a frequency of 3×10^{-3} and characterized according to HOPWOOD's method[9]. One of the mutants, a threonine B auxotroph, was exposed to a second mutagenic treatment with 0.1 mg of NTG per ml for 5 hours. Among the survivors, mutants modified in antibiotic production were detected with the agar block method[10], the culture medium being: corn protein (Roquette frères Lille) 1.2 g, soya bean flour (Société Industrielle des Oléagineux, Paris) 1.1 g, ammonium chloride 0.4 g, calcium carbonate 1.5 g, magnesium sulfate 0.02 g, dextrose 2.5 g, agar 2.5 g per 100 ml.

The activities of the colonies were detected on *Bacillus pumilus*, low antibiotic producers were obtained with a frequency of 1.2×10^{-3}. One of them, designated after purification as $A_{67}M_{15}$, was still threonine B auxotrophic and, under the standard conditions, produced no paromomycin. The fermentation broth had a very low activity, in the range of 10 μg/ml expressed as paromomycin.

Fermentation Procedure

The antibiotic production studies were carried out under aerobic conditions for 120 hours at 30°C in shaken flasks using liquid medium of the following composition: corn protein 2.4 g, soya bean flour 1.1 g, ammonium chloride 0.4 g, calcium carbonate 1.5 g, magnesium sulfate 0.02 g, dextrose 5 g, soya oil 0.5 g per 100 ml.

Extraction and Purification Procedure

At harvest, the fermentation broths were centrifuged and the clear supernatant recovered. The antibiotics were isolated by adsorbtion on a column of Amberlite IRC 50 resin (NH_4^+ form) followed by washing with distilled water and elution with 2 N ammonia. The eluate concentrated under vacuum and freeze-dried gave a crude mixture which was purified by adsorption on a column of Amberlite XE 64 resin (NH_4^+ form) and elution with a linear concentration gradient of ammonia (0~0.4 N). Effluent fractions were tested by TLC (solvent 1) and bioautography. Homogeneous active fractions were pooled and freeze-dried. Ultimate purification of the new compounds was obtained by chromatography on a silica gel column, using a mixture of methanol - ammonia (4: 1) as eluant. The fractions were tested as above, pooled and evaporated to dryness (Table 1).

Identification of Compounds

TLC: Thin-layer chromatography was performed on precoated Merck silica gel plates (60 F 254) or on Schleicher-Schüll plates (F 1500 LS 254), employing solvent 1: chloroform - methanol - 11 N ammonium hydroxide - water (1:4:2:1); solvent 2: chloroform - methanol - 11 N ammonium hydroxide (2:2:1); solvent 3: methanol - 11 N ammonium hydroxide (4:1). Detection was by spraying with 0.1% ninhydrin (Merck reagent) or 0.2% ethanolic naphthoresorcinol-sulphuric acid mixture (10: 1) and heating for 15 minutes at 100°C.

Table 1. Extraction of the new compounds, yield of isolated pure compound from fermented broth.

Strain	Compound	Yield (mg/liter)
S. fradiae	Y_1	5
	Y_2	64
S. rimosus f. *paromomycinus* thr B Par$^-$	X_1	16
	X_2	430

PC: Paper chromatography was performed on Schleicher-Schüll 1043 Mgl sheets, employing as solvent: methyl ethyl ketone - *t*-butyl alcohol - methanol - 11 N ammonium hydroxide - water (160: 30: 10: 35: 25). Detection was by spraying ninhydrin or bioautography on agar plates seeded with *B. pumilus*.

Mass spectroscopy: Mass spectroscopy measurements were obtained by field desorption on a Varian MAT 311 A apparatus.

NMR: Natural abundance ^{13}C NMR spectra were obtained on a Bruker W H 90 apparatus.

Mild acid hydrolysis was performed in 100 volumes of N hydrochloric acid, either for 1 hour at 80°C, or 24 hours at 65°C.

Strong acid hydrolysis was performed in 100 volumes of 8 N hydrochloric acid, for 1 or 2 hours at 100°C.

