CHEMICAL STRUCTURE OF STREPTOTHRICINS

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The general formula, proposed earlier for the natural streptothricins E, D, C, B, A and for the biosynthetic streptothricin X was shown to be valid.

1. The presence of a common moiety in streptothricins F, E, D, C, B and A was demonstrated by isolation of N-*guan*-streptolidyl gulosaminide from preparations of all these antibiotics.

2. It was shown that streptothricins D, C, B, A and X contain unbranched peptide chains, in which residues of L-β-lysine are linked by their ε-amino groups, whereas β-amino groups are free.

The first member of this group of antibiotics, streptothricin (now known as streptothricin F), was discovered by WAKSMAN and WOODRUFF in 1942[1]. The chemical investigation of streptothricins proceeded rather slowly. After long and tedious investigations by American, British and Japanese scientists, the structures of five ninhydrin-positive compounds isolated from the products of total and partial hydrolysis of streptothricin were established. These were: unusual amino acids L-β-lysine[3,4] and streptolidine (roseonine)[5,6], a rare amino sugar, gulosamine (2-amino-2-deoxy-α-D-gulose), a product of its degradation -1,6-anhydro-β-D-gulosamine[7] and N-*guan*-streptolidyl gulosaminide, a product of partial hydrolysis of the antibiotic[8]. Only in 1961～1962 American[9] and British[6] scientists independently suggested the formula I ($n=1$) for streptothricin F.

Shortly after the discovery of streptothricin many other similar antibiotics were reported; namely streptolin, geomycin, phytobacteriomycin, racemomycin, pleocidin, polymycin, *etc.* (the comprehensive review of literature, up to 1960, is given in monography[2]). Soon it was shown[10,11] that all these antibiotics were mixtures of closely related compounds. Their chemical investigation for a long time was hampered by the absence of a reliable general method of isolation. As no reliable methods of isolation of homogeneous compounds were available and the studies were carried out on mixtures, the conclusions drawn were not always correct. Thus the formula proposed by American authors for streptolin with $n=2$[9] had later to be changed (Fig. 1, $n=3$)[12].

In 1964, RESHETOV and KHOKHLOV[14,20,21] elaborated an effective general procedure for separation of streptothricin mixtures based on ion-exchange chromatography on carboxymethylcellulose with a sodium chloride gradient. By means of this method they demonstrated that all the available preparations of antibiotics of this type were mixtures of six streptothricins differing in the number of L-β-lysine residues (1 in

streptothricin F, 2 in streptothricin E and so on to 6 in streptothricin A). Recently a group of Japanese scientists modified the method of ion-exchange chromatography on carboxymethylcellulose (by replacing sodium chloride with the volatile pyridine-acetic acid buffer) and also elaborated gel-chomatographic techniques (dextran gel LH-20)[15,16]. Using these procedures they separated individual racemomycins A, C, B and D, and demonstrated their identity to streptothricins F, E, D and C. They showed by the same methods the identity of antibiotics yazumycins A and C[17] to racemomycins A and C[18]. Thus they confirmed the idea that all the streptothricin antibiotics are mixtures of the same components differing by the length of their peptide chain.

As a result of quantitative determination of L-β-lysine, streptolidine and gulosamine in the hydrolysates of each individual streptothricin KHOKHLOV and co-workers[12,13,19] suggested for the 6 studied antibiotics the general formula I, which was based on three assumptions:

1) all the streptothricins contain the same common moiety;

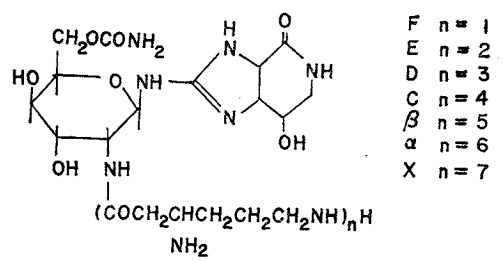
2) they differ in the number of β-lysine residues in the unbranched peptide chain;

3) all the β-lysine residues are linked by their ε-amino groups leaving the β-amino groups free.

