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Structure of the Peptidoglycan in *Escherichia coli* B and *Bacillus megaterium* KM. Stereospecific Synthesis of Two *meso*-Diaminopimelic Acid Peptides with the Tetrapeptide Subunit of Bacterial Cell Wall Peptidoglycan*

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ABSTRACT: The synthesis of the tetrapeptide L-Ala-γ-D-Glu-(L)*meso*-diaminopimelic acid(L)-D-Ala (I) which has the same stereochemical structure as that recently postulated for the tetrapeptide subunit isolated from the cell wall peptidoglycans of *Escherichia coli* and *Bacillus megaterium* was achieved. A new and general approach to the stereoselective substitution of one amino group and one carboxyl group of *meso*-diaminopimelic acid was developed. To offer further proof of the proposed structure for the natural peptide moiety, the isomeric tetrapeptide D-Ala-γ-L-Glu-(D)*meso*-diaminopimelic acid(L)-L-Ala (V) differing only in the position of the C-terminal alanine residue, was also synthesized.

These two synthetic tetrapeptides, I and V, were compared by high-voltage electrophoresis at pH 4. The difference of mobilities observed corresponds to the different positions of the C-terminal alanine residue. In these conditions the bacterial tetrapeptide had the same mobility as the synthetic tetrapeptide L-Ala-γ-D-Glu-(L)*meso*-diaminopimelic acid(L)-D-Ala (I).

The main structural features of the cell wall peptidoglycan of *Escherichia coli* B have been established by Weidel and coworkers (for a general review, see Weidel and Pelzer, 1964). According to these authors the principal repeating unit in this peptidoglycan is represented by the disaccharide tetrapeptide GNAc-MurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala,[1] briefly designated C_6. A disaccharide tripeptide, designated C_5, differing from C_6 by the absence of the C-terminal D-alanine residue, is produced after "endogenous" enzymatic degradation of the peptidoglycan. A dimer, designated C_3, formed by two C_6 units linked by a peptide bond was also isolated by these authors (Figure 1).

In the proposed structure of the tetrapeptide subunit, three points remained unknown, namely (a) which carboxyl group of D-glutamic acid is linked to *meso*-diaminopimelic acid residue, (b) which amino group of *meso*-diaminopimelic acid is attached to the D-glutamic acid residue, and (c) which carboxyl group of the *meso*-diaminopimelic acid is linked to the D-alanine residue.

From the optical rotation measurement of the mono-DNP-*meso*-DAP isolated from the dinitrophenylated C_6 unit, Diringer and Jusic (1966) demonstrated that the amino group adjacent to the L-asymmetric carbon of *meso*-diaminopimelic acid was linked to the D-glutamic residue. A similar result was obtained by Bricas *et al.* (1967) in the case of the *Bacillus megaterium* KM peptidoglycan, by comparing the mono-DNP-*meso*-DAP isolated from dinitrophenylated C_6 unit of this peptidoglycan, with an authentic mono-DNP-(D)*meso*-DAP derivative prepared by stereospecific synthesis.

Recently Diringer (1968) and van Heijenoort *et al.* (1969) established by the hydrazinolysis of peptide fragments from *E. coli* and *B. megaterium* peptidoglycans that the γ-carboxyl group of D-glutamic acid is linked to *meso*-diaminopimelic acid. To provide further proof we synthesized the two isomeric tripeptides L-Ala-α-D-Glu-(L)*meso*-DAP and L-Ala-γ-D-Glu-(L)*meso*-DAP (Dezélée and Bricas, 1968). The electrophoretic mobility at pH 4.0 of the natural tripeptides isolated after enzymatic degradation of the disaccharide tripeptide C_5 of *E. coli* and *B. megaterium* peptidoglycans, is similar to that of the γ-glutamyl synthetic isomer (van Heijenoort *et al.*, 1969).

Finally an indirect proof for the position of the C-terminal D-alanine was brought by van Heijenoort *et al.* (1969) by use of the Edman degradation carried out on the tetrapeptide subunits isolated from the cell wall peptidoglycans of these two bacteria. The first stage degradation indicated that C-terminal D-alanine is linked to the carboxyl group adjacent to the L-asymmetric carbon of *meso*-diaminopimelic acid. Thus the configurational structure of tetrapeptide subunit of the *E. coli* and *B. megaterium* cell wall peptidoglycans is L-Ala-γ-D-Glu-(L)*meso*-DAP(L)-D-Ala.

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[1] The usual symbols of protein and peptide chemistry were used in this paper: *meso*-DAP, *meso*-diaminopimelic acid; Z-benzyloxycarbonyl; Boc, *t*-butyloxycarbonyl; OBzl, benzyl ester; OSU, *N*-hydroxysuccinimide ester. The *N*-acetylglucosamine and *N*-acetylmuramic acid were abbreviated, respectively: GNAc and MurNAc. For the designation of the substitutions on the amino and carboxyl groups of the *meso*-diaminopimelic acid we use our usual abbreviation (Bricas *et al.*, 1962): in order to specify on which asymmetrical carbons of *meso*-DAP are placed the substituted amino groups, we advocate the use of the notation (L) or (D) following the designation of the substituents and before the abbreviation *meso*-DAP. Similarly we propose to write (L) or (D) before the substituents and after *meso*-DAP, in order to distinguish between the carboxyl substituted groups.