Alkaline hydrolysis was performed in 4 N aqueous sodium hydroxide for 2 hours at 100°C.

Sulphuric methanolysis was performed in 100 volumes of a 5: 1 mixture of anhydrous methanol and 36 N sulfuric acid under reflux for 15 minutes.

Successive Hydrolytic Treatment

In one typical experiment, 500 mg of pure X_2 sulfate corresponding to 363 mg anhydrous base was methanolysed and, after cooling, the liberated paromamine was precipitated as sulfate by the addition of triethylamine. The precipitate was separated, washed with methanol, dried and 202 mg was obtained. This is a yield of 86% on a stoichiometric basis from deaminoparomomycin.

$[\alpha]_D$ +106.6° (*c* 1, 0.1 N H_2SO_4 calculated for the free base; authentic sample of paromamine under the same conditions +110°).

Anal. Calcd for $C_{12}H_{25}N_3O_7 \cdot 0.5\ H_2SO_4$: S 4.3%
Found: S 4.1~4.3%.

After separation of paromamine from the methanolysis experiment, the mother liquor was evaporated to dryness under vacuum, and the syrupy residue was then purified by chromatography on a 5 × 25 cm column of Dowex 50 (NH_4^+) resin, and eluted by 0.1 N ammonia. The effluents were tested by TLC and the fractions containing the main homogeneous compound were pooled and evaporated to dryness yielding 176 mg of methyl 2-amino-2-deoxyglucosylriboside

Anal. Calcd for $C_{12}H_{23}O_9N$: C 44.3 H 7.15 N 4.3.
Found: C 43.05 H 7.0 N 4.65.

On silica plate TLC, solvent 3, the compound gives two spots (Rf 0.53 and 0.59) attributable to the α and β anomers.

The methyl riboside was further examined. Deamination was obtained by treating 10 mg of the methyl riboside with 250 mg of silver nitrite in 4 ml of ice cold 20% acetic acid for 1 hour. After standing 48 hours at room temperature, 0.5 ml concentrated hydrochloric acid was added, the silver chloride filtered, and the solution evaporated to dryness under vacuum. By mild acid hydrolysis of the residue, ribose was liberated and identified by TLC.

On strong acid hydrolysis of the methyl riboside a spot of aminosugar appears. In one experiment a sample of methyl riboside was treated for 2 hours at 100°C in 8 N hydrochloric acid. After cooling and filtration the crude hydrochloride was precipitated by addition of 5 ml of acetone, and then purified by adsorption on Dowex 50W × 8(H^+) resin, elution by N hydrochloric acid, evaporation of the main homogeneous fraction and crystallization in 160 volumes of water - ethanol, 1: 15 mixture at 5°C. The compound is indistinguishable from authentic 2-amino-2-deoxy-D-glucose hydrochloride. Specific rotation and mutarotation behaviour were determined in 0.1 N hydrochloric acid on the isolated compound at 0.5% concentration and a sample of authentic glucosamine was submitted to the same treatment. Rotations were, for the isolated compound, +94.4° and +77.8° immediately and after 2 hours, and for the authentic sample, respectively +95.4° and +77.1°. Both compounds are indistinguishable by TLC, solvent 2, Rf: 0.25.

In vitro Antimicrobial Activity

MIC's of the new compounds, in comparison to paromomycin and neomycin, were determined, with the dilution method, against a group of standard Gram-positive and Gram-negative strains. The minimal inhibitory concentrations were detected after 24 hours of incubation at 37°C.

Results and Discussion

The four new compounds are white amorphous substances. They are water soluble, basic, ninhydrin positive, non reducing, and were isolated either as free bases or sulfate salts. In their physicochemical properties they are closely related to their parent compounds, respectively paromomycin for X_1 and X_2 and neomycin for Y_1 and Y_2, but clearly distinct from them.