However there were no data in the literature straight-forwardly confirming any of these assumptions. For example, though β-lysine peptides had been isolated from the products of streptothricin mild hydrolysis[6,9,22], their structures were not proved unequivocally. BROCKMANN and CÖLLIN[22] tried to elucidate the nature of the peptide bond by means of acid hydrolysis of DNP-derivatives of peptides isolated from crude preparations of geomycin (F, E, D, C). However it was found that dinitrophenylation does not proceed completely under the conditions used. This is clear from the fact that they could not detect *bis*-DNP-β-lysine in the hydrolysates of DNP-geomycin, therefore they came to the conclusion that the peptide chain was linked to the rest of molecule by the ε-amino group of the terminal β-lysine. However ROSTOVTSEVA, KIRYUSHKIN and KHOKHLOV isolated a β-lysine dipeptide from the hydrolysate of streptothricin D (identical to streptolin). The structure of this dipeptide was clearly established by means of mass-spectrometric comparison of its triacetate to the specially synthesized triacetates of the two isomeric β-lysyl-β-lysines, in which the amino acids were linked by ε- and by β-amino group respectively[23,24,25,26]. It was shown that in the isolated dipeptide β-lysine residues are linked *via* ε-amino group which is in accordance with the suggested general formula. However this method of comparison could not be used in the case of more complex β-lysine peptides. Therefore the new methods were needed for the confirmation of the general formula.

Here we present data confirming the above-mentioned assumptions.

Fig. 1.