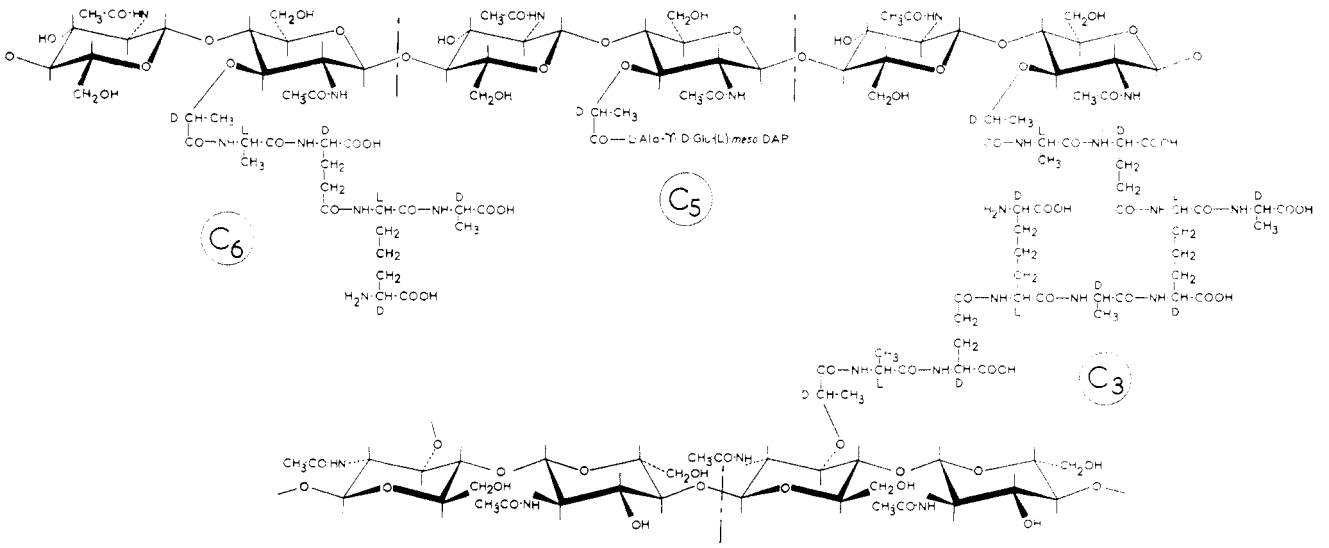

FIGURE 1: Structure of a fragment of the *E. coli* B peptidoglycan representing the disaccharide tripeptide: C_5, the disaccharide tetrapeptide: C_6 and the dimer of C_6, bis(disaccharide tetrapeptide): C_8.

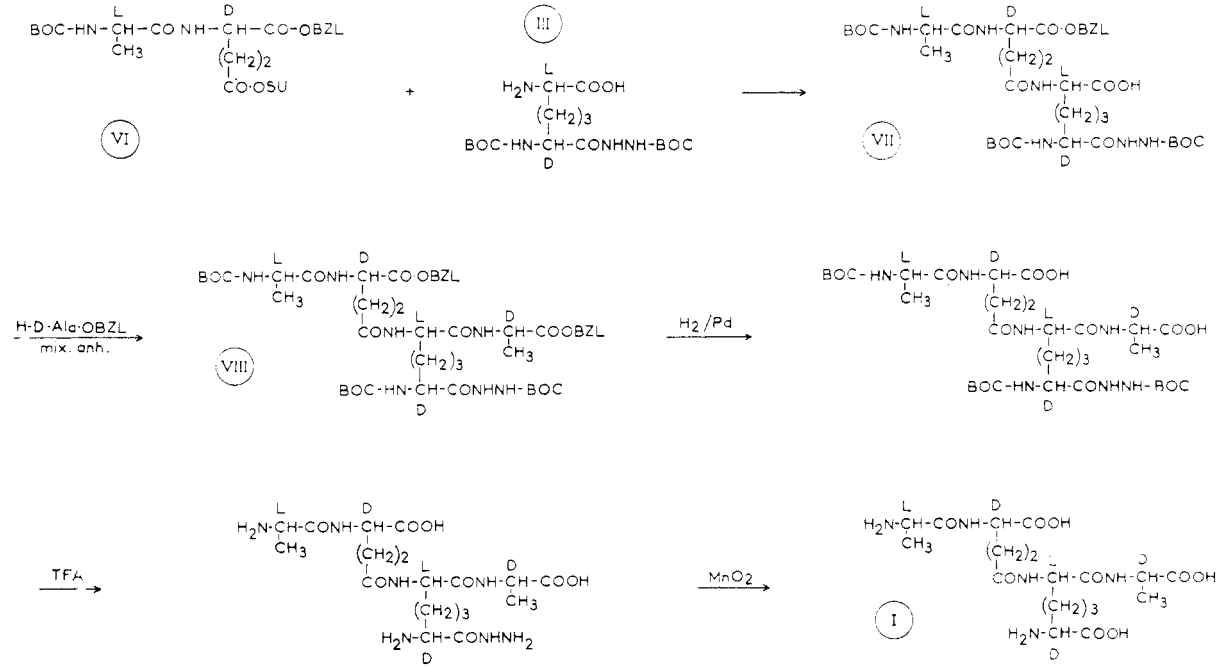

FIGURE 2: Synthesis of the tetrapeptide: L-Ala-γ-D-Glu-(L)*meso*-DAP(L)-D-Ala (I).

