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γ-Glutamyl and D- or L-Peptide Linkages in Mycobacillin, a Cyclic Peptide Antibiotic

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Mycobacillin lacks amino groups but contains two free α-carboxyl groups, indicating the presence of two side-chain peptide linkages. The five aspartic acid residues of mycobacillin are all in α-peptide linkage whereas the two glutamic acid residues are in γ-linkage. Mycobacillin does not react with hydroxylamine to give hydroxamate, indicating the absence of anhydride, lactone and ester linkages. This is also confirmed by i.r. spectroscopy and titration of the molecule. Of the 15 peptides obtained from partial hydrolysates of mycobacillin, 12 contain aspartic acid. Results obtained by treatment of hydrolysates of aspartic acid-containing peptides with D-amino acid oxidase and L-glutamate decarboxylase (containing L-aspartate decarboxylase activity) indicate that residue 5 is L-aspartic acid and residues 2, 8, 11 and 13 are D-aspartic acid. The D- or L-peptide sequence and nature of peptide linkages in mycobacillin are proposed on the basis of these findings and the amino acid sequence reported earlier.

It is now generally accepted that the predominant chemical bond in proteins is the α-peptide linkage. Studies of naturally occurring small peptides and peptide antibiotics indicate that some of these have peptide linkages involving the side chains of amino acids, e.g. glutathione (Kendall, 1929), bacitracin (Swallow & Abraham, 1959) and peptides from human urine (Buchanan, Haley & Markiw, 1962). Also other bonds, such as ester, anhydride or lactone, are found between adjacent parts of the same or different polypeptide chains; e.g. peptide antibiotics like actinomycin (Brockmann, 1960), telomycin (Sheehan *et al.* 1963) and staphylomycin factor S (Vanderhaeghe & Parmentier, 1960) are characterized by the presence of lactone linkages. In addition to these, peptide antibiotics also contain some D-amino acids, e.g. bacitracin (Swallow & Abraham, 1959) and tyrocidine (Paladini & Craig, 1954).

Mycobacillin, obtained from culture filtrates of *Bacillus subtilis* B_3 (Majumder & Bose, 1958), is an antifungal cyclic peptide antibiotic with 13 residues of seven different amino acids whose sequence irrespective of D- or L-configuration has been worked out (Majumder & Bose, 1960*a*).

It contains four residues of D-aspartic acid, two of D-glutamic acid, two of L-tyrosine and one each of L-aspartic acid, L-serine, L-leucine, L-alanine and L-proline (Banerjee & Bose, 1963). In the present paper attempts have been made to determine the nature of the peptide linkages, the presence of any side-chain bonds, such as ester, lactone or anhydride, and the sequential arrangement of stereoisomers of amino acids in the molecule.

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MATERIALS AND METHODS

Materials. Mycobacillin was isolated from the culture broth of the producer strain *B. subtilis* B_3 (Majumder & Bose, 1960*b*). Poly-α-L-aspartic acid, α-L-glutamyl-L-alanine and γ-hydroxy-α-aminobutyric acid, L-glutamic acid, D-amino acid oxidase (hog kidney), catalase and FAD were purchased from Sigma Chemical Co., St Louis, Mo. U.S.A. Glutamate decarboxylase from *Clostridium welchii* was also purchased from Sigma Chemical Co. This preparation which also contains L-aspartate decarboxylase activity was tested on D-, L- and DL-glutamic acid and -aspartic acid and also on the other constituent L-amino acids of mycobacillin. The decarboxylation is strictly stereospecific and the preparation does not attack any other amino acids of mycobacillin except L-aspartic acid, which is converted into L-alanine. γ-Amino-δ-hydroxyvalerate was prepared from L-glutamic acid by the method of Chibnall, Haselbach, Mangan & Rees (1958). Hydrazine hydrate (80%), obtained from E. Merck A.-G., Darmstadt, Germany, was further distilled to 99% purity by the method described by Locker (1954).

Assay of α-carboxyl groups. The assay is based on the method of Barakat, Elwahab & Elsadr (1955), further extended by Chappelle & Luck (1957) for quantitative decarboxylation of carboxyl groups of proteins and peptides.

