# BIOSYNTHESIS OF STREPTOMYCIN
## ENZYMATIC FORMATION OF DIHYDROSTREPTOMYCIN 6-PHOSPHATE FROM DIHYDROSTREPTOSYL STREPTIDINE 6-PHOSPHATE

BERNHARD KNIEP* and HANS GRISEBACH

Lehrstuhl für Biochemie der Pflanzen, Biologisches Institut II der Universität Freiburg, Schänzlestr. 1, D-7800 Freiburg, Germany

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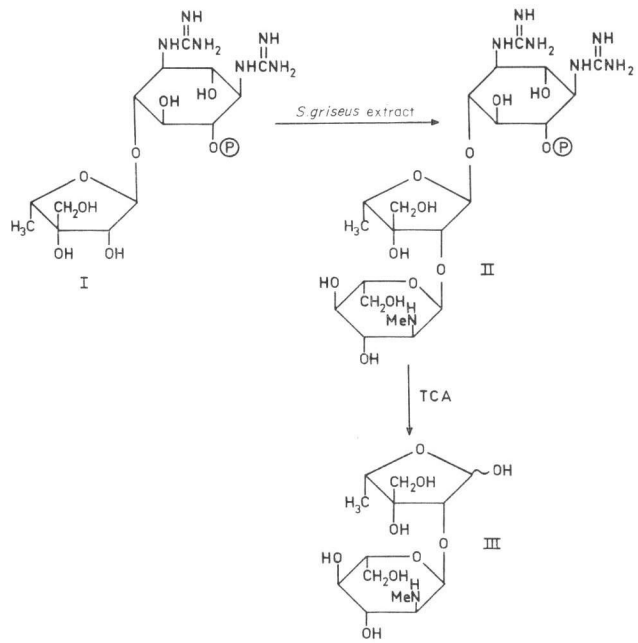
Incubation of O-α-L-dihydrostreptose (1→4) streptidine 6-phosphate (**I**) with a protein-free extract from *Streptomyces griseus* as endogenous donor and a cell-free extract from this organism led to formation of dihydrostreptomycin 6-phosphate (**II**). The product was identified by paper chromatography and by its degradation to dihydrostreptobiosamine (**III**). **II** was not formed when either the donor solution or the dialysed cell-free extract was omitted. The results corroborate the role of **I** as intermediate in streptomycin biosynthesis. The synthesis of **I** from dTDP-L-dihydrostreptose, streptidine 6-phosphate and a dihydrostreptosyltransferase from *S. griseus* has been shown previously.

Recently an enzyme has been detected which is involved in the assembly of the streptomycin molecule in *Streptomyces griseus*[1]. Transfer of the dihydrostreptose moiety from dTDP-L-dihydrostreptose[2] to streptidine 6-phosphate was shown to give O-α-L-dihydrostreptose (1→4) streptidine

Fig. 1. Conversion of dihydrostreptosyl streptidine 6-phosphate (**I**) to dihydrostreptomycin 6-phosphate (**II**) and degradation of **II** with trichloroacetic acid (TCA) to dihydrostreptobiosamine (**III**).




* Present address: Serva Feinbiochemica GmbH & Co., Carl-Benz-Str. 7, D-6900 Heidelberg 1, Germany.

6-phosphate (I)[3] (Fig. 1), a possible intermediate in streptomycin biosynthesis. The transferase from *S. griseus*[4] catalysing formation of I has been highly purified. Its activity runs parallel to that of dTDP-dihydrostreptose synthase and arginine : x-amidinotransferase and reaches a maximum after 50 hours of fermentation, just before appearance of streptomycin in the medium[4].

To further establish the role of I in streptomycin biosynthesis we have investigated its conversion to other products. We now wish to report the formation of dihydrostreptomycin 6-phosphate from I and a cell-free preparation from *S. griseus*.

## Materials and Methods

Microorganism:

*Streptomyces griseus* strain N 2-3-11 from Kaken Chem. Co., Tokyo, was grown in shake cultures as described previously[9]. The cells were harvested after 48 hours of fermentation.