The general scheme for these structures is represented in Figure 1.

To confirm the postulated structure for the peptide moiety of the C_6 unit of the peptidoglycans of these two bacteria, we synthesized the tetrapeptide L-Ala-γ-D-Glu-(L)*meso*-DAP(L)-D-Ala (I) corresponding to the proposed configurational structure.

For the synthesis of this tetrapeptide (Figure 2) a derivative of *meso*-diaminopimelic acid protected on the amino group and carboxyl group adjacent to the D-asymmetric carbon, *t*-butyloxycarbonyl-(D)*meso*DAP(D) - NHNH - *t* - butyloxycarbonyl (III) was prepared stereospecifically by a new procedure (Figure 3).

In order to compare the natural tetrapeptide with the synthetic structural isomers, an other isomeric tetrapeptide differing from I only in the position of the C-terminal alanine residue was required. The two enantiomorphs L-Ala-γ-D-Glu-(L)*meso*-DAP(D)-D-Ala (II) and D-Ala-γ-L-Glu-D-*meso*-DAP(L)-L-Ala (V) would correspond to this structural requirement and of course would have the same electrophoretic behaviour.

However we preferred to synthesize the tetrapeptide V

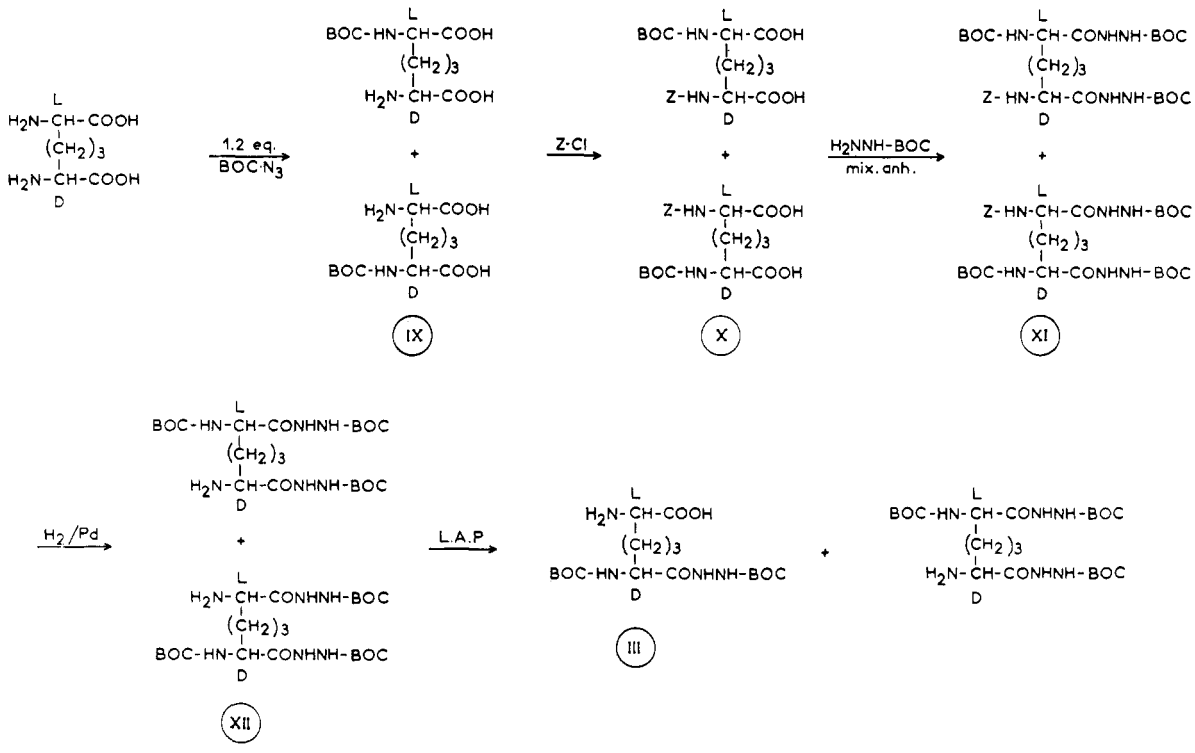

FIGURE 3: New method of preparation of stereospecific *N*-monoacylated derivatives of *meso*-DAP: Synthesis of *t*-butyloxycarbonyl- (*D*) *meso*-DAP(*D*)-NHNH-*t*-butyloxycarbonyl (III).