The suspension of 10% (w/v) *N*-bromosuccinimide (0.5ml) and 10% (w/v) succinimide in 1M-sodium acetate–acetic acid buffer, pH4.7, (1.5ml) was pipetted into the side arm and 0.5ml of a 40% (w/v) KI solution was added to the inner well of a Warburg flask. Crystalline mycobacillin (3.2μmol in 0.5ml) in 1M-sodium acetate–acetic acid buffer, pH4.7, and $PdCl_2$ (10μmol in 0.2ml) solution were added to the main compartment of the flask. The total volume of solution added was 3.2ml. After equilibration for 20min at 37°C, the reaction was initiated by tipping the *N*-bromosuccinimide suspension into the reaction compartment. The amount of CO_2 evolved was determined manometrically.

Sodium hypobromite oxidation of mycobacillin. The method is based on the observation by Haurowitz & Bursa (1949) that glutamic acid linked through γ-linkage in a peptide yields succinic acid after oxidation with sodium hypobromite and subsequent hydrolysis.

Mycobacillin (0.2g) was oxidized at 0°C with 24ml of 1% (v/v) bromine in 0.5M-NaOH. After 5min the mixture was acidified with acetic acid, and 2g of KI was immediately added. The volume of the reaction mixture was decreased to 4–5ml by evaporation *in vacuo*. After the solution had been adjusted to pH3.0 with 2M-HCl it was extracted repeatedly with peroxide-free ether. The residue was hydrolysed with 2vol. of conc. HCl under reflux for 4h. The hydrolysate was evaporated to a small volume and re-extracted with ether. The second ether extract was evaporated to dryness and the residue dissolved in a small volume of water and subjected to paper chromatography with water-saturated butan-1-ol–formic acid (5:1, v/v) as solvent system.

Hydrazinolysis of mycobacillin and identification of aspartyl- and glutamyl-hydrazide. Peptides and proteins on hydrazinolysis yield corresponding amino acid hydrazides except for the *C*-terminal residue. Mycobacillin (10mg) was introduced into a tube with a partly drawn out neck and dried overnight in a vacuum desiccator. Anhydrous hydrazine (2ml) was added to the tube, which was immediately sealed and heated for 8h at 100°C. The excess of hydrazine was then evaporated in a vacuum desiccator over H_2SO_4 and the residue was dissolved in water, shaken for 10h at 0°C with Amberlite IRC50 (H^+ form), decanted and filtered. This process was repeated three times. The filtrate, containing hydrazides of aspartic acid and glutamic acid, was concentrated *in vacuo* and then spotted on Whatman no. 1 filter paper and subjected to paper electrophoresis. For identification, α-hydrazides of aspartic acid and glutamic acid were prepared from poly-α-L-aspartic acid and α-L-glutamylalanine and γ-hydrazide of glutamic acid from L-glutamic acid by the method of Smulson & Neal (1965).

Susceptibility of peptide linkages of mycobacillin to hydrolysis. The method is based on the greater susceptibility of γ-linkages to temperature as found by Hopkins (1929) with glutathione.

A 10mg sample of mycobacillin was suspended in water at pH5 in a sealed tube and heated at 100°C for different periods. Mycobacillin, a cyclic peptide that gives no colour with ninhydrin, first shows an appearance of blue colour with ninhydrin after 4h hydrolysis. The solution was then dried *in vacuo* and chromatographed in two dimensions with (*a*) water-saturated phenol and (*b*) butan-1-ol–acetic acid–water (4:1:1, by vol.). The two spots, so obtained, were eluted, hydrolysed completely with 10M-HCl and rechromatographed in two dimensions with the same solvent system and the constituent amino acids were identified.

Reduction of esterified mycobacillin and identification and determination of the hydroxy acids obtained from aspartic acid and glutamic acid residues. Dicarboxylic amino acids in a peptide or protein can be esterified at the free carboxyl group and then reduced with $LiBH_4$ to give hydroxy amino acids that indicate the position of the original free carboxyl group.