Analytical methods:

Descending paper chromatography was performed on Whatman 3 MM paper with (1) methylethylketone - acetic acid - saturated boric acid (8: 1: 1, v/v) and ascending chromatography with (2) phenol - water (8: 2, w/v) in ammonia atmosphere[5]. High-voltage paper electrophoresis was carried out on Macherey and Nagel paper Nr. 214 with the buffer system (3) pyridine - acetic acid - water (3: 10: 487, v/v). Dihydrostreptomycin 6-phosphate was detected with an $\alpha$-naphthol spray reagent[6] and dihydrostreptobiosamine with the ELSON-MORGAN reagent[10].

For measurement of radioactivity the paper was cut into 1 cm wide strips and counted in a toluene liquid scintillation fluid.

Synthesis of substrates and reference compounds:

[U-$^{14}$C]-Dihydrostreptosyl streptidine 6-phosphate (I) was obtained from dTDP-[U-$^{14}$C]-L-dihydrostreptose[2] and streptidine 6-phosphate with a purified dihydrostreptosyltransferase from *S. griseus*[4]. The incubation mixture contained in a total volume of 7.5 ml: dTDP-[U-$^{14}$C]-L-dihydrostreptose (5.3 nmol, 1.2 $\mu$Ci), streptidine 6-phosphate (156 nmol), glycine-NaOH, pH 8.1 (50 $\mu$mol), EDTA (0.26 $\mu$mol), $MgCl_2$ (1.56 $\mu$mol), and 1.6 ml of a dihydrostreptosyltransferase preparation (DEAE-Sepharose fraction[4]). After incubation for 20 minutes at 0°C the reaction was terminated by addition of 1.6 ml of acetic acid. After removal of the protein by centrifugation the supernatant was subjected to paper electrophoresis in buffer system No. 3. After electrophoresis for 3 hours at 50 V/cm the zone containing I was detected by scanning the paper for radioactivity. It was cut out and eluted with water. The eluate was concentrated by freeze-drying to give 0.34 $\mu$Ci of I. Dihydrostreptomycin 6-phosphate was obtained from dihydrostreptomycin with ATP and streptomycin 6-kinase[12,13]. Dihydrostreptobiosamine[11] was obtained from dihydrostreptomycin by hydrolysis with 3 M trichloroacetic acid for 30 minutes at 95°C.

Preparation of cell-free and protein-free extracts:

Freshly harvested *S. griseus* cells were washed twice with 50 mM Tris-HCl (pH 7.8) containing mercaptoethanol (5 mM) and EDTA (1 mM). The wet cells were then suspended in the same weight of the buffer and disintegrated by sonication as described previously[2]. One half of the cell-free extract was dialysed for 4 hours against 20 mM Tris-HCl (pH 7.8) in 20% (v/v) glycerol containing EDTA (1 mM) and mercaptoethanol (5 mM). The other half was transfered to a Centriflo membrane cone (Amicon Corp., Lexington, U.S.A.) and centrifuged for 30 minutes at 1,000 *g*. The protein-free filtrate was freeze-dried and the residue dissolved in 1/10 of the original volume of water.

Incubation for transfer:

The incubation mixture contained in a total volume of 220 $\mu$l: [U-$^{14}$C]-dihydrostreptosyl streptidine 6-phosphate (0.265 nmol, 60 nCi), $MgCl_2$ (2 $\mu$mol), 50 $\mu$l of Centriflo extract and 100 $\mu$l of dialysed *S. griseus* extract. After incubation for 60 minutes at 30°C the reaction was terminated either by heating for 3 minutes at 100°C or by addition of 50 $\mu$l of 3 M trichloroacetic acid and heating for 30 minutes at 95°C (hydrolysis of reaction product).

Detection of N-methylglucosamine in protein-free filtrate by gas-liquid chromatography-mass spectrometry:

The protein-free freeze-dried sample was hydrolysed with 2 ml 2 NHCl for 2 hours at 100°C. HCl was removed *in vacuo*. The residue was treated with sodium borohydride and acetic anhydride-pyridine to form the alditol peracetates[14,15].