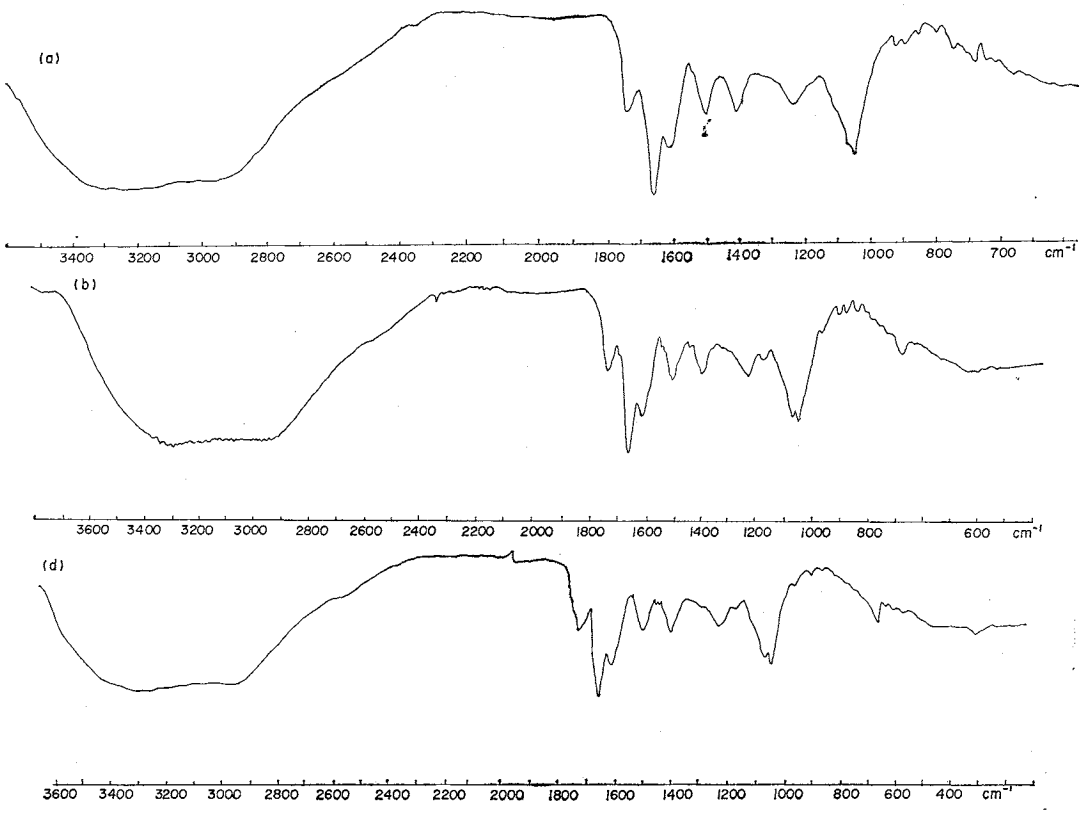


We isolated N-*guan*-streptolidyl gulosaminide from partial hydrolysates of streptothricins E, D, C, B and A respectively and compared the products to a compound prepared from streptothricin F according to CARTER *et al.*[3] To isolate this compound from the hydrolysates of more complex streptothricins we used not only chromatography on cellulose in solvent system *t*-BuOH - AcOH - H_2O (2 : 1 : 1)[3], but also the method developed by us for the separation of β-lysine peptides[28]. The elementary analysis and TLC on cellulose of different samples of streptolidyl gulosaminide showed them to be identical. The Rf values were as follows: 0.39 in system MeOH - $CHCl_3$ - conc. NH_4OH (2 : 1 : 1) (system No. 1), 0.22 in system *n*-BuOH - Pyr - AcOH - H_2O - *t*-BuOH (15 : 10 : 3 : 12 : 4) (System No. 2), 0.23 in system *t*-BuOH - AcOH - H_2O (2 : 1 : 1) (System No. 3). IR-spectra of the samples were identical and contained the following characteristic absorption bonds: 1736 cm^{-1} (carboxyl), 1660 cm^{-1} (trisubstituted guanidine group) (Fig. 2).

Streptolidine and traces of gulosamine, 1, 6-anhydrogulosamine and original streptolidyl gulosaminide were detected in hydrolysates (5.7 N HCl, 110°C 20 hours) chromatographically (Fig. 3). After prolonged hydrolysis (40 hours) streptolidine only was detected.

Thus the presence of N-*guan*-streptolidyl gulosaminide in all streptothricins (F,

Fig. 2. IR-spectra of N-*guan*-streptolidyl gulosaminide hydrochloride, isolated from streptothricins F(*a*), E, D(*b*), C, B(*d*) and A.



E, D, C, B, A) was demonstrated*.

To test the two other assumptions on the structure of peptide chains we attempted to modify the streptothricin peptide chains and elucidate the structure of the hydrolysis products.

However dinitrophenylation and reductive methylation of streptothricins do not proceed to completion and we always find in the hydrolysate some intact β-lysine; this might give rise to a conclusion that the antibiotic contains a branched peptide chain. Attempts to deaminate the antibiotics by nitrous acid or ninhydrin led to the disappearance of intact β-lysine, but simultaneously gulosamine and streptolidine disappeared also. So a conclusion about the absence of β-lysine seemed somewhat unreliable. Therefore for the structural elucidation of peptide chains we had to work out a special procedure of splitting off the chain from the antibiotic molecule to give peptides with the same number of β-lysine residues as previously contained in the antibiotic[28]. Isolated peptides were completely dinitrophenylated** and hydrolyzed.

The presence in hydrolysates of β,ε-bis-DNP-β-lysine and β-DNP-β-lysine and absence of unsubstituted lysine and ε-DNP-β-lysine were shown by TLC on cellulose in

Fig. 3. Paper chromatography of the products of hydrolysis of streptothricins in the system *n*-BuOH - Pyr - AcOH - water - *tert*-BuOH (15 : 10 : 3 : 12 : 4).

1. L-β-Lysine; 2. streptolidine; 3. N-*guan*-streptolidyl gulosaminide; 4. the hydrolysate of streptolidyl gulosaminide.

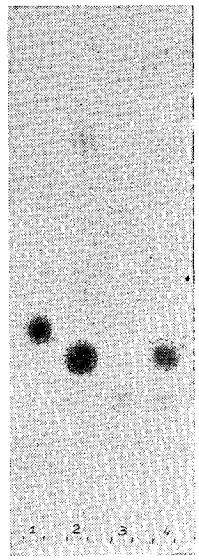
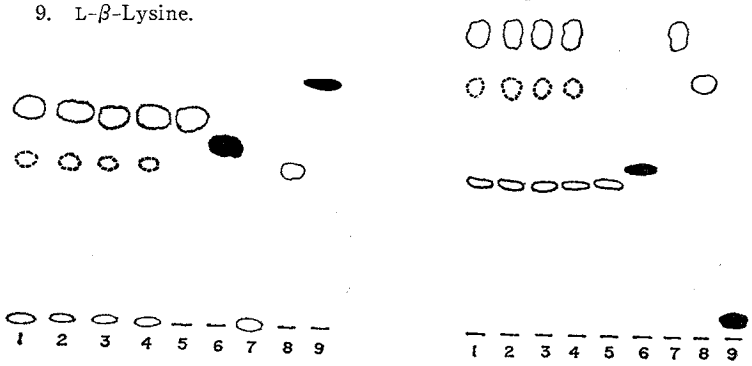


Fig. 4. TLC of the hydrolysates of DNP-derivatives of L-β-lysine peptides on the cellulose in solvent systems: (a) 1.5 M phosphate buffer (pH 6.5), (b) *i*-AmOH - Pyr - H_2O (25 : 28 : 21).