A 100mg (60μmol) sample of mycobacillin was dissolved in 20ml of methanol and methanolic HCl was added to make the solution 0.1M with respect to HCl. After different intervals, samples were taken out, precipitated with acetone–ether (1:1, v/v), washed with the same mixture and dissolved in water. The extent of esterification was then determined by the Zeisel method of Pregl (1937). Mycobacillin requires about 120min for esterification (97%) by methanolic HCl at 30°C.

The ester hydrochloride, precipitated and washed with ether–acetone, was then dried and suspended in tetrahydrofuran (10ml). $LiBH_4$ (100mg) was added and the mixture was refluxed for 6h at 60–70°C. At the end of the reaction the cooled product was mixed with an excess of methanolic HCl and stirred for 1h. Acetone–ether (1:1, v/v) was then added and the pH was adjusted to 3–4, when the reduced product was precipitated. It was washed with acetone and ether separately and dried.

The reduced product was hydrolysed with 10M-HCl–formic acid (1:1, v/v) for 10h and dried. The dried product was dissolved in 1M-HCl and chromatographed on Dowex 50 (H^+ form) and eluted with 1M-HCl.

Amino acids were measured by the ninhydrin method and hydroxy acids by the periodate–formaldehyde procedure of Rees (1958).

Each amino acid and hydroxy acid was identified by co-chromatography with an authentic sample.

Titration of mycobacillin. The presence of lactone linkages was determined by titration. A 1% solution of mycobacillin at pH7 was titrated with KOH and HCl solutions and the pH determined with a Beckmann pH-meter. Mycobacillin was then pretreated with 0.1M-KOH for 30min and again titrated with HCl.

Infrared spectroscopy of mycobacillin. The i.r. spectrum of mycobacillin was taken in Nujol mulls by a Perkin–Elmer 1378 Infracord spectrometer.

Partial hydrolysis of mycobacillin and peptides obtained from the hydrolysate. Mycobacillin was hydrolysed with 11.4M-HCl for 7 days at 37°C and fractionated by paper chromatography as described by Majumder & Bose (1960*a*). The 15 peptides, so isolated, were further hydrolysed with 5.7M-HCl at 110°C for 18h and the constituent amino acids identified. The hydrolysate of each peptide that contained aspartic acid was redissolved in water and evaporated under reduced pressure to remove HCl, this procedure being repeated three times. Finally the residue was dissolved in 0.5ml of water for subsequent experiments.

Oxidation of D-*aspartic acid in hydrolysates.* A 0.2ml sample of each peptide hydrolysate was incubated with D-amino acid oxidase (hog kidney) for 90min. The incubation mixture contained 1.0ml of 1M-sodium pyrophosphate buffer, pH8.3, 0.1ml of FAD (0.1mol), 0.1ml of

catalase (2mg/ml) and 0.2ml of D-amino acid oxidase (24mg/ml) and the volume was made up to 3.0ml with water. The reaction was terminated by the addition of 4vol. of 50% (v/v) ethanol and the mixture was centrifuged. The supernatant was then deionized on a column of Dowex 50 (H^+ form) and dried *in vacuo*. The residue was dissolved in 0.1ml of 10% (v/v) propan-2-ol and chromatographed in two dimensions with (*a*) water-saturated phenol and (*b*) butan-1-ol–acetic acid–water (4:1:1, by vol.). The D-amino acid oxidase-treated peptide hydrolysates were chromatographed together with the untreated ones and the amino acids were identified.

Decarboxylation of L-*aspartic acid in hydrolysates.* A 0.2ml sample of each peptide hydrolysate was incubated with L-glutamate decarboxylase (containing L-aspartate decarboxylase activity) for 4h. The incubation mixture contained 1.5ml of 0.2M-sodium acetate buffer, pH5.0, and 0.5ml of L-glutamate decarboxylase (26mg/ml) and the volume was made up to 2.5ml with water. The reaction was terminated with 4vol. of 50% (v/v) ethanol. Amino acids were recovered and identified in the same manner as in the oxidase treatment.

Hydrazinolysis of L-*aspartate decarboxylase-sensitive peptides.* Hydrazinolysis was carried out to determine the *C*-terminal amino acids of those aspartic acid-containing peptides that were decarboxylated by L-glutamate decarboxylase.

