# SUB-UNIT ASSEMBLY IN THE BIOSYNTHESIS OF NEOMYCIN

## THE SYNTHESIS OF 5-*O*-*β*-D-RIBOFURANOSYL AND 4-*O*-*β*-D-RIBOFURANOSYL-2,6-DIDEOXYSTREPTAMINES*

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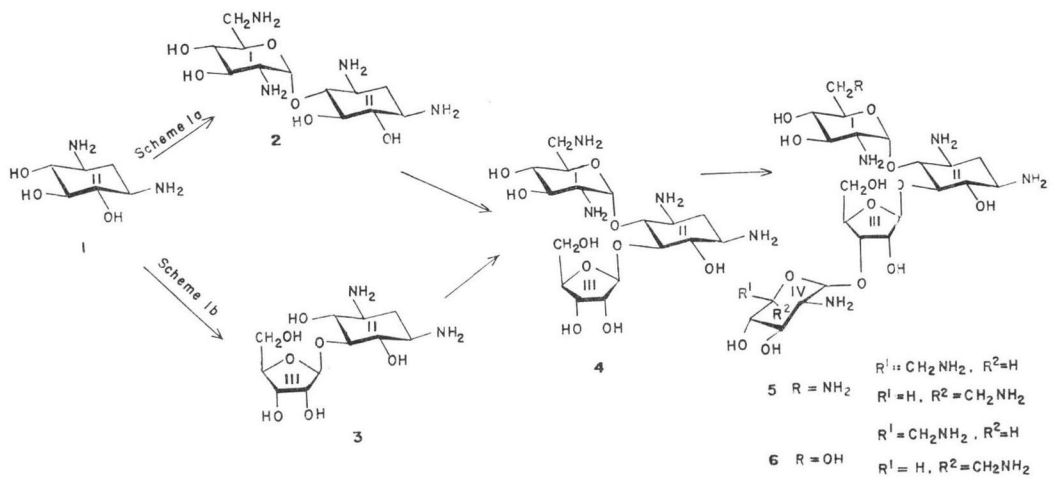
The preparation of the deoxy- analogues of two pseudodisaccharide fragments of neomycin, 5-*O*-*β*-D-ribofuranosyl-2,6-dideoxy-streptamine and 6-deoxyneamine is described. When added to the growth medium of a deoxystreptamine-idiotroph of *Streptomyces rimosus* forma *paromomycinus* only the latter was incorporated into antibiotic, suggesting an obligatory order for the assembly of sub-units. 4-*O*-*β*-D-Ribofuranosyl-2,6-dideoxystreptamine was also prepared. When added to the growth medium of a deoxystreptamine-idiotroph of *Streptomyces fradiae* it was converted into the 6-deoxyneomycins, apparently after hydrolysis to 2,6-dideoxystreptamine. The structure of the protected derivatives of the ribofuranosyl 2,6-dideoxystreptamines, potentially useful intermediates for the synthesis of novel antibiotics, was shown by using $^{13}C$ NMR spectroscopy.

The synthesis of a deoxy-analogue of the central pseudodisaccharide (rings II, III) of neomycin (**5**) has a twofold importance; firstly as a starting point for chemical syntheses of analogues of antibiotics such as neomycin, ribostamycin, paromomycin, lividomycin and butirosin and secondly as an analogue of 5-*O*-*β*-D-ribofuranosyl 2-deoxystreptamine (**3**) for biosynthetic studies.

Fig. 1. Biosynthetic pathways to the neomycins




* This paper is dedicated to Prof. E. LEDERER on the occasion of his 70th birthday.

