TWO DIASTEREOISOMERIC α, β-DIAMINOBUTYRIC ACIDS FROM AMPHOMYCIN

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(Received for publication February 2, 1970)

Acid hydrolysis of the peptide antibiotic amphomycin liberates a group of five basic amino acids. The major components of this mixture were isolated in pure form and identified as L-*threo*-α, β-diaminobutyric acid and D-*erythro*-α, β-diaminobutyric acid. Both occur in the parent molecule. The basic amino acids present in minor amounts are also discussed.

In the first paper on amphomycin HEINEMANN, KAPLAN, MUIR and HOOPER[1] reported the presence of ninhydrin-positive components after hydrolysis. The weak ninhydrin reaction of the antibiotic itself was overlooked. In an early communication on crystallomycin by LOMAKINA and BRAZHNIKOVA[2] a C_7 basic amino acid was reported as building component and the similarity or perhaps identity of crystallomycin with amphomycin was suggested. This might be the explanation for the erroneous statement of SHOJI, KOZUKI, OKAMOTO, SAKAZAKI and OTSUKA[3] that amphomycin does not contain α, β-diaminobutyric acid. The isolation, however, of a mixture of diastereoisomeric α, β-diaminobutyric acids from amphomycin was reported recently from this laboratory[4]. A more detailed investigation had to be undertaken to clarify the absolute configuration of the individual α, β-diaminobutyric acids and to examine whether or not both occur in the molecule of the antibiotic or one of them is the product of racemization during hydrolysis.

In this connection it should be noted that two diastereoisomeric α, β-diaminobutyric acids were obtained from aspartocin by HAUSMANN, BORDERS and LANCASTER[5]. Their elegant elucidation of the stereochemistry of these acids rests in part on an assumed preferred conformation due to the repulsion of identically charged groups. The α, β-diaminobutyric acid pair from amphomycin was isolated by us in pure form and they were found to be identical with the corresponding amino acids from aspartocin. The assignment of configurations in this case was based on experimental evidence and confirmed the findings of HAUSMANN and his associates[5]. The ninhydrin values in quantitative amino acid analysis[6] were established for the individual isomers. Thus it became possible to show that both occur, one mole of each, in the parent molecule. In the aspartocin studies[5] some of these problems were left open.

Interestingly, two moles of α, β-diaminobutyric acid were found also in glumamycin and according to FUJINO, INOUE, UEYANAGI and MIYAKE[7] both have D-*erythro* configuration. In view of our investigations, which also include the determination of the specific rotation of the individual diastereoisomers, the assignment of configuration

by FUJINO, *et al.*[7], needs to be revised. The specific rotation of the amino acid isolated by them is also that of a mixture of the L-*threo* and D-*erythro* isomers.

On amino acid analysis of amphomycin five basic components appear on the short column of the Beckman Spinco-120C amino acid analyzer[6]. Counting from the position of the ammonia peak these appear at −11, −9, −7, +5 and +10 minutes, and were named provisionally as −11, −9, *etc.*, compounds. Subsequently, the −9 component was identified as L-*threo*, the −7 material as D-*erythro*-α,β-diaminobutyric acid.

A large sample of amphomycin (Ca salt)* was hydrolyzed with constant boiling hydrochloric acid. After removal of the solvents and of the fatty acids[8] the mixture of amino acid hydrochlorides was chromatographed[4] on a Dowex 50W-X12 column and the fractions containing the basic amino acids were combined. Countercurrent distribution of this material in a system of butanol - 0.001 N hydrochloric acid through 60 transfers led to almost no separation of the basic amino acids, but evaporation of the solvent from the contents of the main band and addition of ethanol to the concentrate resulted in the crystallization of a considerable part of the α,β-diaminobutyric acid. Elemental analysis revealed that the crystals are the monohydrochloride monohydrate of the basic amino acid, like in the case of the material obtained from aspartocin by MARTIN and HAUSMANN[9] except that the specific rotation of the product from amphomycin was somewhat lower (+14.2°) than that of the crystals from aspartocin (+16.6°)[9]. The nmr spectrum of our product on comparison with the spectra reported by HAUSMANN and his associates[5] revealed that an about equimolar mixture of the *threo* and *erythro* isomers was at hand. The mixture was oxydized with hydrogen peroxide according to DAKIN[10] under the conditions applied in ref. 7. Ion exchange chromatography of the degradation mixture yielded D-alanine, thus establishing the D-configuration for the β-center. Accordingly, the crystalline mixture consists of about equal amounts of the L-*threo*- and D-*erythro*-α,β-diaminobutyric acid.