A 10μmol sample of each peptide was dried overnight in a vacuum desiccator and incubated with 2 drops of anhydrous hydrazine in a sealed tube at 60°C for 2h. The excess of hydrazine was then removed *in vacuo* over H_2SO_4. The dried mass was dissolved in water and shaken overnight with Amberlite IRC 50 (H^+ form) at 0°C. After removal of resin, the aqueous layer was shaken with benzaldehyde (1.2ml) and pyridine (0.012ml) overnight in a stoppered cylinder. The aqueous layer was extracted four times with ether, dried and the amino acids were identified by paper chromatography as above.

RESULTS

Free α-carboxyl groups of mycobacillin. Decarboxylation of mycobacillin with *N*-bromosuccinimide in Warburg manometric flasks indicates that 3.27μmol of mycobacillin yields 150.3μl of CO_2. The substrate used/CO_2 evolved molar ratio is approx. 2.06, showing that two free α-carboxyl groups are present per molecule of mycobacillin (Table 1).

Occurrence of a γ-glutamyl linkage. Oxidation of mycobacillin with sodium hypobromite followed by hydrolysis and chromatography gave only one spot when developed with Bromocresol Green. This was

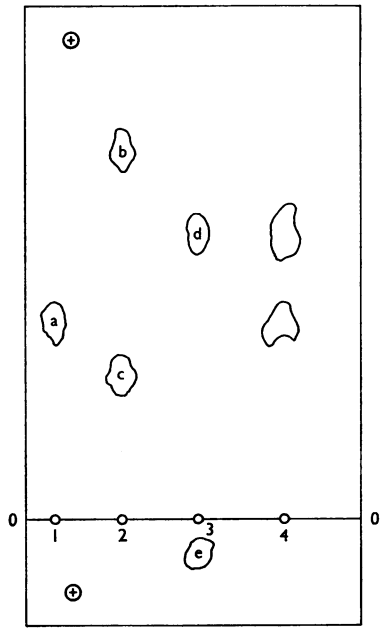


Fig. 1. Paper electrophoretogram of hydrazides of aspartic acid and glutamic acid. Anhydrous hydrazine (2ml) was added to 10mg of peptide and heated for 8h at 100°C. Excess of hydrazine was removed *in vacuo* over H_2SO_4, and the residue was dissolved in water and spotted on the chromatogram. For mycobacillin, hydrazides of other amino acids except those of aspartic acid and glutamic acid were removed by treatment with Amberlite IRC 50 resin. Electrophoresis was conducted on Whatman no. 1 paper at pH4 in a buffer containing 23ml of acetic acid and 6ml of pyridine/l for 2h at 15V/cm. 1, γ-Glutamyl hydrazide (*a*). 2, Hydrazides from α-L-glutamylalanine: (*b*) alanine, (*c*) α-glutamylhydrazide. 3, Hydrazides from poly α-L-aspartic acid: (*d*) α-aspartylhydrazide, (*e*) aspartic acid. 4, Hydrazides from mycobacillin (IRC-treated).

Table 1. *Decarboxylation of mycobacillin with* N-*bromosuccinimide*

Reaction was carried out in acetate buffer, pH4.7; mycobacillin, being insoluble at this pH, was kept in suspension with the addition of a detergent, cetyltrimethylammonium bromide (1.2%); $PdCl_2$ (10μmol) was used as a catalyst. Appropriate controls were run without substrate and with the addition of detergent. Reaction was complete within 150min.

Substrate	Amount added (μmol)	CO_2 evolved (μl)	$\frac{\mu\text{mol of } CO_2 \text{ evolved}}{\mu\text{mol of substrate}}$
Mycobacillin	3.27	150.3	2.06
L-Alanine	1.00	2.3	1.00
D-Glutamic acid	1.00	22.4	1.00