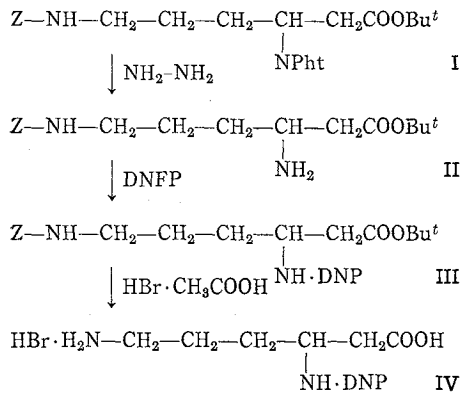
1. The hydrolysate of DNP-derivative of L-β-lysine dipeptide.
2. The hydrolysate of DNP-derivative of L-β-lysine tripeptide.
3. The hydrolysate of DNP-derivative of L-β-lysine pentapeptide.
4. The hydrolysate of DNP-derivative of L-β-lysine hexapeptide.
5. N^{β}-DNP-L-β-lysine.
6. N^{ε}-DNP-L-β-lysine.
7. N^{β},N^{ε}-bis-DNP-L-β-lysine.
8. Dinitrophenol.
9. L-β-Lysine.



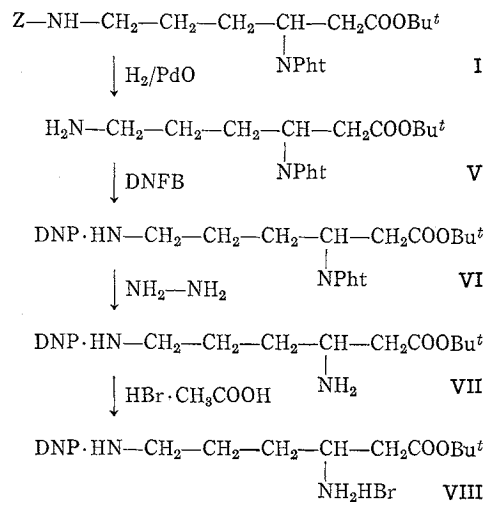
* The presence of streptolidyl gulosaminide in the structure of biosynthetic streptothricin X[29] is clear from the TLC of a mixture of streptolidine-containing compounds, isolated from the products of partial hydrolysis of the antibiotic[28]. However no streptolidyl gulosaminide could be isolated in the individual state as streptothricin X was available in very small amounts only.

** Because streptothricin X and β-lysine heptapeptide obtained from it were almost unavailable, we dinitrophenylated the mixture, which contained in addition to heptapeptide also L-β-lysine and its di-, tri-, tetra-, penta- and hexapeptides.

Scheme 1



Scheme 2



systems: Pyr - *i*-AmOH - H_2O (25 : 28 : 21) (system No. 4), BuOH saturated with 0.1 % aqueous NH_4OH (System No. 5) and 1.5 M phosphate buffer (pH 6.5) (System No. 6) (Fig. 4).

For comparative studies the corresponding β-DNP- and ε-DNP-derivatives of L-β-lysine were synthesized from earlier described N^{ε}-carbobenzoxy-N^{β}-phthalyl-L-β-lysine *tert*-butyl ester[27] according to schemes 1 and 2.

Thus we demonstrated that all the streptothricins contained unbranched peptide chains in which the β-lysine residues were linked by their ε-amino groups. As a result the general formula, proposed earlier for the natural streptothricins E, D, C, B and A and for the biosynthetic streptothricin X, was shown to be valid.

Experimental

As the physical and chemical properties of all the streptothricins are very similar, their structures were determined by very much the same methods. For brevity's sake we describe here the experimental procedures on individual substances.

1. N^{ε}-Carbobenzoxy-L-β-lysine *tert*-butyl ester (II)

N^{ε}-Carbobenzoxy-N^{β}-phthalyl-L-β-lysine *tert*-butyl ester (I) (20 mg) was dissolved in ethanol (0.5 ml) and fresh distilled hydrazine (0.05 ml) was added to the solution. The mixture was allowed to stand at the room temperature for 24 hours; the reaction was monitored by TLC on silica gel (150 mesh) with the systems:

Bz - EtAc (1 : 1) (System No. 7),

MeCOEt - Pyr - H_2O - AcOH (70 : 15 : 15 : 2) (System No. 8).