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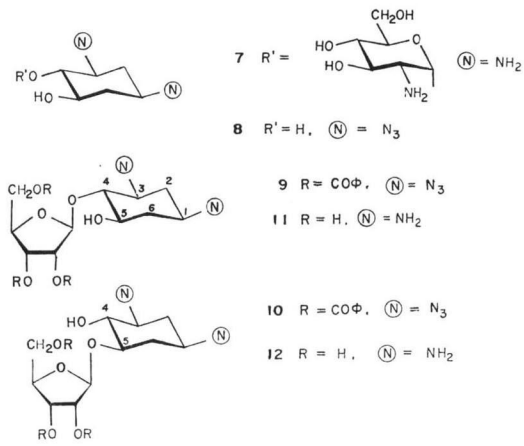
The aminocyclitol antibiotics, neomycin* (**5**), paromomycin (**6**) and ribostamycin (**4**) are all produced by *Streptomyces* and are closely related structurally. They are probably produced by minor modifications of the same biosynthetic pathway[1]. Thus the neomycin producing organism *Streptomyces fradiae* has been shown to produce trace quantities of the paromomycins in addition to the neomycins[2], while a deoxystreptamine-idiotroph** of *Streptomyces rimosus* forma *paromomycinus* which requires 2-deoxystreptamine (**1**) for paromomycin biosynthesis, will produce neomycin if neamine (**2**) is added to the incubation medium[4]. Similarly, a deoxystreptamine-idiotroph of the ribostamycin-producing organism *Streptomyces ribosidificus* will convert neamine (**2**) into ribostamycin (**4**)[5]. The latter experiments suggest that the biosynthesis of the neomycins from their sub-units proceeds by the sequence; 2-deoxystreptamine (**1**) (ring II), neamine (**2**) (rings I, II), ribostamycin (**4**) (rings I, II, III), neomycin (**5**) (rings I, II, III, IV) (Scheme 1a, in Fig. 1).

Fig. 2. Chemical synthesis of 4-*O* and 5-*O*-$\beta$-D-ribofuranosyl-2,6-dideoxystreptamines




In this paper we describe the preparation of the deoxy-analogues: 6-deoxyneamine (**7**) (rings I, II) and 5-*O*-$\beta$-D-ribofuranosyl-2,6-dideoxystreptamine (**12**) (rings II, III) and use them to establish whether this is an obligatory route, or whether an alternative route in which the deoxystreptamine is first ribosylated at C-5 may coexist (Scheme 1b, in Fig. 1).

## Experimental

### General Methods

Paper chromatography was carried out on Whatman 3 MM paper using the solvents described in the text. Detection of antibiotics was either by the method of PAN and DUTCHER[6] which detects amino and amido groups, or by bioautography. A strip 1 cm wide was cut from the chromatogram, cut into 4 cm pieces and placed on the surface of Oxoid diagnostic sensitivity agar (3 mm in depth) and containing 2.5% by volume of an overnight broth culture of *Escherichia coli* (Bristol). The plates were incubated overnight and the zones of inhibition measured.

Electrophoresis was effected in a Gilson High Voltage machine, Model D using pyridine - acetic acid - water (1: 10: 280 by vol.) (pH 3.9) on Whatman 3 MM paper.

Mass spectra were performed by Dr. D. L. CORINA of the University of Southampton.

Proton noise decoupled $^{13}C$ NMR spectra were recorded in $CDCl_3$ solution at a frequency of 22.63 MHz by means of a BRUKER HX FT NMR spectrometer. Chemical shifts are given in parts per million with respect to internal $Me_4Si$.

Optical rotation were measured on a "Quick" polarimeter (Roussel Jouan).

The ribose content of the antibiotics was determined using the technique of DUTCHER *et al.*[7]

### 6-Deoxyneamine (7)

6-Deoxyneomycin[8] (100 mg) was refluxed in 0.35 M anhydrous methanolic hydrogen chloride

* The terms neomycin, paromomycin and the respective deoxy analogues represent mixtures of isomers which are separable by chromatography into the components neomycin B and neomycin C, *etc.*

** NAGAOKA and DEMAIN[3] have proposed that the term idiotroph is used to describe a mutant requiring a special nutrient to produce a product peculiar to that organism.