Table 2. *Peptides from hydrolysis of mycobacillin at* pH 5

Mycobacillin (10 mg) was suspended in 5 ml of water adjusted to pH 5 at 100°C for 4 h. The hydrolysate, after concentration *in vacuo*, was chromatographed in two dimensions. The ninhydrin-positive spots, after elution, were hydrolysed with 10 M-HCl and the constituent amino acids were identified by chromatography in two dimensions with (*a*) phenol-saturated water and (*b*) butan-1-ol–acetic acid–water (4:1:1, by vol.). Mycobacillin is cyclo(-Glu-Asp-Ala-Asp-Pro-Asp-Glu-Tyr-Asp-Tyr-Ser-Asp-Leu-)

Spot no.	R_F value: Solvent (*a*)	R_F value: Solvent (*b*)	Amino acids
1	0.24	0.34	Glutamic acid, aspartic acid, proline, alanine
2	0.52	0.61	Glutamic acid, aspartic acid, leucine, serine, tyrosine

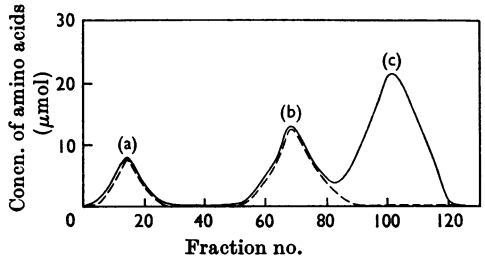


Fig. 2. Elution pattern of hydrolysates obtained from reduced esterified mycobacillin. Mycobacillin was esterified at the free carboxyl groups by methanolic HCl. The ester was reduced by refluxing with $LiBH_4$ in tetrahydrofuran for 6 h. The reduced product was then hydrolysed with 10 M-HCl–formic acid (1:1, v/v) for 10 h and dried *in vacuo*. The dried product was then dissolved in 1 M-HCl and adsorbed on a Dowex 50 (H^+ form) column and eluted with 1 M-HCl. (a) Serine; (b) δ-hydroxy-γ-aminovaleric acid; (c) α-amino-γ-hydroxybutyric acid. ——, Determined by ninhydrin method; ----, determined by periodate oxidation.

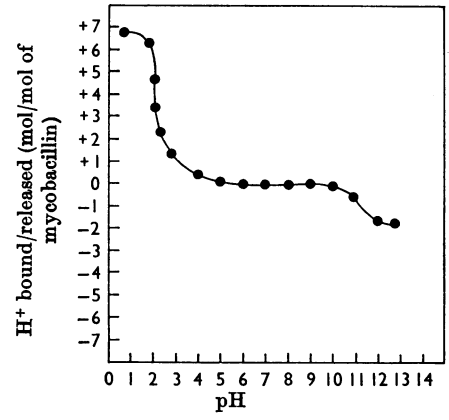


Fig. 3. Titration curve of mycobacillin. A 1% solution of mycobacillin at pH 7 was titrated with KOH and HCl and the amount of bound and free H^+ was determined at various pH values.

identified as succinic acid by its R_F value and subsequently by co-chromatography with an authentic sample. Thus the presence of at least one γ-linked glutamic acid is indicated.

Characterization of aspartic acid and glutamic acid hydrazides from mycobacillin. Hydrazides of aspartic acid and glutamic acid after separation from other amino acid hydrazides of mycobacillin by treatment with Amberlite IRC 50 and paper electrophoresis gave two ninhydrin-positive spots. They were identified as the α-hydrazide of aspartic acid and the γ-hydrazide of glutamic acid (Fig. 1).

Relative stability of glutamyl linkages in mycobacillin to hydrolysis. When hydrolysed at pH 5 and 100°C for 4 h mycobacillin gives two ninhydrin-positive spots on two-dimensional chromatography. The presence of two fractions and identification of their constituent amino acids (Table 2) clearly indicate that this heating is just sufficient to rupture only glutamyl linkages of the mycobacillin molecule.

Identification and determination of α-amino-γ-hydroxybutyric acid and γ-amino-δ-hydroxyvaleric acid obtained from the reduced ester of mycobacillin. α-Amino-γ-hydroxybutyric acid and γ-amino-δ-hydroxyvaleric acid, obtained by the lithium borohydride reduction of esterified aspartic acid and glutamic acid residues of mycobacillin, were separated by chromatography on Dowex 50. The hydroxy acids were confirmed by co-chromatography with authentic samples. The α-amino-γ-hydroxybutyric acid/γ-amino δ-hydroxy-valeric acid molar ratio was approx. 5:2 (Fig. 2), indicating that both glutamic acid residues in mycobacillin are γ-linked whereas all aspartic acids are α-linked.