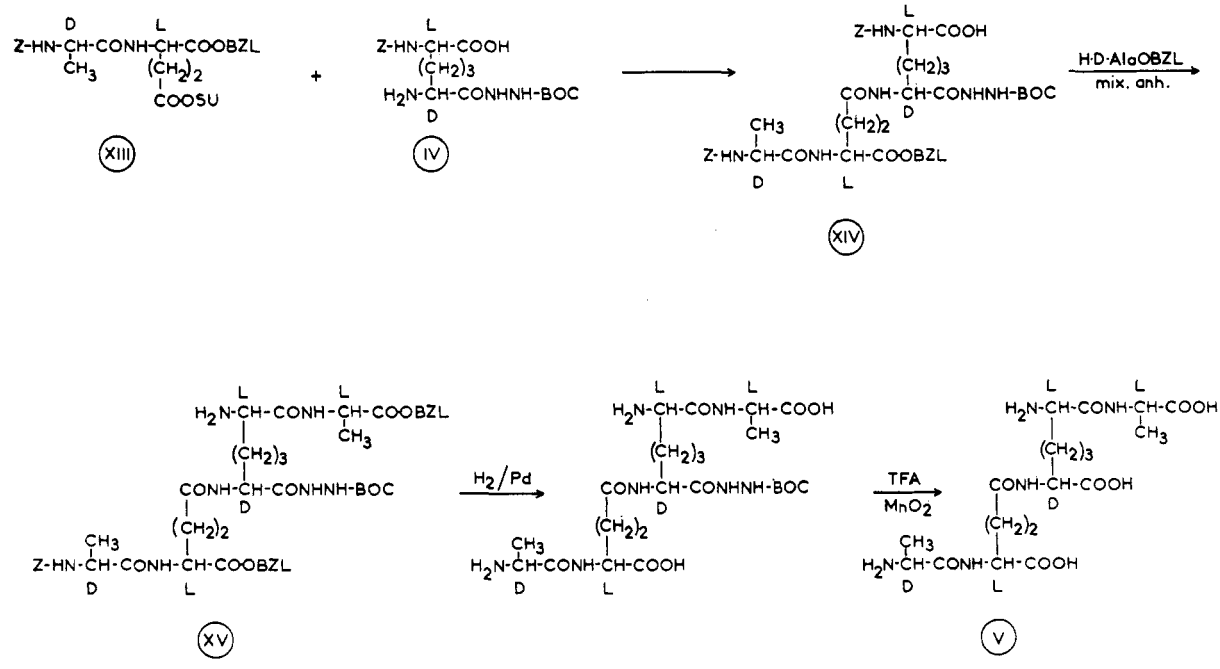

FIGURE 4: Synthesis of the tetrapeptide: D-Ala-γ-L-Glu-(D)*meso*-DAP(L)-L-Ala (V).

(Figure 4) which was much easier to prepare starting from the intermediate Z-(L)*meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl (IV) previously obtained (Bricas *et al.*, 1967).

The expected difference of p*K* values of the free carboxyl group of the *meso*-diaminopimelic acid residue in peptides I and V should produce a difference in the electrophoretic mobility of these peptides in the range pH 3–5.

Thus evidence for the position of C-terminal alanine residue

in the natural tetrapeptide could be given by comparison of its electrophoretic mobility with that of the two synthetic tetrapeptides I and V.

Material and Methods

meso-Diaminopimelic acid (Koch–Light Laboratories Ltd., Colnbrook, England) was purified by repeated recrystallizations from 6 N aqueous HCl.

The natural tetrapeptides subunit samples from *E. coli* and *B. megaterium* were those isolated by van Heijenoort *et al.* (1969). Elemental analysis were performed by the Centre de Microanalyse du C.N.R.S. (Gif-sur-Yvette). Total amino acid analysis were carried out with a Technicon amino acid analyzer. Melting points were taken in capillary tubes and are uncorrected. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. High-voltage paper electrophoretic separations were performed in a Savant apparatus at pH 4.0 in water–pyridine–acetic acid (976:6:23) buffer or at pH 6.4 in water–pyridine–acetic acid (986:100:4) buffer, on Whatman No. 3MM paper. Thin-layer chromatograms were run on silica gel (E. Merck, A. G. Darmstadt, Germany).[2] Spots were revealed with ninhydrin when free amino groups were present, with chlorine (Reindel and Hoppe, 1954; Mazur *et al.*, 1962), in case of acylated amino acid derivatives or acylated peptides, and with Folin and Ciocalteu's reagent when hydrazide groups were present (Guttmann and Boissonnas, 1960).

Experimental Section

Synthesis of Peptide. L-*Ala*-γ-D-*Glu*-(L)*meso*-*DAP*(L)-D-*Ala* (*I*). The tetrapeptide I was prepared by the following steps (Figure 2): preparation of III a derivative of *meso*-diaminopimelic acid protected on the amino and carboxyl groups adjacent to the D-asymmetric carbon; coupling of III with γ-*N*-hydroxysuccinimide ester of *t*-butyloxycarbonyl-L-Ala-α-D-GluOBzl (VI); coupling of tripeptide, *t*-butyloxycarbonyl-L-Ala-γ-D-Glu(α-OBzl)-(L)-*t*-butyloxycarbonyl-(D)*meso*-DAP-(D)-NHNH-*t*-butyloxycarbonyl (VII) so obtained, with D-alanine benzyl ester by the method of Anderson *et al.* (1966) to preclude racemization; liberation of the resulting protected tetrapeptide, *t*-butyloxycarbonyl-L-Ala-γ-D-Glu(α-OBzl)-(L)-*t*-butyloxycarbonyl-(D)*meso*-DAP(L)-D-Ala-OBzl-(D)-NHNH-*t*-butyloxycarbonyl (VIII), by catalytic hydrogenation, acidolysis with trifluoroacetic acid and oxidation with MnO_2 (Kelly, 1963).