The mother liquor of the above crystallization was rechromatographed on a Dowex 50W-X12 column with hydrochloric acid used in increasing concentration for elution. A peak containing the L-*threo* isomer separated from a second peak in which the D-*erythro* compound was still accompanied by the "minor" basic amino acids −11, +5 and +10. From the first band L-*threo*-α,β-diaminobutyric acid was secured by crystallization as the monohydrochloride monohydrate. The material from the second peak was subjected to countercurrent distribution in the system butanol - acetic acid - water (4 : 1 : 5) through 2,440 transfers. The separation of D-*erythro*-α,β-diaminobutyric acid was achieved this way, but the minor components remained in one group. The isolation of these "minor" basic amino acids is being attempted and hopefully will be reported separately. It may be interesting to note that the same basic amino acids, in about the same proportion, can be found in a hydrolysate of aspartocin**.

The samples of the individual diastereoisomers of α,β-diaminobutyric acid were sufficient for their characterization. In addition to analytical values and ninhydrin

* The authors express their gratitude to Dr. L. SZABO of H. Lundbeck and Co., Copenhagen-Valby, Denmark, for the generous sample of the antibiotic.

** The authors thank Dr. E. E. PATTERSON of Lederle Laboratories, Pearl River, New York, for the sample of aspartocin used for this comparison.

values[6], the optical rotatory dispersion curves were taken to firmly establish the configuration at the α-carbons[11]. The ORD spectra were in harmony with the previous conclusion, that the −9 compound is an L-amino acid, while the material with −7 minutes elution time has the D-configuration.

The presence in the hydrolysate of two diastereoisomers does not answer the question about the occurrence of both diastereisomers or of only one of them in the antibiotic: the second could result from racemization during hydrolysis. The same question was raised by HAUSMANN and his co-workers[5] for aspartocin. Clarity was brought into this picture through racemization experiments. A sample of synthetic *erythro* diaminobutyric acid*** was exposed to the hydrolysis conditions used for quantitative amino acid analysis (6 N HCl, 110°C, 16 hours). About 18 % of the *erythro* isomer was converted to the *threo* form. Obviously this degree of racemization cannot explain the presence of the two diastereoisomers in about equal amounts in the hydrolysate of the antibiotic, and therefore both must be constituents of the parent molecule. The isolation of D-alanine from the DAKIN degradation of the equimolar mixture of diastereoisomers indicated that during hydrolysis no racemization took place on their β-carbon atom. Nonetheless, a second racemization experiment was carried out to ascertain that only the α-center is racemized. (Otherwise the optical purity of the isolated diastereoisomers would be questionable.) The pure isomers were exposed to 6 N DCl (in D_2O) at 110°C for about a week. The nmr spectra reveal complete exchange of the α-proton to deuterium (disappearance of the doublet at 4.60 ppm in the case of the *threo* and at 4.49 ppm in the case of the *erythro* compound). The methyl signals, a pair of doublets, of both isomers were present with almost equal areas in both spectra, thus demonstrating that no exchange and therefore no racemization occurred at the β-carbon.

Deamination experiments[7] of amphomycin proved that the β-amino group of D-*erythro*-α, β-diaminobutyric acid residue is free, while both amino groups of the *threo* isomer are acylated in the antibiotic.**** The structure of the "minor" basic amino acids is still uncertain, but the nmr spectrum of their mixture shows that an isopropyl group is present in two of them.

