CONFIGURATION OF THE β-METHYLASPARTIC ACID RESIDUE IN AMPHOMYCIN

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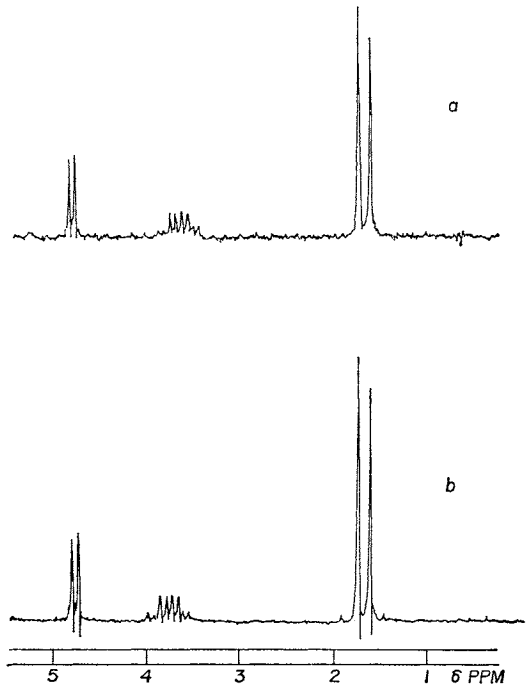
The β-methylaspartic acid residue in amphomycin has the L-*threo* configuration. Since β-methylaspartic acids are readily racemized under the usual conditions of acid hydrolysis, the assignment of configuration was based on β-methylaspartic acid liberated under exceptionally mild acid hydrolysis.

The presence of β-methylaspartic acid among the constituents of the peptide antibiotic amphomycin[1] was already reported[2]. The studies here described were aimed at the configuration of the β-methylaspartic acid residue in the antibiotic.

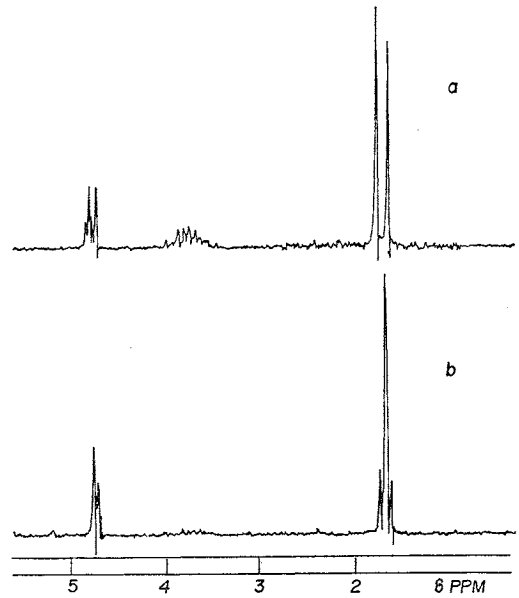
Hydrolysis of amphomycin with constant boiling hydrochloric acid under reflux for 16 hours results in a mixture of fatty acids[3] and amino acids. The separation of these constituents was described earlier.[2] The first major peak from the ion-exchange chromatogram (cf. Fig. 3 in ref. 2) consists of aspartic acid and β-methylaspartic acid, as indicated by comparison on paper chromatograms with authentic samples and by the n.m.r. spectrum of the mixture. For a more detailed study this mixture was rechromatographed on a column of Dowex 50W-X12 in H^+ cycle; 0.36 N hydrochloric acid was used for elution. The aspartic acid-β-methylaspartic acid containing eluent was collected in six fractions. The last of these contained only β-methylaspartic acid and no aspartic acid. The specific rotation of this material ($[\alpha]_D^{25}$ +13.9° (*c* 2, 5 N HCl)) was in excellent agreement with the value reported in the literature[4] for L-*threo*-β-methylaspartic acid. The n.m.r. spectrum is shown in Fig. 1a. An examination of the n.m.r. spectra of all six fractions and comparison with the spectra of authentic samples revealed, however, that the earliest

Fig. 1. 60 MHz n.m.r. spectrum (in 2 N DCl) of a) L-*threo*-β-methylaspartic acid; and b) L-*erythro*-β-methylaspartic acid.



fractions contain—in addition to aspartic acid—the *erythro* rather than the *threo* isomer. Integration of the appropriate areas, with consideration of the amounts in individual fractions, allowed the conclusion that aspartic acid and β-methylaspartic acid are present in a 3 : 1 ratio in the hydrolysate and that the β-methylaspartic acid is a mixture of about equal amounts of the *threo* and *erythro* isomers. The specific rotations of the individual fractions excluded the D-isomers. Separation of β-methylaspartic acid from aspartic acid in the first two fractions by countercurrent distribution and examination of the n.m.r. (Fig. 1b) and ORD spectra unequivocally proved that the L-*erythro* isomer was in hand. Two possibilities remained: 1) L-*threo*- and L-*erythro*-β-methyl- aspartic acid are both constituents of amphomycin; or 2) one of the diastereoisomers is formed by racemization during hydrolysis.