Derivative III was obtained by a new method represented in Figure 3, differing from the method for the stereospecific preparation of mono-N-acylated *meso*-diaminopimelic derivatives previously described (Bricas *et al.*, 1967). Starting from the racemic mixture of mono-*t*-butyloxycarbonyl-*meso*-DAP (IX) we prepared the racemic mono-*t*-butyloxycarbonyl-*meso*-DAP-bis(NHNH-*t*-butyloxycarbonyl) (XII) which was submitted to the action of leucine aminopeptidase.

On these derivatives leucine aminopeptidase hydrolyzed stereospecifically the hydrazide bond of the carboxyl belonging to the L moiety of the *meso*-diaminopimelic acid provided that α-amino group adjacent to the L-asymmetric carbon was free.

Indeed it had been previously shown that only α-hydrazides of L-α-amino acids or hydrazides belonging on the L moiety of the *meso*-diaminopimelic acid are hydrolyzed by this enzyme (Nicot and Bricas, 1963b; Bricas and Nicot, 1966).

The racemic derivative XIII was thus resolved to yield derivative III and *t*-butyloxycarbonyl-(L)*meso*-DAP-bis-(NHNH-*t*-butyloxycarbonyl) which were easily separated by fractional crystallization.

This method is more direct than the preferential monoacylation of *meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl, previously described (Bricas *et al.*, 1967).

Mono-t-butyloxycarbonyl-meso-DAP (Racemic) (IX). *meso*-Diaminopimelic acid hydrochloride (9.6 g, 32 mmoles) was dissolved in 80 ml of water and 80 ml of dioxan. The pH was adjusted at 9.5 and 6 ml (42 mmoles) of *t*-butyloxycarbonyl-azide (Carpino, 1959) was added. The mixture was stirred overnight at room temperature, maintaining the pH at 9.5 with a pH-Stat adding 2 N NaOH. The solution was then evaporated *in vacuo* and the aqueous solution was extracted twice with 30 ml of ether. The aqueous layer was acidified to pH 3.0 with 2 N HCl and extracted three times with 50 ml of ethyl acetate. The aqueous layer was then kept at 4° overnight and yields hydrophobic crystals which were filtered and washed with 3 N acetic acid: yield, 6.25 g, 56%; mp <250°. *Anal.* Calcd for $C_{12}H_{22}N_2O_6 \cdot 1.5\ H_2O$: C, 45.4; H, 7.95; N, 8.85. Found: C, 45.4; H, 7.76; N, 8.95. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A;[2] R_F 0.45 (revealed with ninhydrin and with chlorine).

Z-butyloxycarbonyl-meso-DAP ((X) Dicyclohexylammonium Salt) (Racemic). IX (4.2 g, 13 mmoles) was suspended in 50 ml of water and 13 ml (26 mmoles) of 2 N NaOH was added; 2.13 ml (15 mmoles) of benzyloxycarbonyl chloride was then added alternatively with 2 N NaOH so that the pH remained in the alkaline range; during this operation the mixture was ice cooled and was rigorously shaken with a Vibromixer. After 3 hr the reaction was complete; the solution was extracted twice with 30 ml of ether, and the aqueous layer was acidified to pH 3.0 with 2 N HCl and extracted three times with 50 ml of ethyl acetate. The organic layers were pooled and dried over anhydrous $MgSO_4$; from the filtered solution the solvent was removed *in vacuo*, the residual oil was dissolved in 50 ml of ether, and 5.2 ml (26 mmoles) of dicyclohexylamine was added. Crystals were obtained from the solution when it was cooled to 4°: yield 7.9 g, 78%; mp 152°. *Anal.* Calcd for $C_{44}H_{74}N_4O_8$ (785.09): C, 67.1; H, 9.48; N, 7.12. Found: C, 67.1; H, 9.27; N, 7.06. The compound travels as a single spot in solvent system A, on silica gel thin-layer chromatography, R_F 0.78 (revealed with chlorine).