Experimental

Hydrolysis of amphomycin and preliminary separation of amino acids. The Ca-salt of the antibiotic (10.0 g) was hydrolyzed as described earlier[8]. After extraction of the hydrolysate with ether, the aqueous layer was evaporated to dryness. A preliminary separation[4] of the acidic, neutral and basic amino acids was carried out with a Dowex 50W-X12 column (4×30 cm) in H^+ cycle. The basic amino acids were eluted when the concentration of the HCl was raised to 2 N (cf. Fig. 3 in ref. 4). Evaporation of the appropriate fractions left a residue of 4.3 g.

Attempted countercurrent distribution of the basic amino acids. The mixture of basic amino acids (hydrochlorides) was placed into the first 3 tubes of a 60 tube CRAIG apparatus

*** The authors thank Dr. JOHANNES MEIENHOFER of the Children's Cancer Research Foundation, Boston, Massachusetts, for this synthetic material.

**** In our interpretation of the data of ref. 7 glumamycin, too, contains one mole L-*threo*- and one mole D-*erythro*-α, β-diaminobutyric acid residues, the β-amino group of the latter being free.

(10 ml phases) and distributed in the solvent system *n*-butanol - 0.001 N HCl through 160 transfers (single withdrawal). A weight curve indicated that almost all the material was in tubes 1~13. Quantitative amino acid analysis showed little difference in composition of the individual tubes.

α, β-Diaminobutyric acid (mixture of *threo* and *erythro* isomers). The contents of tubes 1~13 were pooled, the solvent removed with a stream of nitrogen; the residue, a syrup, was dissolved in small volume of aqueous ethanol and absolute ethanol was added until a permanent turbidity was reached. After crystallization started gradually more absolute ethanol was added, to a final volume of about 50 ml. The crystals were collected, washed with ethanol and dried in air (1.0 g). Part of the crystalline material (0.80 g) was dissolved in water (4 ml) and ethanol (16 ml) was added while warming the solution and seeding; crystals appeared. The purified product was filtered, and washed with a mixture of ethanol (16 ml) and water (4 ml) and finally with absolute ethanol (30 ml). The air dry material (0.50 g) sinters at 180°C and melts with dec. 193~194°C. $[\alpha]_D^{25}$ +14.2° (*c* 1.34, 5 N HCl).

Anal.: Calc'd for $C_4H_{10}N_2O_2 \cdot HCl \cdot H_2O$: C 27.84, H 7.59, N 16.24, Cl 20.54.
Found: C 27.84, H 7.54, N 16.02, Cl 20.39.

A second crop was obtained on dilution of the mother liquor and washings with absolute ethanol (50 ml): 0.18 g, with same m.p. as the first crop.

Separation of the basic amino acids by ion exchange chromatography. The mother liquors of the crystallization (cf. previous paragraph) were pooled and evaporated to a syrup. This was dissolved in water (30 ml) and applied to a column (4×43 cm) of Dowex 50-X12 cation exchange resin in H^+ cycle. Dilute hydrochloric acid was used for elution, the concentration of the acid was gradually increased from 1.2 N to 2.4 N. Fractions of 200 ml were collected. A peak appeared when the concentration of hydrochloric acid reached 1.8 N. The residue weight of the individual fractions dropped to almost zero after 15 fractions taken at this HCl concentration. A second peak was then eluted with 2.1 N HCl.

L-*Threo*-α, β-diaminobutyric acid (monohydrochloride-monohydrate). The first peak contained inorganic material and L-*threo*-α, β-diaminobutyric acid, identified by its nmr spectrum. The evaporation residue was dissolved in a minimum volume of water, diluted with absolute ethanol and neutralized with about 1 ml of pyridine. The crystals were collected and washed with absolute ethanol (0.23 g). Recrystallization from water (1 ml) and ethanol (10 ml) gave the monohydrochloride-monohydrate of the pure isomer (0.15 g). M.p. 213~214°C (dec.). $[\alpha]_D^{25}$ +27.1° (*c* 2, 5 N HCl) (or calculated for anhydrous monohydrochloride +30.7°).

Anal.: Found: C 28.11, H 7.68, N 16.28, Cl 20.67, loss of wt. on drying *in vacuo* at 110°C 12.0 %, calc'd for one mole H_2O: 10.5 %.

