

## LAST STEP IN THE BIOSYNTHESIS OF STREPTOMYCIN: N-METHYLATION OF N-DEMETHYLSTREPTOMYCIN

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Addition of dihydro-N-demethylstreptomycin (DH-NDMS) or N-demethylstreptomycin (NDMS) in a concentration of 10 mg/ml to a growing culture of *S. griseus* blocked the *de novo* biosynthesis of streptomycin (SM). A simultaneous addition of  $^{14}\text{CH}_3$ -L-methionine, however, led to specific N-methylation of the added compounds, thus giving the authentic antibiotics dihydrostreptomycin (DHS) and SM. By using the quantitative specific reaction of SM with bisulfite an electrophoresis system was developed capable of separating SM and DHS.

The antibiotic streptomycin (SM) consists of the three moieties streptidine, L-streptose, and N-methyl-2-deoxy-2-amino-L-glucose linked together with  $\alpha$ -L-glycosidic bonds. D-Glucose is the main precursor of all the three moieties.

The general pattern of the biosynthesis is now well established. In some cases the enzyme systems responsible for the single steps have been isolated and studied in detail (for a review see DEMAIN *et al*<sup>1</sup>). It has been demonstrated that the N-methyl-group of the glucosamine moiety is derived from L-methionine<sup>2,3</sup>. This compound is not a precursor to other groups of the SM molecule. Based on these observations NDMS was isolated from a culture of *Streptomyces griseus*, in which the natural methylation processes were partially inhibited by addition of ethionine to a synthetic substrate<sup>4</sup>. It has been proposed that the methylation process of the L-glucosamine moiety of the SM molecule could be the last step in the biosynthesis of the antibiotic. With N-demethylstreptomycin (NDMS) and dihydro-N-demethylstreptomycin (DH-NDMS) available we decided to investigate if these two compounds can function as methyl-acceptors when added to a growing culture of *S. griseus*. If this was the case SM and DHS would be synthesized.

### Materials and Methods

#### Cultural Conditions

The strain used in this study was a relatively high yielding strain of *S. griseus* producing more than 2,000  $\mu\text{g/ml}$ , our No. THS-1. In this study a well-defined substrate was used for preparation of the inoculum and production medium<sup>5</sup>. The fermentation experiments were performed in the conventional way using a two-stage fermentation in shake flasks on a rotary shaker. After 72 hours of growth in the second stage NDMS or DH-NDMS (a kind gift from Novo Industri A/S, Denmark) was added to give a concentration of 10 mg per ml. Simultaneously  $^{14}\text{CH}_3$ -L-methionine (Amersham, UK) was added (0.18  $\mu\text{Ci/ml}$ ). The fermentation was continued for a further 100 hours.

#### Isolation

The SMs were extracted as described<sup>6</sup>, and the aqueous extracts ( $\text{H}_2\text{SO}_4$ ) were neutralized with triethylamine, decolourized with carbon and evaporated under vacuum to a small volume. The SMs were precipitated by slowly adding the solution to 10 vol. of  $\text{CH}_3\text{OH}$  under stirring. The

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precipitate was isolated by filtration, washed carefully with  $\text{CH}_3\text{OH}$ , and dried under vacuum.

The purity of the products thus obtained was estimated using the maltol method<sup>7)</sup> and a guanidino specific method<sup>8)</sup>. The purity varied from 70% to 75%, the impurities were streptidine (SD) and inorganic salts. Estimation of the  $^{14}\text{C}$  incorporation was performed using a liquid scintillation counter (Beckman).

#### Electrophoresis and Radio Scanning

Separation of the streptomycins, SM from DHS and SD was obtained by using high-voltage electrophoresis<sup>9)</sup>. In order to separate SM from DHS an aqueous solution of the mixture was added an excess of sodium bisulfite at  $24^\circ\text{C}$  at pH 4, 4~6 hours before electrophoresis. Chemical development of the SMs was performed with the diacetyl- $\alpha$ -naphthol reagent<sup>10,11)</sup>.

Distribution of the incorporation of  $^{14}\text{CH}_3$  in the components extracted was carried out on electrophoretograms with a Packard radioscanner.

### Results and Discussion

SM contains a potential aldehyde group which readily reacts with bisulfite forming an  $\alpha$ -hydroxy sulfonic acid. In DHS, the aldehyde group is replaced by a primary alcohol, hence no reaction with bisulfite occurs.