Most of the structural studies are described here in the case of compound X_2. Observations pertaining to compounds X_1, Y_1 and Y_2 for which similar studies were performed, are briefly summarized in Tables 1, 2, 3 and 4.

The FD mass spectrum of X_2 gives a value of 617^+ for the molecular peak, instead of 616^+ for paromomycin, and in the ^{13}C NMR spectrum of X_2, carbon 6''' is found at 63.6 ppm in the $-CH_2OH$ area, like carbon 6', while one carbon is missing in the C-N area, by comparison with paromomycin. These data suggest that compound X_2 could have the structure of paromomycin with one amino group replaced by one hydroxyl group.

Moreover, from comparison of the NMR spectra of compound X_2 with the spectra of neomycin B and neomycin C (epimers at carbon 5''') it appears that the new compound is similar at carbon 5''' to the orientation of neomycin C, and the aminosugar of compound X_2 is, thus probably of D-glucose configuration, with X_1 being its L-idose counterpart.

Table 2 shows that the rotations and the chromatographic mobilities of the new compounds X_1 and X_2 are in a ratio similar to the ratio of the corresponding values of paromomycin I and paromomycin II.

Table 2. Physicochemical characteristics of compounds X_1, X_2 and of paromomycins I, II.

	Compound X_1	Compound X_2	Paromomycin I	Paromomycin II
TLC SS* plate Solvent 1 Rf	0.45	0.45	0.29	0.35
TLC MK* plate Solvent 3 Rf	0.13	0.19	—	—
PC Solv. A 65 H 30°C	19.5 cm	14.5 cm	16 cm	9.7 cm
$[\alpha]_D$ (*c* 1, 0.1 N H_2SO_4)	+54.1°	+111.4°	+77.9•	+114.5°

* SS: Schleicher-Schüll, MK: Merck.

More structural information was obtained from the hydrolysis studies and fragments isolated from the new compounds.

While compound X_2 remains unaltered by alkaline treatment, after mild or strong acid hydrolysis chromatographic examination shows the liberation of deoxystreptamine, 2-amino-2-deoxyglucose, paromamine, ribose and spots of unknown compounds. Paromamine was isolated after sulphuric acid methanolysis. The compound is indistinguishable from authentic paromamine by TLC and PC, and is also devoid of significant antibiotic activity.

Treatment of the paromamine mother liquors yields the other moiety as methyl 2-amino-2-deoxy-glucosylriboside. After strong acid hydrolysis 2-amino-2-deoxy-D-glucose(glucosamine) is isolated and identified by rotation values and TLC. After deamination and acid treatment of the methyl riboside, the ribose fragment was obtained and identified by TLC.

Strong acid hydrolysis of compound X_2 yields also, according to chromatographic examination, a spot for deoxystreptamine, similar to the spot obtained from paromomycin itself submitted to the same treatment, confirming thus the presence of this building block in the paromamine moiety of X_2. The deoxystreptamine component was not isolated.

Table 3. Fragments identified after hydrolytic treatment of the new compounds.

	Compound X_1	Compound X_2	Compound Y_1	Compound Y_2
Methanolysis	Paromamine Riboside I	Paromamine Riboside II	Neamine Riboside I	Neamine Riboside II
Riboside hydrolysis	Ribose 2-Amino-2-deoxy-idose*	Ribose 2-Amino-2-deoxy-glucose	Ribose 2-Amino-2-deoxy-idose*	Ribose 2-Amino-2-deoxy-glucose

* In both cases of the idose derivatives, a significant amount of the same degradation product is formed during the hydrolysis. By contrast the glucose derivatives are stable.

Table 4. MIC (μg/ml) for compounds X_1, X_2, Y_2 and parent antibiotics.