D-*Erythro*-α, β-diaminobutyric acid monohydrochloride monohydrate. The material from the second peak of the ion exchange chromatogram (eluted with 2.1 N HCl, cf. above), was shown by quantitative amino acid analysis[6] to contain the basic amino acids of amphomycin with the exception of the −9 component. Countercurrent distribution of this mixture (0.69 g) was carried out in a 520-tube CRAIG apparatus with 3 ml phases of the solvent system *n*-butanol - acetic acid - water (4 : 1 : 5). The mixture was placed into the first two tubes and distributed through 600 transfers and then by recycling through additional 1,840 transfers. The α, β-diaminobutyric acid, which was the main component, was removed from a band corresponding to a distribution coefficient of 0.12. A faster moving band contained the "minor" basic amino acids. Quantitative amino acid analysis (short column[6]) was used for detection.

The solvent was removed with a stream of nitrogen from the α, β-diaminobutyric acid containing fractions and the residue treated with ethanol. The white precipitate was collected, washed with ethanol and dried (0.23 g). Analysis indicated that it consisted of

about 90 % of monoacetate of the basic amino acid and only of about 10 % of the hydrochloride (Obviously the chloride ions were gradually displaced by acetate ions from the solvent). A part (58 mg) of the product was dissolved in N HCl (0.33 ml), the solution diluted with absolute ethanol (5 ml) and pyridine (1 drop). Nice needles formed. The crystals were dried at room temperature over P_2O_5 (54 mg). M.p. 200°C (dec.). $[\alpha]_D$ −9° (*c* 2.44, 5 N HCl).

Anal.: Found: C 28.19, H 7.57, N 16.31, Cl 20.74.

Ninhydrin values. Samples of the pure diastereoisomers were applied to the short column of the Beckman-Spinco 120C amino acid analyzer[6]. The value (C_{HW}) for one μmol of *threo*-α,β-diaminobutyric acid was found to be conspicuously small: 7.2. The *erythro* isomer gave a some what higher value, 21, but still small when compared, *e. g.* with that for lysine (72), determined under the same conditions.

ORD spectra were taken on a Carey 60 spectropolarimeter in 1.5 N HCl and were found to be similar to those described by HAUSMANN and his associates[5]. The molar rotation at the peak (225 mμ) of L-*threo*-α,β-diaminobutyric acid is 2,200° and at the trough (225 mμ) of D-*erythro*-α,β-diaminobutyric acid is −1,900°.

Racemization experiments.

(a) A sample (30 mg) of synthetic *erythro*-α,β-diaminobutyric acid was dissolved in 6 N HCl (about 1 ml) and kept in an evacuated sealed ampoule at 110°C for 16 hours. After evaporation of the hydrochloric acid the residue was dissolved in 2 N DCl and the nmr spectrum recorded on a Varian A60 instrument. Integration values of the methyl peak showed that 18 % of the starting material was converted to the *threo* isomer.

(b) The pure *threo* isomer (30 mg) was dissolved in about 0.5 ml 6 N DCl and the nmr spectrum recorded. After heating the sample in an evacuated sealed ampoule at 110°C for a week the nmr spectrum was again recorded. The doublet at 4.60 ppm corresponding to the α-protons disappeared on heating. The experiment repeated with a sample of the *erythro* isomer from amphomycin lead similarly to the disappearance of the signal of the α-protons at 4.49 ppm. In both cases the β-protons gave a multiplet centered at 4.15 ppm while the methyl-proton doublets of both isomers were present after heating.

DAKIN degradation of the mixture of the diastereoisomeric α,β-diaminobutyric acids from amphomycin. The crystalline material from the short countercurrent distribution (0.42 g) was oxidized with H_2O_2 as described in ref. 7. The alanine isolated by ion exchange chromatography and crystallization had $[\alpha]_D^{25}$ −13° (*c* 1.6, 5 N HCl); Reported for L-alanine[12] $[\alpha]_D^{25}$ +14.6° (*c* 2, 5 N HCl).

Acknowledgements

The authors thank Mr. J. ALICINO for the microanalyses and Mrs. DELORES J. GAUT for the amino acid analyses. This work was supported by a grant from the U. S. Public Health Service (NIH No. AI-07515-04).

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