Mono-t-butyloxycarbonyl-meso-DAP-bis(NHNH-t-butyloxycarbonyl) Oxalate Salt (Racemic) (XII). *t*-Butyloxycarbonyl-Z-*meso*-DAP (X) (dicyclohexylammonium salt) (7.9 g, 10.8 mmoles) was desalted with HCl in tetrahydrofuran as described by Klieger *et al.* (1961). The *t*-butyloxycarbonyl-Z-*meso*-DAP obtained as an oily residue was dissolved in 30 ml of tetrahydrofuran and cooled to −10° with stirring; 2.80 ml (10.8 mmoles) of isobutyl chlorocarbonate and 2.96 ml (10.8 mmoles) of triethylamine were then added. After 5 min, a solution of 4.2 g (30 mmoles) of *t*-butyl carbazate in 15 ml of tetrahydrofuran was added. The

[2] The following solvent systems were used: A, 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v), B, 1-butanol–pyridine–acetic acid–water (60:6.6:13.3:20, v/v).

mixture was stirred overnight at room temperature. Tetrahydrofuran was then removed *in vacuo* and the residue was taken up in 50 ml of water and 100 ml of ethyl acetate. The organic layer was washed with 10% citric acid solution (four times), water (twice), 1 M $NaHCO_3$ (eight times), and water (twice). It was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The resulting oily residue, *t*-butyloxycarbonyl-Z-*meso*-DAP-bis(NHNH *t*-butyloxycarbonyl) (XI) could not be crystallized. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.91 (revealed with Folin–Ciocalteu's reagent and with chlorine).

The product was hydrogenated in 30 ml of methanol, and 1 ml of acetic acid with 1 g of palladium black. After completion of the reaction (about 6 hr), the catalyst was removed and the solution evaporated to dryness *in vacuo*. The oily residue was crystallized from ether–hexane; yield from X, 4.88 g of crystals, 79%. A sample was recrystallized as the oxalate salt, mp 133–134°. *Anal.* Calcd for $C_{22}H_{42}N_6O_8C_2H_2O_4$ (608.66): C, 47.4; H, 7.29; N, 13.80. Found: C, 47.4; H, 7.33; N, 13.24. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.74 (revealed with ninhydrine, with chlorine and with Folin–Ciocalteu's reagent).

t-Butyloxycarbonyl-(D)*meso-DAP*(D)*-NHNH-t-butyloxycarbonyl* (*III*). XII (2 g, 3.85 mmoles) was dissolved in 80 ml of water, the pH was adjusted at 8.6, and the temperature was maintained at 37°. A solution of calf lens leucine aminopeptidase (2.5 ml) (VEB Arzneimittelwerk, Dresden, East Germany), containing 0.4 mg of protein/ml and activated by Mg^{2+} as previously described (Dezélée and Bricas, 1967), was added to the substrate solution. The pH was kept at 8.6 by the recording pH-Stat adding 1 N NaOH. At the end of the reaction (2 hr) the pH was adjusted at 5.0 with 2 N HCl and the solution was concentrated to dryness *in vacuo*. The residue was dried by repeated evaporation *in vacuo* with ethanol–benzene. The residue was then triturated with 10 ml of ethanol, and the remaining precipitate was filtrated and washed with 5 m lof ethanol. The filtered solution was concentrated to about 5 ml and 40 ml of ether was added; crystals were thus obtained; yield 0.71 g (hygroscopic); yield from starting material 45.5% (91% of theoretical yield). A sample was recrystallized from ethanol for analysis, mp 183–185° dec. *Anal.* Calcd for $C_{17}H_{32}N_4O_7 \cdot HCl \cdot 2H_2O$ (476.95): C, 41.3; H, 7.94; N, 11.3. Found: C, 41.8; H, 7.52; N, 11.2; $[\alpha]_D^{25}$ +11.5° (*c* 1.5, methanol). The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.57 (revealed with ninhydrin, with chlorine and with Folin–Ciocalteu's reagent).

Action of the L*-Amino Acid Oxidase on the Compound* (*III*). The stereochemical structure of III was controlled by its oxidation with L-amino acid oxidase of *Crotalus adamanteus* venom: 1 ml of a 4.54×10^{-3} M solution of III was placed in a Warburg vessel with 1 ml of Tris buffer (0.1 M, pH 7.5) and 0.1 ml of catalase (Boehringer and Soehne). In the side arm was placed 0.5 ml of a 2-mg/ml purified L-amino acid oxidase solution (Sigma). The incubation was carried out at 37° in presence of 20% KOH solution. The quantities of oxygen absorbed were calculated from manometric measurements and were corrected for the absorption of a blank containing only enzyme and buffer. A 5×10^{-3} M solution of L-glutamic acid (1 ml) was incubated in the same conditions as the reference solution. The rate of oxidation of III was faster than that of L-glutamic acid in the first 12 hr and is achieved after consumption of 52 μl of oxygen, *i.e.*, 2.32 μM (100% of theory). As expected from the results of Work (1955) concerning the substitutions on the amino and carboxyl groups of the D moiety of *meso*-diaminopimelic acid, compound III was oxidized with an appreciable rate by the L-amino acid oxidase. The stereochemical structure of derivative III was thus confirmed in the limits of the accuracy of the method.