The precipitate of phthalylhydrazide was filtered off and washed with absolute ethanol. The filtrate was then evaporated under reduced pressure, the residue was dissolved in chloroform and washed with water and saturated NaCl solution. The chloroform solution was dried over Na_2SO_4. After evaporation of the chloroform a chromatographically homogeneous oily residue (II) (Rf 0.80 in System No. 8) was obtained. The yield was 18 mg. Conc. H_2SO_4 and ninhydrin solution in ethanol with collidine and acetic acid were used for detection.

2. N^{ε}-Carbobenzoxy-N^{β}-DNP-L-β-lysine *tert*-butyl ester (III)

To a solution of 18 mg of compound II in 0.5 ml of ethanol 5 drops of 0.5 % aqueous solution of fresh distilled triethylamine and 0.3 ml of 10 % ethanolic 2,4-dinitrofluorobenzene were added. The reaction mixture was shaken for 5 hours at room temperature in the dark. Then it was diluted with 5 ml water and compound III, containing traces of DNFB

was extracted with ether. Since the presence of DNFB does not interfere with further synthesis, it was not removed and the ether was evaporated.

3. N^{β}-DNP-L-β-lysine hydrobromide (**IV**)

The crude preparation **III** obtained above was dissolved in 0.5 ml of glacial acetic acid saturated with dry HBr and allowed to stand for 20 minutes. Then the reaction mixture was evaporated, the residue was dissolved in water and repeatedly washed with ether. Aqueous solution was evaporated and the residue was dried under reduced pressure (P_2O_5) to give 9.7 mg of chromatographically homogeneous compound **IV** (Rf 0.55 in system 4; 0.38 in system 5, 0.76 in system 6).

4. N^{β}-Phthalyl-L-β-lysine *tert*-butyl ester (**V**)

A solution of I (20 mg) in methanol (1 ml) was hydrogenated over PdO at room temperature for 5 hours. The reaction was monitored by TLC on silica gel using Systems 7 and 8. When hydrogenation was finished the catalyst was filtered off and methanol evaporated. The residue was dissolved in benzene, washed with saturated solution of sodium bicarbonate and water dried over Na_2SO_4. As a result, 13 mg of homogeneous compound **V** with Rf value of 0.75 in System 8, was obtained.

5. N^{β}-Phthalyl-N^{ε}-DNP-L-β-lysine *tert*-butyl ester (**VI**)

The compound **VI** was prepared by the procedure similar to that described for **III** (see 2). After shaking for 5 hours aspartic acid was added to the reaction mixture to remove the excess of DNFB and shaking was continued for 2 hours more. Then the solution was diluted with water and extracted with ether. After evaporation of ether the residue was purified by thin-layer electrophoresis (Pyr - AcOH - water (30 : 1 : 270), pH 6.5, U 1,000 v, 1 hour). DNP-aspartic acid moves to anode, and the ester **VI** stays at the origin. Then **VI** was eluted from the electrophoretogram with acetone and absolute ethanol. After solvent evaporation chromatographically homogeneous **VI** was obtained.

6. N^{ε}-DNP-L-β-lysine *tert*-butyl ester (**VII**)

Chromatographically pure compound **VII** was obtained by the same procedure as described above for **II** (see 1). The reaction was monitored by TLC using Systems 4 and 6.

7. N^{ε}-DNP-L-β-lysine hydrobromide (**VIII**)

Compound **VIII** was prepared according to the procedure 3 to give 5.3 mg of chromatographically homogeneous compound **VIII** (Rf 0.61 in System 4, 0.42 in System 5 and 0.65 in System 6).

8. N^{ε},N^{β}-bis DNP-L-β-lysine (**IX**)

A mixture of 2 mg L-β-lysine, 1 ml 0.5 % aqueous triethylamine and 0.1 ml 10 % ethanolic DNFB was shaken for 7 hours at room temperature in the dark. Then the solution was diluted with water and washed with ether. The water solution was evaporated and the residue was purified by thin-layer electrophoresis on cellulose (Pyr-Ac-buffer (pH 6.5), U 900 v, 1 hour). After elution of the start strip with acetone compound **IX** with Rf value of O in System 6, was collected.