Side-chain linkage of mycobacillin. Fig. 3 indicates the titration curve for mycobacillin. It shows two

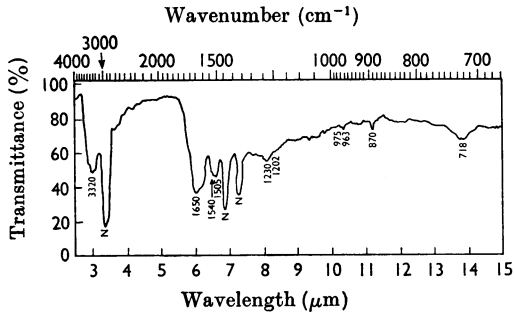


Fig. 4. Infrared spectrum of mycobacillin.

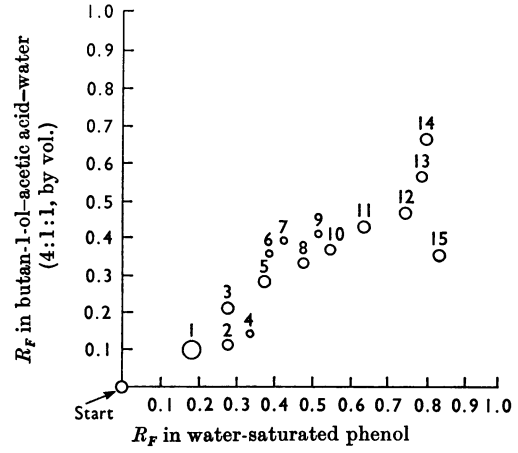


Fig. 5. Two-dimensional chromatogram of peptide mixture from partial hydrolysis of mycobacillin. Mycobacillin was partially hydrolysed with 11.4M-HCl at 37°C for 7 days and the peptides obtained from hydrolysate were separated by two-dimensional chromatography.

inflexions, one in the acid and another in the alkaline region. From the inflexion in the acid region it is found that mycobacillin contains approximately seven free carboxyl groups. It was further observed that treatment with 0.1M alkali for 30min did not alter the curve. Thus no additional carboxyl group was released in alkaline conditions, indicating the absence of any lactone linkages from the molecule. It also appears from the curve that approx. 2mol of H^+ is released/mol of mycobacillin between pH10 and 12, which might be due to the presence of two tyrosine residues/mycobacillin molecule.

Fig. 4 shows the i.r. spectrum of mycobacillin, which appears similar to that of other allied peptides. The spectrum shows the presence of N–H bonds, hydroxyl groups, amide I and amide II bands (Yale, 1943), aromatic or conjugated diene system and free carboxyl groups. The bands characterized by the presence of anhydride, lactone (5.36–5.50μm, 1865–1818cm^{-1}) or ester (5.68–5.81μm, 1761–1721 cm^{-1}) linkages are absent. Mycobacillin does not react with hydroxylamine (Cheronis, Entrikin & Hodnett, 1965) to give any hydroxyamate, indicating the absence of anhydride, lactone or ester linkages.

Positions of D- *and* L-*amino acids.* Fig. 5 shows that 15 well-separated spots were obtained from the partial hydrolysate of mycobacillin. On rechromatography it was observed that each of the peptides was pure except spots 1 and 3, which resolved into two ninhydrin-positive spots and were omitted from subsequent experiments. All the remaining 13 peptides, except peptide 9, were found to contain aspartic acid. Table 3 shows that hydrolysates of peptides 6 and 12 contain aspartic acid after D-amino acid oxidase treatment whereas the rest of the peptides do not. Thus peptides 6 and 12 contain L-aspartic acid and the remaining ten peptides only D-aspartic acid. Hydrolysates of the 12 aspartic acid-containing peptides were treated with L-glutamate decarboxylase (containing L-aspartate decarboxylase activity) and the amino acids in the incubation mixture were identified. In peptides 6 and 12 alanine appeared with consequent disappearance of L-aspartate. Further, the molar proportions of amino acids in peptides 5, 7, 11 and 15 were determined both before and after treatment with decarboxylase and were found to be unaltered (Table 3), thus indicating the absence of L-aspartate. Hydrazinolysis of peptides 6 and 12, which are sensitive to L-aspartate decarboxylase, indicates that tyrosine and serine are their respective *C*-terminal amino acids.

