STUDIES ON STREPTOTHRICINS
VII. DETERMINATION OF THE NINHYDRIN-POSITIVE FRAGMENTS OF STREPTOTHRICIN ANTIBIOTICS

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Khimiya Prirodnykh Soedinenii, Vol. 1, No. 2, pp. 117-122, 1965

In preceding communication, we described the production and properties of a series of streptothricin antibiotics, including new compounds of this group: polymycin A (streptothricin A) ($C_{49}H_{94}N_{18}O_{13}$; $[\alpha]_D^{21} - 9.2°$); polymycin B (streptothricin B) ($C_{43}H_{82}N_{16}O_{12}$; $[\alpha]_D^{21} - 9.6°$); and phytobacteriomycin C (streptothricin C) ($C_{37}H_{70}N_{14}O_{11}$; $[\alpha]_D^{21} - 12.8$) [1]. The empirical formulae of these not widely distributed and difficultly accessible compounds were determined on the basis of the elementary analysis of their salts, their equivalent weights, and the number of their amino groups. At the same time, we showed the existence of six types of streptothricins (A, B, C, D, E, F) possessing characteristic physiochemical and biological properties. In the course of a systematic study of the streptothricins we have developed a method for the quantitative analysis of hydrolyzates of these compounds in an automatic amino acid analyzer.

The present work is devoted to the determination of the amino acid and carbohydrate composition of representatives of the six types of streptothricins: grisemins F and E, phytobacteriomycins D and C, and polymycins B and A.

EXPERIMENTAL

Qualitative analysis of hydrolyzates of the six types of streptothricins. Samples of a few milligrams of the hydrochlorides of the streptothricins of the six types were hydrolyzed with 5.7 N HCl for 24 hr at 105 ± 1°. The hydrolyzates were evaporated to dryness in a rotary evaporator and the last traces of HCl were removed by storage in a vacuum desiccator over alkali.

The qualitative composition of the hydrolyzates was determined by means of radial [2] and horizontal chromatography at an elevated temperature [3, 4] on type "B" paper of the Leningrad Volodarskii mill washed with a 0.1 N solution of Trilon B. The dimensions of the sheets were as follows: for radial chromatography, circles 30 cm in diameter; and for horizontal chromatography, 13 × 18 sheets. The following solvent systems were used:

1) Butanol-formic acid-water (75 : 15 : 10) [5];
2) Butanol-acetic acid-water (10 : 2 : 4) [5];
3) Butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12) [6]; and
4) Methyl ethyl ketone-propionic acid-water (30 : 10 : 12) [7].

The time for radial chromatography was 3-6 hr, depending on the system and the temperature, and that for horizontal chromatography was 2-3 hr at 50-60°.

For the radial chromatography, the lid of a desiccator was used as the chamber, and for horizontal chromatography a stainless steel chamber [3]. The revealing agents were: a 0.5% solution of ninhydrin in acetone, Weber's reagent for substituted guanidines [5], the Elson-Morgan reagent [5], and triphenyltetrazolium chloride [5]. The reference substances were samples of L-β-lysine and streptolidine kindly provided by Prof. H. Brockman and H. Brockman Jr. (German Federal Republic).

Isolation of L-β-lysine, streptolidine and a 1, 6-anhydrohexosamine. A mixture of 4.7 g of phytobacteriomycin and 50 ml of 5.7 N HCl (twice distilled in the presence of $SnCl_2$) was heated in a tube at 105 ± 1° for 24 hr. After the completion of the hydrolysis, the solution was decolorized by shaking with activated carbon (type BAU) and evaporated to dryness at a temperature not above 40°, and the residue was dissolved in 20 ml of water, evaporated to dryness again, and dried with absolute ethanol. The last traces of HCl were removed in a vacuum desiccator in the presence of alkali. The oily hygroscopic hydrolyzate was dissolved in 20-30 ml of water, treated with the anion-exchanger Amberlite IR-45 in the OH form to pH 6-7, evaporated to dryness, and dried with absolute ethanol.

The hydrolysis products were isolated by chromatography on powdered cellulose in a 3.2 × 60 cm column. The adsorbent was powdered cellulose of the firm of Serva Entwicklungslabor (Heidelberg, German Federal Republic); the system was butanol-formic acid-water (75 : 15 : 10); the velocity was 50-70 ml/hr; the fraction volume was 17 ml; and the number of fractions was 156. Analysis was carried out by chromatographing an aliquot of every third fraction at an elevated temperature in system 1 (or system 2).