(10 ml) for 2.5 hours, filtered through Whatman No. 1 filter paper and the filtrate cooled in ice/water. Diethyl ether (4 ml) was added giving a white precipitate of 6-deoxyneamine. The precipitate was collected on a scintered glass filter using Celite Hiflo Supercell filter aid, and then washed with cold methanol and dissolved in water. The filtered solution was evaporated to dryness giving 6-deoxyneamine (**7**), 12.5 mg, Rf 0.35 using methanol - ammonium hydroxide (34%), (4: 1) as solvent on Whatman No. 1 paper. (neamine, Rf 0.25). 6-Deoxyneamine was further purified before use by preparative chromatography in the same solvent.

The mass spectrum of the per-trimethylsilyl derivative, although not showing the molecular ion, was very similar to that of the corresponding derivative of neamine, except that two peaks of the former (*m/e* 255 and 372) were 88 mass units lower than the corresponding peaks of the neamine derivative (*m/e* 343 and 460). This shift corresponds to replacement of the trimethylsiloxy group by hydrogen.

4-*O*-*β*-D-Ribofuranosyl-2,6-dideoxystreptamine (**11**)

1 D-1,3,5/4-1,3-Diazido-4,5-cyclohexanediol[8] (**8**) (2.5 g, 12.6 mmol) and 2,3,5-tri-*O*-benzoyl-D-ribofuranosyl chloride[9] (7.4 g, 15 mmol) were dissolved in dry chloroform (100 ml), mercurous cyanide (2.5 g) and drierite (10 g) added, and the mixture refluxed with stirring for 3 days. The solids were filtered, the filtrate evaporated to dryness in vacuum and dissolved in a little ethanol to give crystalline 4-*O*-(2′,3′,5′-tri-*O*-benzoyl-*β*-D-ribofuranosyl)-1,3-diazido-4,5-cyclohexanediol (**9**) (4.4 g, 53%) recrystallized from ethanol, m.p. 101~103°, $[\alpha]_D^{25}$ +27.5° (*c* 1.5, $CHCl_3$). Found: C, 59.85; H, 4.8; N, 13.1. $C_{32}H_{30}N_6O_9$ requires C, 59.8; H, 4.7; N, 13.07%.

Compound **9** (3 g) was dissolved in ethyl acetate (60 ml) and methanol (20 ml) and hydrogenated over RANEY nickel (8 g) for 18 hours. After removal of the catalyst, the solution was evaporated to dryness in vacuum, redissolved in dry methanol (25 ml) and sodium metal (50 mg) added. After 16 hours, the solution was neutralized with Amberlite IRC 50 ($H^+$ form) and after washing with water, the resin was eluted with 2 N ammonium hydroxide in a small column. The eluate was concentrated in vacuum at 25° giving 4-*O*-*β*-D-ribofuranosyl-2,6-dideoxystreptamine (**11**) (1.21 g, 93%). The product was homogeneous on t.l.c. silica gel (acetone-water-ammonium hydroxide (34%), (80: 20: 1 by vol.) Rf 0.35) and was converted into its hydrochloride m.p. 220°C (dec.), $[\alpha]_D^{25}$ −26° (*c* 1.36, water). Found: C, 37.89; H, 7.01; N, 7.93; Cl, 20.33. $C_{11}H_{24}N_2O_6Cl_2$ requires C, 37.61; H, 6.88; N, 7.97; Cl, 20.18%.

5-*O*-*β*-D-Ribofuranosyl-2,6-dideoxystreptamine (**12**)

The mother liquors after crystallization of **9** were evaporated to dryness and the residue (5.9 g) chromatographed on silica gel (500 g), eluted with hexane - ethyl acetate (7: 3 by vol.) to give 5-*O*-(2′,3′,5′-tri-*O*-benzoyl-*β*-D-ribofuranosyl)-1,3-diazido-4,5-cyclohexandiol (**10**) (2.6 g, 32%) as an amorphous solid, homogeneous on silica gel t.l.c. (hexane - ethyl acetate, 7: 3 by vol.), $[\alpha]_D^{22}$ +16.3° (*c* 1.47, $CHCl_3$).