DISCUSSION

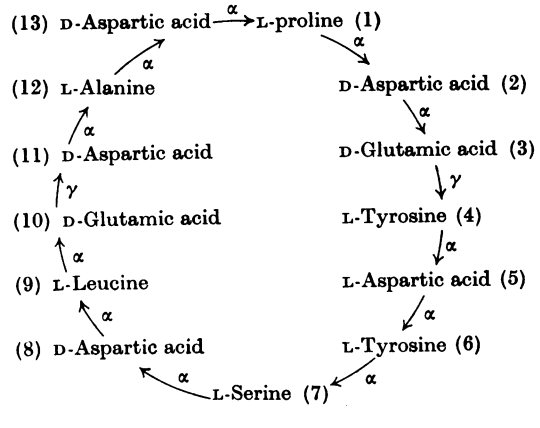
The present work studies the nature of aspartyl, glutamyl and side-chain linkages of mycobacillin. Several approaches have been made to identify the presence of γ- or β-peptide linkages in the molecule. It was observed that the antibiotic when acted upon by *N*-bromosuccinimide in succinimide solution yields 2 mol of CO_2/mol of the compound, indicating the presence of two non-α-peptide linkages in the molecule, which may be either β- or γ- or both. The hypobromite oxidation method was thereafter applied to determine γ-linkages among the non-α-linkages of the molecule. The oxidation method when applied to mycobacillin gave succinic acid, indicating that at least one glutamic acid residue is γ-linked in the molecule. The γ-glutamyl linkage of capsular polyglutamic acid of *Bacillus anthracis* was similarly determined (Haurowitz & Bursa, 1949). However, it was not possible to extend this oxidation method for quantitative determination of

Table 3. *Identification of* D- *or* L-*aspartic acid in the aspartic acid-containing peptides obtained from partial hydrolysates of mycobacillin by* D-*amino acid oxidase (hog kidney) and* L-*glutamate decarboxylase and* L-*aspartate decarboxylase* (Cl. welchii)

The incubation mixture for D-amino acid oxidase contained 0.2 ml of peptide hydrolysate, 1.0 ml of 0.1 M-sodium pyrophosphate buffer, pH 8.3, 0.1 ml of FAD (0.1 mol), 0.1 ml of catalase (2 mg/ml) and 0.2 ml of D-amino acid oxidase (24 mg/ml), the volume was made up 3 ml with water and it was incubated for 90 min. The reaction was terminated with 4 vol. of 50% (v/v) ethanol and the clear supernatant was deionized by passage through a Dowex 50 column, dried and subjected to chromatography. The incubation mixture for L-glutamate decarboxylase contained 0.2 ml of peptide hydrolysate, 1.5 ml of 0.2 M-sodium acetate buffer, pH 5, and 0.5 ml of L-glutamate decarboxylase (26 mg/ml), the volume was made up to 2.5 ml with water and it was incubated for 4 h. The reaction was terminated with 4 vol. of 50% (v/v) ethanol and amino acids were recovered in the same way as in the oxidase treatment.