Strain	Neomycin B	Neomycin C	Compound Y_2	Paromomycin I	Compound X_1	Paromomycin II	Compound X_2
Staphylococcus aureus UC 1061	0.2	1	5	0.5	5	3	40
Streptococcus pyogenes A 561	1	10	>40	20	40	>40	>40
Bacillus subtilis ATCC 6633	0.05	0.5	>40	0.2	2	2	10
Escherichia coli RT	2	10	10	5	40	40	>40
Klebsiella pneumoniae Exp 52 145	0.2	1	2	1	5	5	40
Salmonella typhimurium 420	1	10	40	10	40	40	>40
Enterobacter cloacae 681	0.6	2	3	1	10	10	40
Pseudomonas 3935 Exp	10	>40	>40	20	>40	>40	>40
Serratia 25 32	1	5	10	5	10	>40	>40

The same study performed on compound X_1 (Table 3) gives, in similar yield, the paromamine fragment but a different riboside, the difference in the fragment being located in the aminohexose component. This hydrolytic study gives thus the same location for the sole difference between X_1 and X_2 as suggested by the NMR and rotation data.

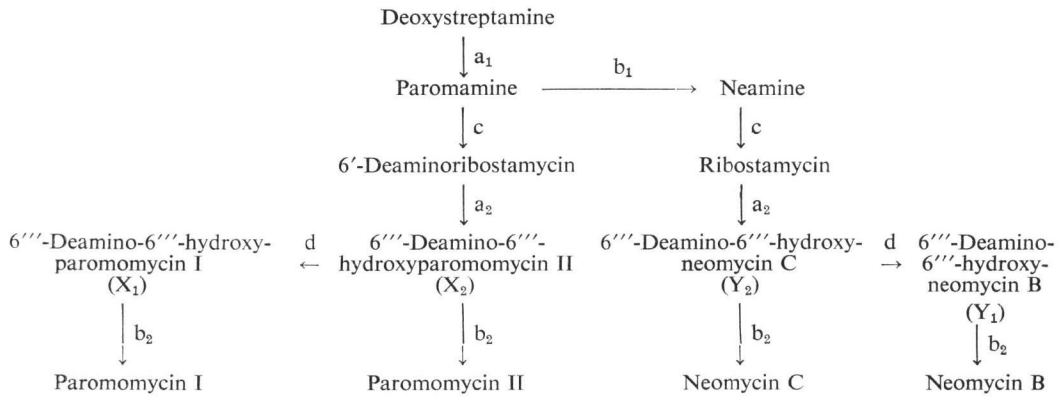
The antimicrobial activities of the new compounds are much lower than the activities of the parent antibiotics. Roughly speaking the activity depends on the number of amino groups in positions-6′ and -6‴, the L-idose series being superior to the D-glucose series (Table 4).

The new compounds, differing respectively from paromomycin and neomycin by one chemical substitution only, might thus be suggested as intermediates in the biosynthesis of these antibiotics. The fact that compounds X_1, and X_2 are accumulated in the paromomycin blocked mutant supports that proposal.

In that hypothesis, the last steps from ribostamycin would be the incorporation of glucosamine leading to the new compound of the neomycin series, followed by the substitution of the 6‴-amino group, leading to neomycin C. A similar pathway can be written for paromomycin II starting from 6′-deamino-6′-hydroxyribostamycin.

In this scheme, the incorporation of both aminohexoses would take place through a very similar reaction: establishment of an α-glycosidic linkage with 2-amino-2-deoxy-D-glucose. The second amino group of the 2,6-diamino-2,6-dideoxyhexose moieties would be introduced at a later step, on paromamine to obtain neamine[6] and on the new compounds described here to obtain the complete antibiotics. This hypothesis gives an explanation to the fact that the free diaminohexose added as a precursor to the fermentation broth is not incorporated, a fact which was shown in the case of neomycin by the Urbana

Scheme 1. Schematic overview of neomycin and paromomycin biosynthetic pathways.
a_1 and a_2: 2-amino-2-deoxy-D-glucose incorporation; b_1 and b_2: 6′- or 6‴-amino group incorporation; c: ribose incorporation; d: D-glucose-L-idose isomerisation.