After the collection of 156 fractions, the column was washed with 500 ml of methanol acidified with formic acid. Fractions Nos. 27-36 containing the 1, 6-anhydrohexosamine, Nos. 79-122 containing L-β-lysine, and the methanolic eluate containing streptolidine were treated in the following way.

The solution was evaporated to dryness at a temperature not above 40°, the residue was dissolved in 5-10 ml of water, the resulting solution was passed through a layer of BAU-Celite 545 (1 : 1) and evaporated to dryness, and the residue was dissolved in methanol and the solution was again evaporated to dryness in the presence of absolute ethanol. Fractions Nos. 27-36 gave about 30 mg of a colorless, glassy, highly hygroscopic hydrochloride of the 1, 6-anhydrohexosamine, and fractions Nos. 79-120 and the methanolic eluate gave colorless pulverulent hygroscopic hydrochlorides of L-β-lysine and streptolidine. A chromatographic comparison of the aminoacids with authentic samples showed their complete identity. The substances obtained were used as standards for calibrating the column of the automatic aminoacid analyzer.

Quantitative analysis of the streptothricin hydrolyzates. Standard solutions of L-β-lysine, streptolidine, glucosamine, and ammonia were prepared. For this purpose, 27.37 mg (125 moles) of L-β-lysine was dissolved in 50 ml of water, and 6.525 mg of streptolidine (25 moles) in 10 ml of water. The standard solutions of glucosamine and ammonia each contained 125 moles in 50 ml of solution. Because of their high hygroscopicity, we were unable to prepare standard solutions of gulosamine and 1, 6-anhydrogulosamine.

For the quantitative analysis, weighed samples of the hydrochlorides of the streptothricins of the six types, and also streptothricin itself (6.3 mg each of streptothricin and grisemin F, 3.0 mg of grisemin E, 7.9 mg of phytobacteriomycin D, 10.2 mg of phytobacteriomycin C, 11.0 mg of polymycin B, and 13.0 mg of polymycin A) were hydrolyzed with 10 ml of 5.7 N HCl (distilled three times in heat-resistant glass over $SnCl_2$) for 24 hr at 105 ± 1° under vacuum. The hydrolyzates were evaporated to dryness in a rotary evaporator. To complete the elimination of the acid, the hydrolyzate was evaporated to dryness three times with dissolution in a small volume of water, after which it was stored in a vacuum desiccator over alkali. The hydrolyzates were dissolved in 10 ml of 0.2 N Na citrate buffer with pH 2.2. 1.0 ml of the solution prepared in this way was transferred to a column (see the next section).

For the analysis, a column 0.9 × 60 cm (working height of the resin) with an ion-exchanger of the type of Amberlite CG-120 fraction B (Evans Electroselenium Ltd., England) was used. The eluting buffer was 0.7 N Na citrate buffer with pH 5.28 ± 0.02. The rate of elution was 30 ml/hr, the rate of addition of the ninhydrin reagent 15 ml/hr, and the column was heated to 50°.

Since the hydrolyzates of the streptothricins contain no compounds capable of being eluted from the column earlier than gulosamine, the feed of ninhydrin can be switched on 1 hr 40 min after the beginning of the experiment. The whole experiment lasted 6 hr 20 min, which makes it possible to carry out another experiment overnight on a given column or to use the instrument for analysis on a 150-cm column.

All the analyses were carried out on an automatic aminoacid analyzer of type 9015/III manufactured by the experimental workshops of the Czechoslovakian Academy of Sciences.

The calculations of the compositions of the hydrolyzates were carried out in the usual way from the C constants that we found for L-β-lysine and streptolidine. To calculate the amount of gulosamine and 1, 6-anhydrogulosamine in the hydrolyzates we made use of the C constant for galactosamine (14.58) given in the handbook for the apparatus which we used.

DISCUSSION OF THE RESULTS

As is well known, the antibiotics of the streptothricin group are constructed of residues of two basic aminoacids – L-β-lysine and streptolidine – and one aminosugar, generally α-D-gulosamine (or, as in racemomycin-O, D-glucosamine). So far as concerns the ratio of these compounds in the various streptothricins, the data available in the literature relate only to streptothricin, streptolin, and racemomycin O, the structure of which can be regarded as established [8, 9]. In streptothricin and racemomycin O, this ratio is 1:1:1, and in streptolin, from the data of the American authors, 2:1:1.