Fig. 2. 60 MHz n.m.r. spectrum of *erythro*-β-methylaspartic acid after heating for 24 hours at 110°C in a) 6 N HCl; and b) 6 N DCl.



In order to test the second mentioned possibility a sample of synthetic DL-*threo*-methylaspartic acid* was exposed to the conditions of acid hydrolysis (6 N HCl, 110°C, 24 hours). The n.m.r. spectrum of the product (Fig. 2a) in 2 N DCl when compared with the spectra of the starting material and of DL-*erythro*-β-methylaspartic acid (Fig. 1b) showed that complete racemization took place. The same extent of racemization was observed when the *erythro* isomers were treated similarly. When the experiment was repeated with 6 N deuterium chloride, the n.m.r. spectrum (Fig. 2b) revealed that the β-proton was completely exchanged to deuterium. Hence, it is the β-center which is racemized during hydrolysis.**

In preliminary experiments[2], a mixture of aspartic acid and β-methylaspartic acid was isolated in crystalline form. The n.m.r. spectrum of this mixture in conjunction with the specific rotation of the material led to the conclusion that the β-methylaspartic acid is the L-*threo* isomer. Yet, it should be noted that in the process of crystallization the more soluble *erythro* isomer was probably left in the mother liquors and therefore the finding of only the *threo* isomer could have been misleading. From the racemization experiments described earlier in this paper it is obvious that both the *erythro* and *threo* isomers were present in the hydrolysate from which the *threo* compound separated. Only hydrolysates prepared under mild conditions which do not lead to significant racemization can give unequivocal information in this

* The procedure of BENOITON, BIRNBAUM, WINITZ and GREENSTEIN[6] was applied for the synthesis of DL-β-methylaspartic acid. However, in contrast to the observations of these authors, the *threo* and *erythro* isomers could be separated by crystallization from water.

** When aspartic acid was heated in 6 N DCl at 110°C for 24 hours, complete exchange of the β-protons occurred; the n.m.r. spectrum consists of a singlet corresponding to the α-protons.

respect. Hydrolysis with 0.25 N acetic acid[6] represents such mild conditions. Also, degradation with 6 N hydrochloric acid on a steam bath for 8 hours was sufficient to liberate most of the β-methylaspartic acid from the antibiotic. Model experiments showed that under these conditions racemization of β-methylaspartic acid does not take place to an extent which would interfere with the assignment of configuration.

When amphomycin was hydrolyzed with 0.25 N acetic acid or with 6 N hydrochloric acid under the conditions described above, isolation of the aspartic acid-β-methylaspartic acid mixture and examination of the n.m.r. spectrum gave convincing evidence that the β-methylaspartic acid residue in amphomycin has the L-*threo* configuration.

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Experimental

Nuclear magnetic resonance spectra were taken on a Varian A-60A spectrometer in 2 N DCl with external TMS as reference. Optical rotatory dispersion spectra were observed on a Cary 60 spectropolarimeter in 1 cm cells at a concentration of 0.1 % in 0.5 N HCl. Optical rotations were measured on a Perkin-Elmer Model 141 automatic polarimeter in 1 dm cells, in 5 N HCl.

Hydrolysis of Amphomycin and Separation of Acidic Amino Acid Residues. L-*threo*-β-Methylaspartic Acid. Amphomycin calcium salt (15 g) was dissolved in 6 N HCl (500 ml) and heated under reflux for 16 hours. After extraction of the fatty acids the solution was evaporated to dryness. The residue was redissolved in a small amount of water and applied to a column of Dowex 50W-X12 (H^+ cycle; 4 cm × 35 cm). After preliminary separation (cf. ref. 2) the fractions containing aspartic acid and methylaspartic acid were combined and rechromatographed on an identical column. The column was eluted with 0.36 N HCl in 200 ml fractions. Aspartic acid and β-methylaspartic acid were detected in fractions 23~28. The n.m.r. spectra showed that fractions 23, 24 and 25 contained aspartic acid and *erythro*-β-methylaspartic acid, fraction 26 consisted of aspartic acid and a mixture of *threo*- and *erythro*-β-methylaspartic acid, fraction 27 contained aspartic acid and *threo*-β-methylaspartic acid, while in fraction 28 (0.19 g) only *threo*-β-methylaspartic acid (cf. also Table 1) was found. The material from fraction 28 was crystallized from ethanol with the addition of some pyridine: 152 mg. On thin-layer plates of silica gel in a system of butanol – acetic acid – water (3 : 1 : 1) the *threo* isomer travels with an Rf value of 0.16. $[\alpha]_D^{24}$ +13.9° (*c* 2, 5 N HCl). Lit.[4] $[\alpha]_D^{24}$ +14.2° (*c* 2, 5 N HCl). The ORD spectrum corresponds to that of an L-amino acid (cf. Ref. 7). $[\alpha]_{228}^{24}$ +1000°.

