

## DEHYDROALANINE RESIDUES IN THIOSTREPTON

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In the molecule of thiostrepton two L-alanine residues are present. Reduction of the antibiotic leads to the formation of additional alanine moieties.

The formation of pyruvic acid on treatment of thiostrepton<sup>1)</sup> with trifluoroacetic acid [or with hydrochloric acid was noted earlier<sup>2)</sup> and was interpreted as evidence for  $\alpha, \alpha$ -disubstituted propionic acid or acylaminoacrylic acid moieties in the molecule of the antibiotic. Such derivatives of alanine are known to occur in several microbial peptides<sup>3)</sup>. The amount of pyruvic acid isolated in the form of its 2,4-dinitrophenylhydrazone suggested that at least two residues of pyruvic acid precursors are present in the structure of thiostrepton. To determine the number and nature of these alanine derivatives, a more detailed study of the formation of pyruvic acid was undertaken. Furthermore, thiostrepton was reduced both with sodium borohydride and by catalytic hydrogenation, the reduced materials were hydrolyzed and the hydrolysates examined. These investigations revealed that in addition to the two L-alanine residues<sup>2)</sup> of thiostrepton, at least three alanine derivatives with a higher state of oxidation are constituents of the molecule.

The previously reported treatment of thiostrepton with trifluoroacetic acid<sup>2)</sup> was repeated, but this time the nmr spectra were observed in the presence of a known amount of *p*-dimethylamino benzaldehyde. This compound was chosen as an internal standard because it is stable in trifluoroacetic acid, and because of the chemical shift (3.54 ppm) of the six proton singlet of its two N-methyl groups. The position of this signal in the nmr spectrum is close to the signal of the pyruvic acid methyl protons gradually emerging at 2.62 ppm, yet does not interfere with the latter. The spectra taken from time to time confirmed the earlier observation (based on the amount of isolated pyruvic acid 2,4-dinitrophenylhydrazone), and integration of the area under the methyl proton signals gave evidence for the formation of exactly two moles of pyruvic acid from one mole of the antibiotic. After removal of the trifluoroacetic acid *in vacuo*, the nmr spectrum of the residue in deuterioacetic acid showed no more pyruvic acid. Therefore, the pyruvic acid must have been present in the reaction mixture as the free acid rather than bound as an acyl group to the rest of the molecule. A new study of the effect of trifluoroacetic acid was carried out on three model compounds:  $\alpha$ -acetylamino- $\alpha$ -hydroxy propionic acid-acetamide complex,  $\alpha, \alpha$ -diacetylaminopropionic acid, and  $\alpha$ -acetylaminoacrylic acid. From these, only  $\alpha$ -

acetylaminoacrylic acid was readily cleaved and formed pyruvic acid\*. The results of the degradation with trifluoroacetic acid suggest two dehydroalanyl residues in thiostrepton.

Because reduction with sodium borohydride gave useful information on the dehydrobutyrine residue of stendomycin<sup>4</sup>), the same approach was applied to thiostrepton. If during the reduction of the antibiotic the two above-mentioned dehydroalanine residues are reduced, amino acid analysis of the reduced material should give a total of four alanine residues: two originally present L-alanines and two D,L-alanines, newly formed. Surprisingly, instead of the expected four alanine residues, 5 to 6 moles\*\* were found per mole of the parent molecule\*\*\*.

In order to further clarify the number of alanine precursors, reduction of the antibiotic by catalytic hydrogenation in the presence of a palladium on charcoal catalyst was also attempted. Notwithstanding the high sulfur content of thiostrepton<sup>2</sup>) hydrogen was absorbed. On the other hand, the reduction did not go to completion. Amino acid analysis of catalytically reduced thiostrepton revealed about 3.8 moles of alanine, and about 0.5 mole of  $\alpha$ -aminobutyric acid. The obviously incomplete catalytic reduction could not help the determination of the number of dehydroalanine residues.

The crystallographic analysis of thiostrepton in the laboratory of Prof. HODGKIN at Oxford University resulted in a preliminary structure depicting most of the molecule<sup>5</sup>). The X-ray study led to the description of three alanine residues which are next neighbors of each other. Earlier isolation of isoleucylalanine<sup>2</sup>) and isoleucylalanine amide<sup>6</sup>) suggested that the central moiety is a dehydroalanyl rather than alanyl residue. Refinement of the X-ray data confirmed this assumption, but from the above-discussed degradation with trifluoroacetic acid, it follows that thiostrepton must also contain an additional dehydroalanine residue in the part of the molecule not yet defined by the X-ray studies. On the other hand, the results of borohydride reduction experiments suggest that not only a dehydroalanyl residue is still missing from this partial structure, but that three to four alanine derivatives with higher oxidation state are present in thiostrepton. The assumption of two dehydroalanine and one  $\alpha, \alpha$ -diaminopropionic acid residues gives an elemental composition which fits best the values of elemental analysis<sup>2</sup>) of the antibiotic.