Peptide no.	Amino acids present	Amino acids present after oxidase treatment	Amino acids present after decarboxylase treatment	*C*-Terminal amino acid	Molar proportion of amino acids: Before treatment	Molar proportion of amino acids: After treatment	Configuration of aspartic acid
1*	Asp, Glu	—	—	—	—	—	—
2	Asp, Ser	Ser	Asp, Ser	—	—	—	D
3*	Asp, Glu, Ala	—	—	—	—	—	—
4	Asp, Glu	Glu	Asp, Glu	—	—	—	D
5	Asp, Ala	Ala	Asp, Ala	—	(1:1)	(1:1)†	D
6	Asp, Tyr (1:1)	Asp, Tyr	Ala, Tyr	Tyr	(1:1)	(1:1)‡	L
7	Asp, Ala	Ala	Asp, Ala	—	(1:1)	(1:1)†	D
8	Asp, Glu, Tyr	Glu, Tyr	Asp, Glu, Tyr	—	—	—	D
10	Asp, Glu, Leu	Glu, Leu	Asp, Glu, Leu	—	—	—	D
11	Asp, Glu, Ala, Leu	Glu, Ala, Leu	Asp, Glu, Ala, Leu	—	(1:1:1:1)	(1:1:1:1)†	D
12	Asp, Ser, Tyr (1:1:1)	Asp, Ser, Tyr	Ala, Ser, Tyr	Ser	(1:1:1)	(1:1:1)‡	L
13	Asp, Glu, Ser, Leu	Glu, Ser, Leu	Asp, Glu, Ser, Leu	—	—	—	D
14	Asp, Ser, Leu	Ser, Leu	Asp, Ser, Leu	—	—	—	D
15	Asp, Ala, Pro	Ala, Pro	Asp, Ala, Pro	—	(1:1:1)	(1:1:1)†	D

* Chromatographically impure.
† Molar proportion determined after decarboxylase treatment.
‡ Molar proportion determined after amino acid oxidase treatment.



(→, CO–NH linkages)

Mycobacillin

Scheme 1.

the exact number of γ-glutamyl residues in mycobacillin, so the technique of hydrazinolysis was adopted. Mycobacillin, on hydrazinolysis, yields only the α-hydrazide of aspartic acid and γ-hydrazide of glutamic acid. This shows that the aspartic acids are all α-linked whereas the two glutamic acids are γ-linked. In this way, Kakimoto & Armstrong (1961) and Ito & Strominger (1964) also identified β-aspartyl and γ-glutamyl linkages in β-aspartyl-histidine and in cell-wall mucopeptide precursors. However, in the case of the γ-linkage of glutamic acid this conclusion is open to criticism because of the fact that $\alpha \rightleftharpoons \gamma$ interconversion may occur under the condition of hydrazinolysis in a non-aqueous solvent (Clayton & Kenner, 1953), so the peptide linkages of aspartic acid and glutamic acid residues were further confirmed by isolating and determining hydroxy amino acids obtained from reduced mycobacillin by the procedure described by Chibnall *et al.* (1958). The methyl ester of mycobacillin, when reduced with lithium borohydride, yields α-amino-γ-hydroxybutyric acid and γ-amino-δ-hydroxyvaleric acid; β- and γ-esters of aspartic acid and glutamic acid should give α-amino-γ-hydroxybutyric acid and α-amino-δ-hydroxyvaleric acid and α-esters should give β-amino-γ-hydroxybutyric acid and γ-amino-δ-hydroxyvaleric acid. Therefore this method conclusively proves that all aspartic acid residues are α-linked and glutamic acids are γ-linked. This observation is also supported by the fact that the glutamyl linkages are more susceptible to hydrolysis as found by Hopkins (1929) and Kendall, Mason & McKensie (1930) in the case of glutathione. No ester, anhydride or lactone groups could be detected either by hydroxylamine reaction or by i.r. spectroscopy. It was further observed from the titration curve of mycobacillin that pretreatment with alkali does not liberate any additional carboxyl group, which suggests the absence of any lactone linkage from the molecule.

It has also been found that treatment of hydrolysates of aspartic acid-containing peptides obtained from partial hydrolysis of mycobacillin with D-amino acid oxidase or L-glutamate decarboxylase (containing L-aspartate decarboxylase activity) revealed L-aspartic acid only in peptides 6 and 12 and D-aspartic acid in others. Hydrazinolysis shows that peptide 6 is aspartyl-tyrosine and peptide 12 is (Asp,Tyr)Ser. Majumder & Bose (1960*b*) have shown by reaction with fluorodinitrobenzene that peptides with similar R_F values both had *N*-terminal aspartic acid. Hence the single L-aspartic acid in mycobacillin must occupy position 5 in the complete structure of the molecule shown in Scheme 1.

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