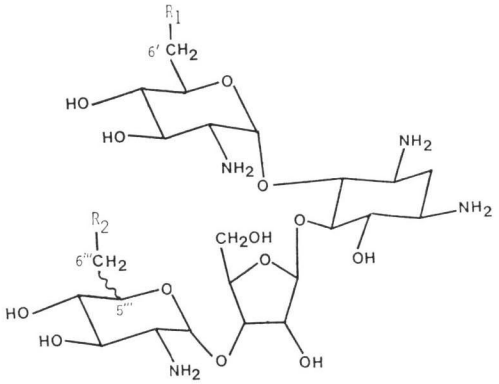
group[11] and in the case of paromomycin by the Jena group[12].

The scheme given in Scheme 1 is the simplest which includes all the presently known compounds.

The 5‴-isomerization step, giving, respectively neomycin B and paromomycin I could take place either at the 6‴-hydroxy stage or at the 6‴-amino stage. From the observations described here, we suggest that the reaction takes place as the penultimate step, involving the primary alcohol or a derivative of the primary alcohol, but not the corresponding amine. At that stage of the biosynthesis, in the case of neomycin, C-6 of the first hexose already bears an amino group. This could play a role in the fact that the D-glucose⇌L-idose isomerisation works only on the second hexose group. In the case of paromomycin, on the contrary, both hexoses are still in the hydroxyl form at C-6 and nevertheless the isomerisation works only at the second hexose group. The specificity of the enzymes responsible for the isomerisation includes thus the group attached by a glycosidic bond at C-1 of the hexose, *viz.*, ribose in the accepted case, and deoxystreptamine in the rejected case.

One can also underline that the isomerization, which remains to be elucidated, should, in this proposal, be similar to the 5-epimerisation already described in the biosynthesis of other natural saccharides which appears there also at a late, but not ultimate stage of the biosynthesis. In the case of heparin[13], the epimerisation of D-glucuronic acid to L-iduronic acid involves the loss of the hydrogen atom at C-5, and in the case of alginic acid the epimerisation of D-mannuronic

Fig. 1. Structure of the paromomycins, the neomycins and the new compounds X_1, X_2, Y_1 and Y_2.



	5‴-CH_2-R_2	R_1	R_2
Compound X_1	α	OH	OH
Compound X_2	β	OH	OH
Compound Y_1	α	NH_2	OH
Compound Y_2	β	NH_2	OH
Neomycin B	α	NH_2	NH_2
Neomycin C	β	NH_2	NH_2
Paromomycin I	α	OH	NH_2
Paromomycin II	β	OH	NH_2

acid to L-guluronic acid is catalysed by 5-epimerase and the reaction includes also the loss of hydrogen at C-5[14].

In usual fermentations of neomycin producing strains it is well known that the ratio of epimer B to epimer C varies from 3~10[3] and for paromomycin the ratio of epimer I to epimer II varies from 10 to 20. In the case of the new compounds described here, this ratio is about 0.08 for epimer Y_1 to epimer Y_2 and 0.04 for epimer X_1 to epimer X_2. Thus, for the antibiotics the L-idose form is favoured over the D-glucose form, while for the precursors, the D-glucose form is favoured. These facts are presumably the result of the respective kinetics and the respective mechanisms of the two last steps in both biosyntheses (steps d and b_2, Scheme 1).

The conformation of the L-idose ring in neosamine B is a long standing and still unsolved problem, discussed already by RINEHART in 1964[15]. In recent publication[1] the substituted L-idose is conventionally written in 4C1 conformation (Fig. 1), in which the bulky substituents at C-1 and C-5 are axial. As in the case of heparin[16], the L-idose ring of neomycin B and paromomycin I could be in the 1C4 conformation in which C-1 and C-5 substituents are equatorial.

One should finally add, that according to MAJUMDAR and MAJUMDAR[17], *Streptomyces fradiae* produces neomycin as a phosphoamido derivative whose phosphate bond is cleaved at the excretion step. Some of the biosynthetic steps suggested here could also take place on phosphorylated intermediates.

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