9. 2,4-Dinitrophenyl derivative L-β-lysine tripeptide, isolated from the products of partial hydrolysis of streptothricin D

The 2,4-dinitrophenyl derivative of L-β-lysine tripeptide was prepared with 2, 4-dinitrofluorobenzene as described above (see 8). It was purified by thin-layer electrophoresis (HCOOH - AcOH - H_2O (5 : 30 : 65), U 900 v, 1 hour). Completely dinitrophenylated tripeptide which stayed at the start, was eluted with acetone. After acetone evaporation the residue was hydrolyzed with 0.5 ml 5.7 N HCl at 110°C for 17 hours in a sealed tube.

The hydrolysate was concentrated under reduced pressure and then excess dried to remove HCl over NaOH. The residue was examined by TLC on cellulose in Systems 4, 5 and 6.

10. Isolation of N-*guan*-streptolidyl gulosaminide from the streptothricin D hydrolysate

Hundred mg of streptothricin D in 5 ml 5.7 N HCl was hydrolyzed in a sealed tube (50°C, 96 hours). The hydrolysate was decolourized by shaking with charcoal, evaporated

(40°C) and dried under reduced pressure (NaOH). The the residue was dissolved in 5 ml water, adjusted to pH 6~7 with Amberlite IR-45 (OH^- form) and dried by repeated co-evaporations with a mixture of methanol and absolute ethanol.

The streptolidine-containing products were separated from the mixture of L-β-lysine peptides as described elsewhere[28].

The mixture of streptolidine-containing compounds was chromatographed on a column (1×100 cm) of Cellulose CC 31 using the system *n*-BuOH - Pyr - AcOH - water (15 : 10 : 3 : 12). The flow rate was 4.5 ml/hour.

Fractions (1.5 ml) were examined by TLC on cellulose (MN 300 or "Filtrak") with Systems 2 and 3. Fractions 56~80 contained streptolidine, while fractions 81~89 were a mixture of streptolidine and N-*guan*-streptolidyl gulosaminide; fractions 90~120 were N-*guan*-streptolidyl gulosaminide and fractions 123~150 were a mixture of streptothricin inactivation products. Fractions 90~120 were evaporated to dryness under reduced pressure. Then the resulting streptolidyl gulosaminide dissolved in 5 ml water was passed through a column of Dowex 1×8 (Cl^-). After evaporation of the eluate the residue was dried by repeated co-evaporation with a mixture of methanol and absolute ethanol and then dried under reduced pressure (P_2O_5). Ten mg of streptolidyl gulosaminide hydrochloride (Rf 0.35 in System 1, 0.22 in System 2, 0.23 in System 3) were obtained; mp 220°C (decomp.).

11. Isolation of N-*guan*-streptolidyl gulosaminide from the products of partial hydrolysis of streptothricin B

Acid hydrolysis of 50 mg of streptothricin B was carried out in a sealed tube (25 ml of 5.7 N HCl, 50°C, 96 hours). Then the hydrolysate was treated as described above for streptothricin D. The hydrolysate was applied to a column (1×100 cm) with cellulose powder CC 31 and eluted with *tert*-BuOH - AcOH - water (2 : 1 : 1). The flow rate was 2.5 ml/hour. Fractions were collected 1 ml. The elution was monitored by TLC on cellulose using Systems 2 and 3.

Fractions 60~91 contained the L-β-lysine, fractions 95~97 streptolidine, fractions 100~113 L-β-lysine dipeptide, fractions 115~124 L-β-lysine tripeptide, and some β-lysyl-β-lysine, fractions 125~128 streptolidyl gulosaminide and small amount of a tripeptide, fractions 129~137 the pure streptolidyl gulosaminide and fractions 138~198 contained streptothricin B and some shortened streptothricins, resulting from the partial hydrolysis, inactivation products.

The fractions 129~137 were evaporated to dryness and then the residue dissolved in 5 ml of water was passed through Dowex 1×8 (Cl^-). Water was evaporated and residue was dryed under reduced pressure to give 5 mg streptolidyl gulosaminide hydrochloride.

12. N-*guan*-Streptolidyl gulosaminide sulfate

The aqueous solution of streptolidyl gulosaminide hydrochloride was passed through a column of Amberlite IRA-400 (SO_4^{--}). Water eluate was concentrated and streptolidyl gulosaminide was precipitated with methanol. The fine precipitate was centrifugated, washed with methanol and then dried over P_2O_5.

13. The IR-spectrum (KBr) was measured on a Model UR-10-spectrophotometer

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