Compound **10** was hydrogenated and deacetylated as described for its isomer to give 5-*O*-*β*-D-ribofuranosyl-2,6-dideoxystreptamine (**12**) (85%) isolated as the sulphate, homogeneous on t.l.c. (acetone - water - ammonium hydroxide (34%), (80: 20: 1 by vol.), Rf 0.20) m.p. 215°C (dec.), $[\alpha]_D^{25}$ −57.2° (*c* 1.1, water). Found: C, 34.8; H, 6.6; N, 7.2; S, 8.7. $C_{11}H_{24}N_2O_{10}S$ requires C, 35.09; H, 6.4; N, 7.4; S, 8.5%.

Organisms and Culture Methods

Deoxystreptamine idiotrophs of *Streptomyces rimosus* forma *paromomycinus* (A.T.C.C. 21484) and *Streptomyces fradiae* (A.T.C.C. 21401) were obtained from the American Type Culture Collection.

Tests for Antibiotic Production

The test used was a modification of that used by SHIER *et al.*[10] Nutrient agar plates supplemented with the substance to be tested were prepared. The deoxystreptamine-idiotrophs were streaked on the plates and incubated at 30°C for 3 days. The plates were then overlaid with agar seeded with *E. coli* (Bristol) and incubated overnight at 37°C. The plates were inspected for a zone of inhibition of *E. coli* growth around the *Streptomyces*. No inhibition zone was observed when the *Streptomyces* were grown on plain nutrient agar.

Antibiotic Production in Broth Culture

The deoxystreptamine-idiotroph of *S. fradiae* was grown in SEBEK[11] complex medium for 2~3 days at 30°C on a rotary-shaker (150 rotations per minute). A 5% inoculum was used to inoculate SEBEK complex medium which was supplemented with 125 μg/ml 4-*O*-β-D-ribofuranosyl-2,6-dideoxystreptamine (**11**). The cultures were incubated at 30°C on a rotary-shaker as before; antibiotic activity was maximal after 5 days and was equal to 80 μg deoxyneomycin/ml. Antibiotic was extracted using Amberlite IRC-50 ($NH_4^+$ form) and was further purified using preparative paper chromatography with methanol - ammonium hydroxide (34%) (4: 1) as solvent.

## Results and Discussion

In the course of our program for the synthesis of novel aminoglycoside antibiotics we required a pseudodisaccharide containing rings II and III which could accept addition of further subunits. Although 5-*O*-β-ribofuranosyl-2-deoxystreptamine (**3**) has recently been described[12], as an intermediate it has the disadvantage of having two equivalent hydroxyl groups in the deoxystreptamine ring so that further substitution could lead to several possible diastereoisomers. The chiral deoxyanalogue (**12**) which lacks the hydroxyl group at C-6 does not have this disadvantage and we therefore decided to prepare it, particularly since we have recently shown[8] that the 6-deoxyneomycins have a very similar antimicrobial activity to that of the neomycins indicating that this modification has little or no effect on biological activity.

The starting material for the synthesis of 5-*O*-β-D-ribofuranosyl-2,6-dideoxystreptamine (**12**) was 1 D-1,3,5/4-1,3-diazido-4,5-cyclohexanediol (**8**) prepared from quinic acid[8]. When the former was treated with a mixture of 2,3,5-tri-*O*-benzoyl-α- and β-ribofuranosyl chlorides in the presence of mercurous chloride two products were formed which were separated by fractional crystallization and chromatography. Unambiguous assignment of their structures as 5-*O*-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)-1,3-diazido-4,5-cyclohexanediol (**10**) and 4-*O*-(2′,3′,5′-tri-*O*-benzoyl-β-D-ribofuranosyl)-1,3-diazido-4,5-cyclohexanediol (**9**) was made by proton noise-decoupled $^{13}C$ NMR spectroscopy (Table 1) (Fig. 3).