In a study of the quantitative composition of the hydrolyzates of the six types of streptothricins by means of paper chromatography in the four systems of solvents, the hydrolyzates of all six antibiotics were each found to contain four ninhydrin-positive compounds which, by comparison with authentic samples of the aminoacids (L-β-lysine and streptolidine) and a streptothricin hydrolyzate and also by means of characteristic color reactions (Weber, Elson-Morgan, triphenyltetrazolium chloride), were identified as streptolidine, L-β-lysine, a hexosamine (probably α-D-gulosamine), and a 1, 6-anhydrohexosamine (in order of increasing R_f). Thus, the assumption remained that the difference between the streptothricins of all six types is due to different quantitative ratios of the residues of the three compounds mentioned. Speaking more accurately, this difference might consist in the gradual increase in the dimensions of the molecules by one or several diaminoacid residues in a similar manner to what occurs in the transition from streptothricin to streptolin. It is evident that a determination of the quantitative composition of the streptothricins of the six types had a decisive importance for establishing the structure of the new antibiotics of this group, streptothricins A, B, C, and E.

After preliminary experiments, we determined the optimum conditions for finding the number of aminoacids and carbohydrate residues in the hydrolyzates of the streptothricins in the automatic aminoacid analyzer. These conditions

are close to the method proposed by Kominz for the analysis of fairly complex mixtures of ninhydrin-positive bases also containing an aminosugar [10].

First, L β-lysine, streptolidine, and the strongly hygroscopic 1, 6-anhydrohexosamine were isolated in the form of hydrochlorides from a hydrolyzate of phytobacteriomycin by means of partition chromatography on cellulose and these were used as standards. The positions of the peaks of these compounds in the eluate from the column are shown in Fig. 1.

Hydrolyzates of representatives of the six types of streptothricins were chromatographed under the same conditions (Fig. 2). The accuracy of the method, according to published data [11], is ±3% in the range from 0.16 to 4.0 μmoles for the majority of aminoacids. Consequently, in the analysis of antibiotics containing 5-6 μmoles of L-β-lysine in an aliquot of 1 ml (for example, polymycins A and B), half-volume aliquots (0.5 ml) were used in addition to the usual charges, which made it possible to increase the accuracy of the determination. The results of the calculation of the aminoacid and carbohydrate composition of the six types of streptothricins are given in the Table.

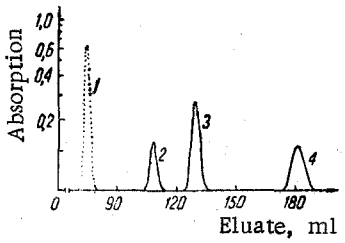


Fig. 1. Positions of the peaks of glucosamine (1), the 1, 6-anhydrohexosamine (2), L-β-lysine (3), and streptolidine (4).

Since in the preparation of the standards, the marked hygroscopicity of the 1, 6-anhydrohexosamine made it impossible to get an accurately weighed sample of it, the C constant for galactosamine (14.58), which is close to the C constant for glucosamine (15.81), was taken as an approximate evaluation of the content of sugars. This assumption is justified since on elution from the column gulosamine occupies the position characteristic of galactosamine.

For each antibiotic, a magnitude reflecting the proportion of hexosamine present in the hydrolyzate in the form of the anhydro derivative was determined. The constancy of this magnitude for all the antibiotics which we studied shows to some extent the constancy of the nature of the aminosugar. With all the streptothricins studied, practically the same ratio of hexosamine residues (total sugars) and streptolidine residues (0.6) was found, which shows the constancy of the yield