*t-Butyloxycarbonyl-*L*-Ala-α-*D*-GluOBzl* (*Dicyclohexylammonium Salt*). To an ice-cold solution of 1 mg (4.2 mmoles) of D-glutamic acid α-benzyl ester (obtained by treatment of the *t*-butyloxycarbonyl-α-D-GluOBzl prepared by the method of Nefkens and Nivard (1964) with HCl–CH_3COOH) and 0.59 ml (4.2 mmoles) of triethylamine in 15 ml of water is added a solution of Boc-L-Ala OSU (0.21 g, 4.2 mmoles) (Anderson *et al.*, 1964) in 15 ml of dimethoxy-1-2-ethane. The mixture was stirred for 12 hr allowing it to rise to room temperature. The solvent was then evaporated *in vacuo*, the remaining aqueous solution was acidified to pH 3.0 with 2 N HCl, and extracted three times with 25 ml of ethyl acetate. The pooled organic layers were washed with water and dried over $MgSO_4$. The ethyl acetate solution was then concentrated to dryness *in vacuo*, and the oily residue was taken up in 25 ml of ether. Dicyclohexylamine (0.83 ml, 4.2 mmoles) was added to the solution, and the precipitate obtained was collected by filtration and recrystallized from hot acetone: yield 2.15 g, 91%; mp 151°; $[\alpha]_D^{25}$ −2.2° (*c* 0.65, methanol). *Anal.* Calcd for $C_{32}H_{51}N_3O_7$ (589.78): C, 65.2; H, 8.72; N, 7.12. Found: C, 64.9; H, 8.72; N, 6.92. The product travels as a single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.87 (revealed with chlorine).

*t-Butyloxycarbonyl-*L*-Ala-γ-*D*-Glu(α-OBzl)-*(L)*-t-butyloxycarbonyl-*(D)*meso-DAP*(D)*-NHNH-t-butyloxycarbonyl* (*VII*). The preceding dicyclohexylammonium salt (225 mg, 0.38 mmole) was desalted as described by Klieger *et al.* (1961). The resulting residue was dissolved in 3 ml of ethyl acetate and cooled to −15° with stirring: 0.042 ml (0.38 mmole) of *N*-methylmorpholine and 0.050 ml (0.38 mmole) of isobutyl chlorocarbonate were added. After 5 min, 60 mg (0.42 mmole) of *N*-hydroxysuccinimide in 2 ml of ethyl acetate was added; the mixture was stirred for 12 hr and allowed to reach room temperature. The solvent was then evaporated *in vacuo* and the residue was triturated with 10 ml of ether. The precipitate was filtrated off and the ethereal solution was concentrated to dryness. The dipeptide *N*-hydroxysuccinimide ester so obtained could not be crystallized and was therefore used without further purification for coupling with derivative III. The preceding ester was dissolved in 4 ml of dimethoxy-1-2-ethane and cooled to 0°; 160 mg (0.40 mmole) of III dissolved in 4 ml of water, and 0.044 ml (0.40 mmole) of *N*-methylmorpholine were then added. The mixture was stirred for 3 hr at room temperature. The solvent was then removed *in vacuo*, and the remaining aqueous solution was acidified to pH 3.0 and extracted four times with ethyl acetate. The combined organic layers were washed with water and dried over $MgSO_4$. The filtered solution was concentrated and hexane was added to crystallize the protected tripeptide VII: yield 225 mg, 71%; mp 108–110°; $[\alpha]_D^{25}$ 14.1° (*c* 0.2, methanol). *Anal.* Calcd for $C_{37}H_{58}N_6O_{13} \cdot H_2O$ (812.93): C, 54.7; H, 7.44;

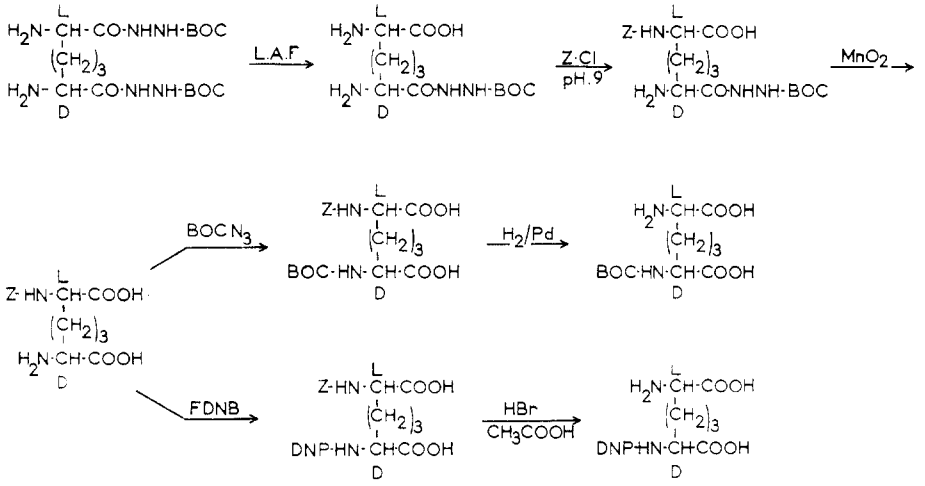

FIGURE 5: Stereospecific synthesis of Z-(L)*meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl and of DNP-(D)*meso*-DAP.