GLC of the acetylated alditols was performed on a glass-wall coated open-tubular (WCOT) column of CP-SIL-5 (Chromapack, Berlin, G.F.R.). The column was operated at 225°C with an injector split ratio of 50: 1 and helium as carrier gas. The retention time for the hexaacetate of N-methyl-glucosaminitol was 5.78 minutes.

Mass spectrometry was performed on a Finningan Model 3200 E quadrupole GC-MS with jet separator and an ionizing voltage of 70 eV coupled to a Finnigan Model 6000 data system.

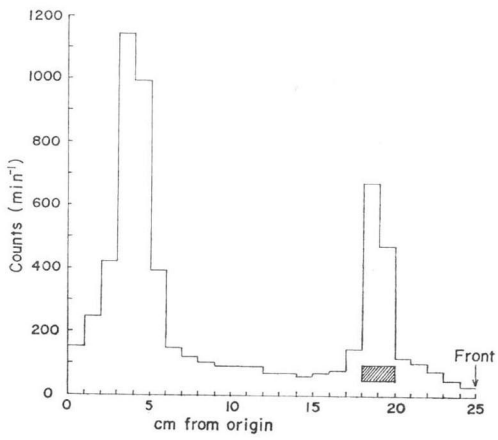
## Results and Discussion

The $^{14}$C-labelled pseudodisaccharide **I** was prepared from dTDP-[U-$^{14}$C]-dihydrostreptose and streptidine 6-phosphate in the presence of the dihydrostreptosyltransferase[4] and was purified by paper electrophoresis. $^{14}$C-labelled **I** was incubated with a concentrate of the low molecular components from a crude extract of *S. griseus* and a cell-free extract of this organism. Separation of the incubation mixture on paper with solvent system No. 2 gave two radioactive products (Fig. 2). The substance with the higher Rf-value (Rf 0.76) co-chromatographed exactly with dihydrostreptomycin 6-phosphate (**II**, Fig. 1). The spot with Rf 0.13 corresponded to nonconverted **I**. **II** was not formed when either the donor solution or the dialysed cell-free extract was omitted or when the incubation was terminated immediately.

To further substantiate that the radioactive product was identical with dihydrostreptomycin 6-phosphate the incubation mixture was treated with trichloroacetic acid, which hydrolyses the linkage between dihydrostreptose and streptidine with formation of dihydrostreptobiosamine[11] (**III**, Fig. 1). Subsequent paper chromatography with solvent system No. 1 showed two radioactive spots. The product with Rf 0.06 co-chromatographed with authentic dihydrostreptobiosamine; the second product (Rf 0.4) corresponded to dihydrostreptose. The hydrolysis mixture was also subjected to paper electrophoresis in buffer system No. 3. Again two radioactive products were found which migrated in electrophoresis together with dihydrostreptobiosamine and dihydrostreptose, respectively.

Fig. 2. Paper chromatogram of incubation mixture with solvent system No. 2.

Shaded area shows position of dihydrostreptomycin 6-phosphate.




The protein-free concentrate of *S. griseus* was hydrolyzed with HCl and the hydrolysate treated with sodium borohydride and acetic anhydride-pyridine to convert sugars to their alditol acetates[14,15]. The alditol acetates were analyzed by gas-liquid chromatography-mass spectrometry and compared with the mass-spectrum of the alditol acetate prepared from a reference sample of N-methyl-L-glucosamine. The occurence of the characteristic fragment ions at *m/e* 116 (100%) and 158 (57%) proved the

presence of N-methylglucosamine in the concentrate. Further experiments (H. P. WAHL and H. GRISEBACH unpublished results) showed that N-methyl-glucosamine is present in the sugar nucleotide fraction of *S. griseus*.

From these results it can be concluded that a transferase present in the cell-free extract from *S. griseus* catalyzes the transfer of a nucleotide-bound N-methyl-L-glucosamine moiety to **I** with formation of dihydrostreptomycin 6-phosphate. This result corroborates the role of the pseudodisaccharide **I** as an intermediate in the biosynthesis of streptomycin. According to previous results the final steps leading from **II** to streptomycin would be the oxidation of dihydrostreptomycin 6-phosphate to streptomycin 6-phosphate[7] and the hydrolysis of the latter to streptomycin[8].

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