Anal.: Calc'd for $C_5H_9NO_4$: C 40.8, H 6.2, N 9.5.
Found: C 40.5, H 6.0, N 9.4.

Separation of Aspartic Acid and L-*erythro*-β-Methylaspartic Acid by Countercurrent Distribution. Fractions 23 and 24 (cf. above) were combined and the components separated by

Table 1. Composition of chromatographic fractions

Fraction Number	Total Weight	$[\alpha]_D$	Per cent [a]		
			Aspartic acid	*e*-MeAsp [b]	*t*-MeAsp [c]
23	0.18 g	+20.3°	67	33	—
24	0.76 g	+19.4°	67	33	—
25	1.53 g	+16.9°	84	16	—
26	2.08 g	+15.6°	84	8	8
27	1.44 g	+13.6°	65	—	35
28	0.19 g	+11.1°	—	—	100

a) calculated from integration of the n.m.r. spectra
b) *erythro*-β-methylaspartic acid
c) *threo*-β-methylaspartic acid

countercurrent distribution. A system of *n*-butanol – acetic acid – water (4 : 1 : 5) was used in a 520 tube CRAIG apparatus with three milliliters each of upper and lower phase. After 530 transfers the apparatus was set to recycle. The distribution was continued for a total of 5,000 transfers, after which a weight curve showed good separation of the two components. Aspartic acid was recovered from tubes 60~103 (K=0.14) while the β-methylaspartic acid was found in tubes 150~220 (K=0.16). The distribution yielded 171 mg of L-*erythro*-β-methylaspartic acid. On thin-layer chromatograms (for condition cf. above) Rf=0.14. $[\alpha]_D^{24}$ +35.0° (*c* 2, 5 N HCl). Lit.[4] $[\alpha]_D$ +42.5° (*c* 2, 3 N HCl). The ORD spectrum corresponds to that of an L-amino acid (cf. Ref. 7). $[\alpha]_{224}^{24}$ +1490°.

Racemization of β-Methylaspartic Acid. DL-*threo*-β-Methylaspartic acid (30 mg) was dissolved in 6 N HCl (1.5 ml) and heated in a sealed ampoule at 110°C for 16 hours. After cooling the solution was evaporated to dryness, redissolved in water, and once again evaporated to dryness. The n.m.r. spectrum of this material in 2 N DCl (Fig. 2a) showed both isomers in about equal amounts. The same result was obtained when the experiment was repeated with DL-*erythro*-β-methylaspartic acid.

Deuterium Exchange of β-Methylaspartic Acid. A mixture of DL-*threo*- and DL-*erythro*-β-methylaspartic acid (30 mg) was dissolved in 6 N DCl (1.5 ml) and heated in a sealed ampoule for 16 hours at 110°C. After cooling, the solution was evaporated to dryness. The n.m.r. spectrum of the residue in 2 N DCl (Fig. 2b) revealed the practically complete exchange of the β-proton to deuterium.

Partial Hydrolysis of Amphomycin with 0.25 N Acetic Acid. A sample of amphomycin (0.75 g) was dissolved in 0.25 N acetic acid (250 ml) and heated under reflux for 16 hours. After cooling, the solution was extracted with ether and ethyl acetate (two 100 ml portions of each) to remove the fatty acids.[3] The water layer was applied to a column of Dowex 1-X8 resin (2.5 cm × 40 cm ; acetate cycle). After elution of the basic and neutral amino acids and peptides with water, aspartic acid and β-methylaspartic acid were eluted with 2 N acetic acid. The n.m.r. spectrum of this material (0.16 g) showed that it was a mixture of aspartic acid and *threo*-β-methylaspartic acid.

Partial Hydrolysis of Amphomycin with 6 N HCl. A sample of amphomycin (1.5 g) was dissolved in 6 N HCl (100 ml) and heated on a steam bath. At two-hour intervals paper chromatograms were run to monitor the release of β-methylaspartic acid. After eight hours, the solution was removed from the steam bath and evaporated under vacuum. The residue was redissolved in water (50 ml) and the resulting solution extracted with ether and ethyl acetate (two 50 ml portions of each). The acidic amino acids (0.23 g) were separated by ionexchange chromatography as described in the preceding paragraph. The n.m.r. spectrum of the mixture showed aspartic acid and *threo*-β-methylaspartic acid.

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