The presence of the β-D-ribofuranosyl moiety at the respective positions C-4 and C-5 of the cyclitol is apparent by comparison of the chemical shifts of the carbon atoms of **9** and **10** with those

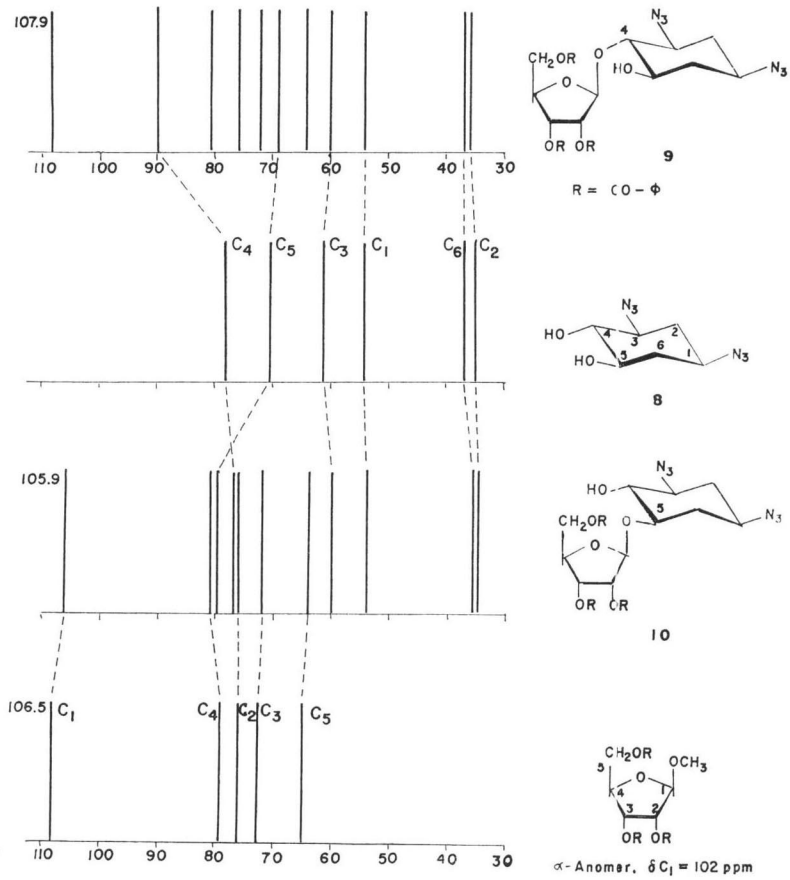
Table 1. Carbon-13 chemical shifts of comparative glycosides

| Carbon atom | Pseudodisaccharide **9** | Pseudodisaccharide **10** | Methyl β-D-tri-*O*-benzoyl ribofuranoside | 1 D-1,3,5/4-1,3-diazido-4,5-cyclohexanediol (**8**) |
|---|---|---|---|---|
| 1 | 54.1 | 54.1 | | 54.2 |
| 2 | 35.8 | 35.2 | | 35.3 |
| 3 | 60.2 | 60.4 | | 61.2 |
| 4 | 90.3 | 77.0 | | 78.3 |
| 5 | 69.1 | 79.6[a] | | 70.6 |
| 6 | 36.8 | 35.6 | | 37.2 |
| 1′ | 108 | 105.9 | 106.5 | |
| 2′ | 75.7 | 75.8 | 75.6 | |
| 3′ | 71.8 | 71.8 | 72.7 | |
| 4′ | 80.3 | 80.6[a] | 79.2 | |
| 5′ | 64.0 | 64.1 | 64.9 | |

a—Assignments may be reversed.

of the unsubstituted 1 D-1,3,5/4-1,3-diazido-4,5-cyclohexanediol (**8**). As shown in Fig. 3, the substituted carbons (C-4 in **9** and C-5 in **10**) are strongly deshielded (+12 ppm and +9 ppm respectively) while the neighboring carbons are slightly shielded (−1,3 and −1,5 ppm). The magnitude of deshieldings is diagnostic for establishing the position of linkage of the ribosyl unit as shown by previous observations of the $^{13}C$ NMR spectra of aminoglycoside antibiotics[13,14].