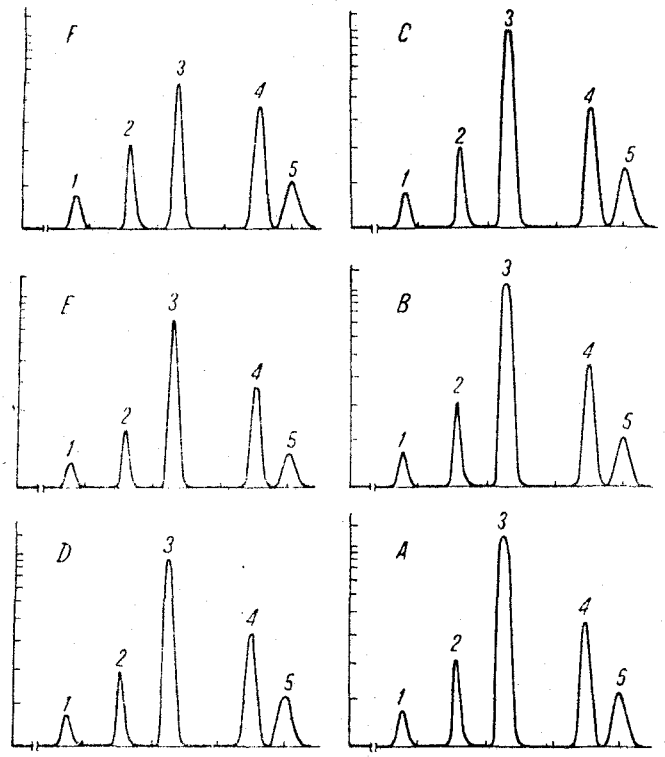


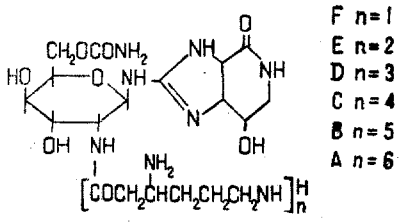
Fig. 2. Chromatographic analysis of hydrolyzates of streptothricins of types A-F: 1) α-D-gulosamine; 2) 1, 6-anhydrohexosamine; 3) L-β-lysine; 4) ammonia; 5) streptolidine. The Figure shows only one curve at 570 mμ for a cell of large dimensions.

of aminosugar in the hydrolysis of each antibiotic (about 55%). In the hydrolysis of streptothricin, which contains 1 mole of α-D-gulosamine, the yield of the latter was 54%; on this basis the conclusion was drawn that the antibiotics concerned contained one hexosamine residue.

The composition of the streptothricins of the six types given in the Table confirms the empirical formulae given

earlier and well explain the monotonic change in their properties. It is also possible to assume that the antibiotic streptolin which, judging from its properties, belongs to type D, must contain not two L-β-lysine residues, as was assumed previously by American workers [8], but three and, consequently, must have the empirical formula of not $C_{25}H_{46}N_{10}O_9$, but $C_{31}H_{58}N_{12}O_{10}$. However, a final answer to this question will be possible only after the analysis of an authentic sample of this compound.

The results obtained, taken in conjunction with published data on the arrangement of the bonds in the common fragment of the streptothricins, suggest that the structure of the six types of streptothricin antibiotics can be represented by the following structural formula



It must be noted that the literature contains individual items of information which can be regarded as confirmation of this common idea relating to the structure of the streptothricins. Thus, in 1961, in a study of the components of pleo-

Composition of the six types of streptothricin antibiotics.

Compound	Streptothricin*			F			E			D		
	1	2	3	1	2	3	1	2	3	1	2	3
L-β-Lysine	0.91	1.0	1	1.69	1.0	1	1.77	2.0	2	3.21	3.0	3
Streptolidine	0.601	0.66	1	1.08	0.63	1	0.738	0.83	1	0.91	0.86	1
Ammonia	1.88	2.06	2	1.25	0.73	1	0.633	0.71	1	0.94	0.88	1
Total sugars	0.489	0.537		0.708	0.418		0.410	0.463		0.553	0.516	
Total sugars (per mole of streptolidine)	0.81			0.66			0.56			0.60		
1,6-Anhydrohexosamine (per mole of hexosamine)	2.94			2.11			1.964			1.992		

Compound	C			B			A		
	1	2	3	1	2	3	1	2	3
L-β-Lysine	4.31	4.0	4	4.96	5.0	5	5.78	6.0	6
Streptolidine	1.14	1.05	1	0.86	0.87	1	1.04	1.07	1
Ammonia	1.05	0.97	1	1.07	1.08	1	1.07	1.11	1
Total sugars	0.625	0.580		0.609	0.613		0.656	0.680	
Total sugars (per mole of streptolidine)	0.55			0.70			0.63		
1,6-Anhydrohexosamine (per mole of hexosamine)	1.984			2.08			2.10		

* 1 – number of micromoles; 2 – calculation of the residues; 3 – rounded-off number of residues

cidin, belonging, according to our results, to types F, E, D, it was shown [12] that these three compounds differ from one another with respect to their content of L-β-lysine from 1 mole in type F pleocidin to 3 moles in type D pleocidin. In 1959, Brockman et al. discovered tri- and tetrapeptides of L-β-lysine in partial hydrolyzates of a mixture of geomycins [13].

SUMMARY

1. A method for the quantitative analysis of the hydrolyzates of streptothricin antibiotics has been developed and has been used to determine the composition of representatives of the six types of this group of substances.

2. The empirical formulae previously established for the new antibiotics have been confirmed and a proposed structure of the six types of streptothricins is discussed.

3. On the basis of the results obtained, the hypothesis put forward that the empirical and structural formula of the antibiotic streptoline proposed by American workers is incorrect. A new empirical formula is proposed for this compound.

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12 December 1964

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