Addition of  $^{14}\text{CH}_3$ -L-methionine to a culture of *S. griseus* containing added NDMS leads to  $^{14}\text{C}$ -labeled SM formed either by *de novo* biosynthesis or by methylation of the NDMS. It is not possible to demonstrate how the labeled SM would have been formed. We, therefore, decided to investigate if a simultaneous addition of  $^{14}\text{CH}_3$ -L-methionine and DH-NDMS to a shake flask culture of *S. griseus* would result in  $^{14}\text{C}$ -labeled DHS. The result is shown in the electrophoretogram (Fig. 1) which also demonstrates that the isolated SM-mixture only shows one spot.

Treatment of the same mixture with sodium bisulfite as described above reduced the mobility of SM by approximately 30% without affecting the mobility of the dihydro-SM's. The part to the right of Fig. 1 shows a radio-scanning of the electrophoretogram of the bisulfite-treated mixture next to it.

Fig. 1. Electrophoretogram of streptomycins (1) DHS, (2) DHS+ $\text{HSO}_3^-$ , (3) SM, (4) SM+ $\text{HSO}_3^-$ , (5) SMs from incubation mixture (see text), (6) SMs from incubation mixture+ $\text{HSO}_3^-$ , (7) Distribution of  $^{14}\text{C}$  in  $\text{HSO}_3^-$ -treated incubation mixture.

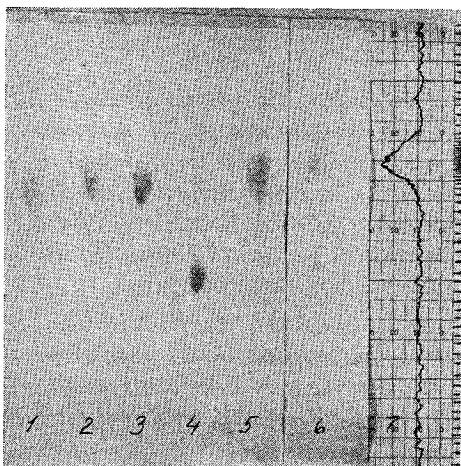
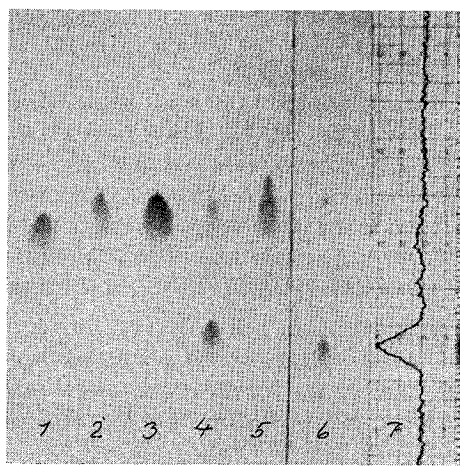


Fig. 2. Electrophoretogram of streptomycins (1) DHS, (2) DHS+ $\text{HSO}_3^-$ , (3) SM, (4) SM+ $\text{HSO}_3^-$ , (5) SMs from incubation mixture (see text), (6) SMs from incubation mixture+ $\text{HSO}_3^-$ , (7) Distribution of  $^{14}\text{C}$  in  $\text{HSO}_3^-$ -treated incubation mixture.



Two conclusions can be drawn from this figure. First, DH-NDMS can function as methyl-acceptor, as only the spot corresponding to DHS and unchanged DH-NDMS is radioactive.

Secondly, the electrophoretogram and radioscan clearly show that the addition of the DH-NDMS under the above conditions stops the *de novo* biosynthesis of SM, as the spot corresponding to the SM-bisulfite reaction product is not radioactive.

Assuming that a similar addition of NDMS also stops the biosynthesis of SM, NDMS was added in the same concentration as DH-NDMS together with the same amount of  $^{14}\text{C}_3\text{-L-methionine}$ .

Fig. 2 shows the controls DHS and SM, treated and untreated with bisulfite. The product isolated from the fermentation broth showed as expected one single spot (SM+SD). Treatment with bisulfite reduced the mobility of SM leaving behind SD and trace amounts of SM.

The radioscan of the electrophoretogram unambiguously demonstrates that part of the added NDMS is methylated by *S. griseus*. The radioscan also indicates that a trace amount of SM may not have reacted with the bisulfite.

The percentage of incorporation of  $^{14}\text{C-L-methionine}$  into the SMs was measured in another experiment. It could be estimated to be about 60%. This high rate of  $^{14}\text{C}$ -incorporation is probably due to the large excess of methyl-acceptor (NDMS and DH-NDMS) relative to the amount of added  $^{14}\text{C-L-methionine}$ .

The conclusion of the experiments described is that both NDMS and DH-NDMS are methylated by *S. griseus*, yielding SM and DHS. This strongly indicates that the last step in the biosynthesis is the N-methylation. It is interesting to notice that our strain of *S. griseus* which only produces SM does not distinguish between NDMS and DH-NDMS with respect to N-methylation.

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