Fig. 3. $^{13}C$ NMR spectrum of compounds used




The $\beta$-orientation of the ribofuranose unit in both pseudodisaccharides **9** and **10** is established by comparison of the chemical shift of C-1′ with that of methyl D-2,3,5-tribenzoyl-ribofuranoside, the anomeric carbons for the $\alpha$- and $\beta$-anomers appearing at 102 and 106.5 ppm respectively.

The pseudodisaccharide derivatives **9** and **10** are ideal intermediates for further glycosylation reactions to give antibiotic-like pseudotrisaccharides, but for the biological tests described in this paper, we required the unprotected ribosyl diaminocyclitols **11** and **12**. This conversion was achieved by catalytic reduction of the diazido groups using RANEY nickel followed by debenzoylation with sodium methoxide.

Over the past few years, several idiotrophs of aminoglycoside antibiotic-producing microorganisms have been isolated which are apparently unable to biosynthesize 2-deoxystreptamine and which only make antibiotic when 2-deoxystreptamine or a suitable cyclitol analogue is added to the growth medium. These mutants have proven useful both for the production of new antibiotics[3,5,8,15~19] and for biosynthetic studies[4,20~23]. It has recently been shown that a deoxystreptamine-idiotroph of *S. rimosus* forma *paromomycinus*, which converts 2-deoxystreptamine into paromomycin, can incorporate neamine intact into neomycin[4]. Because both deoxystreptamine (**1**) (ring II) and neamine (**2**) (rings I, II) are converted into antibiotic, one order for assembly of subunits (Scheme 1a, Fig. 1) is addition of ring I to ring II followed by further glycosylation. The availability of 5-*O*-ribofuranosyl-

Table 2. Antibiotic production by deoxystreptamine-idiotrophs of *Streptomyces* incubated with test pseudodisaccharides.

| Test compound | Zone of inhibition (mm) | |
|---|---|---|
| | *S. fradiae* | *S. rimosus* forma *paromomycinus* |
| 4-*O*-*β*-D-Ribofuranosyl-2,6-dideoxystreptamine (250 *μ*g/ml) | 23 | 25 |
| 5-*O*-*β*-D-Ribofuranosyl-2,6-dideoxystreptamine (250 *μ*g/ml) | 0 | 0 |
| 6-Deoxyneamine (250 *μ*g/ml) | 0 | 25 |
| 2-Deoxystreptamine (125 *μ*g/ml) | 43 | 40 |
| Neamine (50 *μ*g/ml) | 0 | 31 |

Table 3. Chromatographic and electrophoretic properties of the antibiotics*

| Compound | Chromatography | | Electrophoresis |
|---|---|---|---|
| | Solvent a Rf | Solvent b** distance migrated (cm) | distance migrated (cm) |
| Deoxyneomycin | 0.2 | 13<br>21 | 11.5 (towards cathode) |
| Antibiotic from 4-*O*-*β*-ribosyl-2,6-dideoxystreptamine | 0.2 | 13<br>24 | 11.5 (towards cathode) |

Solvent a) is methanol - ammonium hydroxide (34%) (4: 1 by vol.)

Solvent b) is *t*-butanol - butan-2-one - methanol - 6.5 M ammonium hydroxide (3: 16: 1: 6 by vol.); chromatogram were developed for 60 hours using this solvent, which was allowed to run off the end.

Electrophoresis was carried out using 2,000 volts applied for 45 minutes.

* Antibiotics were detected by staining and using a bioautogram.

** This solvent separates the antibiotic mixture into two distinct components.

Table 4. Antimicrobial activity of the antibiotic from the incubation of the deoxystreptamine-idiotroph of *S. fradiae* with 4-*O*-*β*-D-ribofuranosyl-2,6-dideoxystreptamine, compared with that of 6-deoxyneomycin.