### Experimental

Determination of the amount of pyruvic acid formed from thiostrepton. To a solution of thiostrepton (36 mg) in trifluoroacetic acid (0.5 ml) was added *p*-N, N-dimethylaminobenz-

\* This is in variance with the previous observation (cf. ref. 2).

\*\* The calculation was based on the assumption that one mole of threonine and one mole of isoleucine are present in the hydrolysate of the reduced material. This does not exclude the possibility that the latter amino acids are liberated in diminished amount, in which case the above alanine values would be too high.

\*\*\* The possibility that an alanine residue was formed from a cysteinyl moiety during reduction with sodium borohydride (*e.g.*, by elimination and reduction) has not been overlooked. Since X-ray studies (ref. 5) revealed that cysteine is present in the form of a thiazoline, in a model experiment bacitracin A was treated with sodium borohydride; no alanine formation was noted.

aldehyde (3 mg) in trifluoroacetic acid (0.5 ml). An nmr spectrum was taken immediately and spectra were taken after 1, 2, 4, 8, 12, 24 and 48 hours while the solution was kept at room temperature. A Varian 100 Mc spectrometer was used and TMS as the internal standard. The peak emerging at 2.62 ppm reached its maximum after 24 hours. The area of this peak was found by integration to be equal to that of the reference peak.

Reduction of thiostrepton with sodium borohydride. To a solution of thiostrepton (10 g) in a mixture of tetrahydrofuran (400 ml) and methanol (60 ml), sodium borohydride (5 g) was added in a single portion. After about 5 hours at room temperature, 6N HCl was added dropwise to the reaction mixture until pH 4 was shown on wet universal indicator paper. Some solid separated during addition of the acid but was not removed. The solvents were evaporated at room temperature with a stream of nitrogen and the residue dried in air. The reduced material was hydrolyzed under reflux with 6N HCl (500 ml) in an atmosphere of nitrogen for 25 hours. Amino acid analysis of the hydrolysate gave the following results: threonine 0.89, alanine 5.9,  $\alpha$ -aminobutyric acid 1.0, isoleucine 1.0,  $\text{NH}_3$  3.2. Thiostrepton itself shows the following analysis: threonine 0.90, alanine 1.99, isoleucine 1.0.

Isolation of alanine from borohydride-reduced thiostrepton and determination of its specific rotation. Alanine was isolated from the hydrolysate of the sodium borohydride reduced thiostrepton in a manner identical to that described earlier<sup>4)</sup> for the isolation of  $\alpha$ -aminobutyric acid. Fractions no. 25 to 28 from the cation exchange column were pooled and evaporated to dryness. The residue was dissolved in ethanol and treated with pyridine. The white crystalline precipitate (0.305 g) was collected, washed with alcohol and dried. On paper chromatograms and thin-layer chromatograms it was indistinguishable from alanine and was identified by ir and nmr spectra.  $[\alpha]_D^{25} +4.20^\circ$  (c 2, 5N HCl). Lit.<sup>7)</sup> (for L-alanine)  $[\alpha]_D^{25} +14.6^\circ$  (c 2, 5N HCl).

Catalytic hydrogenation of thiostrepton. A palladium on charcoal catalyst (2 g) was suspended in a mixture of tetrahydrofuran (20 ml), absolute ethanol (20 ml) and acetic acid (2 ml) and the suspension was stirred in an atmosphere of hydrogen until no more absorption of the gas was observed. Thiostrepton (1.8 g) was dissolved in a mixture of tetrahydrofuran (80 ml), absolute ethanol (30 ml) and acetic acid (2 ml), and added dropwise to the above suspension of the catalyst. The hydrogenation was continued at room temperature, in a closed system for 36 hours at which time fresh palladium on charcoal catalyst (1 g) was added to the reaction mixture: (The system was carefully flushed with nitrogen before the catalyst was added). The hydrogenation was continued an additional 12 hours at room temperature, the catalyst was removed by filtration and the solvents were evaporated at room temperature under a stream of nitrogen. Amino acid analysis of a hydrolysis sample of the hydrogenated antibiotic gave the following values: threonine 0.93, alanine 2.4,  $\alpha$ -aminobutyric acid, a small amount, isoleucine 1.0,  $\text{NH}_3$  8.2.

In a subsequent experiment the hydrogenation was continued for 7 weeks at room temperature. Several additions of fresh catalyst were made to a total of 9 g. Amino acid analysis of a hydrolysis sample of this reduced antibiotic gave the following values: threonine 1.0, alanine 3.8,  $\alpha$ -aminobutyric acid 0.5, isoleucine 0.9,  $\text{NH}_3$  6.4.

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