In each case the area corresponding to 6-deoxyneomycin B was eluted from a preparative chromatogram developed using solvent b) of table 3.

| Test organism | Minimum inhibitory concentration (*μ*g/ml)* | |
|---|---|---|
| | 6-Deoxyneomycin B | Component B of antibiotic |
| *Escherichia coli* (Bristol) | 2.5 | 2.5 |
| *Proteus mirabilis* | 5 | 10 |
| *Staphylococcus aureus* | 2.5 | 2.5 |
| *Salmonella thyphimurium* | 5 | 10 |
| *Escherichia coli* K12 ML 1629 | >80 | >80 |

* Estimated using an agar dilution technique

2,6-dideoxystreptamine (**12**) (rings II, III) allows us to test whether this route is obligatory or whether an alternative biosynthetic route (Scheme 1b, Fig. 1) in which ring III is first added to ring II may coexist.

When 6-deoxyneamine (**7**) (rings I, II), prepared by methanolysis of 6-deoxyneomycin[8] using established procedures[24], was added to the growth medium of a deoxystreptamine-idiotroph of *S. rimosus* forma *paromomycinus* antibiotic activity was produced (Table 2). In contrast 5-*O*-*β*-D-ribofuranosyl-2,6-dideoxystreptamine (**12**) (rings II, III) was not converted into antibiotic.

Table 5. Chromatography of methanolysis products from the antibiotics.
Antibiotics were subject to methanolysis conditions (as described for deoxyneamine preparation but scaled down) followed by paper chromatography of the products for four hours using methanol-ammonium hydroxide (34%) (4: 1, v/v).

| Methanolysis products | | Rf value* |
|---|---|---|
| From deoxyneomycin A | | 0.4 |
| B | | 0.7 |
| From antibiotic derived from 4-*O*-*β*-ribofuranosyl-2,6-dideoxystreptamine; | A | 0.4 |
| | B | 0.7 |

* Detected using staining technique

The lack of incorporation of 5-*O*-*β*-D-ribofuranosyl-2,6-dideoxystreptamine (**12**) compared with the incorporation of 6-deoxyneamine (**7**) suggests the order of sub-unit addition is obligatory and proceeds by the pathway outlined in Scheme 1a. In view of the existence of ribostamycin and of a *Streptomyces* species which produces both 5-*O*-*β*-D-ribosyl paromamine (rings I, II and III of paromomycins) and paromomycins[25], it seems probable that neomycin-type antibiotics are elaborated by a pathway involving the addition of ring II to ring I followed by ribosylation (ring III) and finally by addition of ring IV.

It is not known why the deoxystreptamine-idiotroph of *S. fradiae* cannot incorporate neamine-like compounds into neomycins although various explanations have been proposed[20]. One attractive possibility is that in the experimental conditions used these potential precursors are unable to enter the region of the cell where sub-unit assembly occurs. It seems probable however that neamine has access to at least some intracellular processess because at high concentration it is toxic to *S. fradiae*.

In the hope that an interesting neomycin analogue might be produced, we tested 4-*O*-*β*-D-ribofuranosyl-2,6-dideoxystreptamine (**11**) for incorporation into antibiotics by idiotrophs of both *S. fradiae* and *S. rimosus* forma *paromomycinus*. In both cases antibiotic was produced (Table 2). The antibiotic from *S. fradiae* was purified and its properties compared with those of the 6-deoxyneomycins. The antibiotics had the same Rf, electrophoretic mobility (Table 3), and antimicrobial spectra (Table 4). Furthermore they had the same ribose content and both gave 6-deoxyneamine and methyl neobiosaminide on methanolysis (Table 5). It must be concluded, therefore that the 4-*O*-*β*-D-ribofuranosyl linkage was unstable in the conditions of the incubation, and that the 2,6-dideoxystreptamine formed was converted into the 6-deoxyneomycins. Whether this hydrolysis was chemical or biological is not known, although in the absence of the organism, the pseudodisaccharide appeared to be stable.

## Added in Proof

Since submitting this manuscript for publication further evidence for the involvement of ribostamycin in neomycin biosynthesis has been published[26].

Acknowledgements

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