N, 10.3. Found: C, 54.9; H, 7.40; N, 10.2. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.93 (revealed with chlorine and with Folin–Ciocalteu's reagent).

t-Butyloxycarbonyl-L-*Ala*-γ-D-*Glu*(α-*OBzl*)-(L)-*t-butyloxycarbonyl*-(D)*meso*-DAP(L)-D-*AlaOBzl*-(D)-*NHNH-t-butyloxycarbonyl* (*VIII*). VII (80 mg, 0.1 mmole) was dissolved in 3 ml of tetrahydrofuran and cooled to −15°. To the stirred solution, 0.013 ml (0.1 mmole) of isobutyl chlorocarbonate and 0.011 ml (0.1 mmole) of *N*-methylmorpholine were added. After 3 min, a solution of 43 mg (0.11 mmole) of D-alaninebenzyl ester *p*-toluenesulfonate (Zervas *et al.*, 1957) and 0.013 ml (0.11 mmole) of *N*-methylmorpholine in 3 ml of tetrahydrofuran were added. The mixture was stirred overnight, allowing it to rise to room temperature. The tetrahydrofuran was then evaporated *in vacuo* and the residue was taken up in ethyl acetate. The organic solution was washed four times with 10% citric acid solution, twice with water, six times with 1 M $NaHCO_3$, and twice with water. The organic layer was dried over $MgSO_4$ and filtered; ethyl acetate was evaporated and the oily residue crystallized in ethanol–hexane: yield 77 mg of crystals, 80%; mp 143°; $[\alpha]_D^{25}$ +6.3 °(*c* 0.1, methanol). *Anal.* Calcd for $C_{47}H_{69}N_7O_{14}$ (956.12): C, 59.0; H, 7.27; N, 10.2. Found: C, 58.7; H, 7.18; N, 10.2. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.97 (revealed with chlorine and with Folin–Ciocalteu's reagent).

L-*Ala*-γ-D-*Glu*-(L)*meso*-*DAP*(L)-D-*Ala* (*I*). The amino- and carboxyl-protecting groups of the preceding tetrapeptide were removed in three steps without isolation of the intermediate, but with control of the reactions by thin-layer chromatography.

VIII (30 mg, 31.4 μmoles) was hydrogenated for 18 hr in 3 ml of acetic acid with 10 mg of palladium black; the catalyst was then filtered off and the acetic acid solution evaporated to dryness *in vacuo*. The compound obtained as an oily residue travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.79 (revealed with chlorine and with Folin–Ciocalteu's reagent). It was dissolved in 1 ml of trifluoroacetic acid and after 15 min at room temperature, 5 ml of ether was added. The precipitate was centrifuged and washed three times with ether; yield from protected tetrapeptide VIII, 11.5 mg, 77%. The tetrapeptide hydrazide obtained travels as a single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.14 (revealed with chlorine, with ninhydrin, and with Folin–Ciocalteu's reagent).

The preceding compound was dissolved in 2 ml of aqueous acetic acid (60% v/v); 10 mg of active MnO_2 (Attenburow *et al.*, 1952) was added and the mixture was stirred 30 min at room temperature. The MnO_2 was then filtered off and washed with aqueous acetic acid; the solution was concentrated to about 0.5 ml and applied to a column of Chelex 100 (Bio-Rad, H^+ form, 1 × 7 cm) equilibrated against 1 N aqueous acetic acid. Elution was effected with 1 N acetic acid, and 2-ml fractions were collected; fractions 2–6, containing ninhydrin-positive material, were pooled and concentrated to dryness. Addition of ethanol to the residue gave crystals which were collected by centrifugation and dried: yield from tetrapeptide hydrazide, 7.5 mg, 70%. The peptide travels as a single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.08 (revealed with ninhydrin); $[\alpha]_D^{25}$ −69° (*c* 0.1 water). A sample (0.5 mg) was hydrolyzed 6 hr at 110° in a sealed tube with 1 ml of 6 N HCl for amino acid analysis: glutamic acid = 1.00, alanine = 2.15, diaminopimelic acid = 0.98.

Synthesis of Peptide D-*Ala*-γ-L-*Glu*-(D)*meso*-*DAP*(L)-L-*Ala* (*V*). As in the case of the tetrapeptide I, the synthesis of peptide V may be divided into three steps (Figure 4): preparation of a derivative of the *meso*-diaminopimelic acid protected on the amino group adjacent to the L-asymmetric carbon and on the carboxyl group adjacent to the D-asymmetric carbon: Z-(L)*meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl (IV); coupling of IV with γ-*N*-hydroxysuccinimide Z-D-Ala-α-L-GluOBzl (XIII), coupling of Z-(L),Z-D-Ala-γ-L-Glu(α-OBzl)-(D)-*meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl (XIV) so obtained, with the L-alanine benzyl ester (Zervas *et al.*, 1957) by the mixed anhydride method, to obtain Z-(L),Z-D-Ala-γ-L-Glu(α-OBzl)-(D)*meso*-DAP(L)-L-AlaOBzl-(D)-NHNH-*t*-butyloxycarbonyl (XV). The free tetrapeptide V was obtained by catalytic hydrogenation, acidolysis by trifluoroacetic acid, and oxidation by MnO_2.

The intermediate derivative IV was previously obtained

(Bricas *et al.*, 1967) and its preparation (see Figure 5) was revised (*vide infra*).

Z-(L)*meso-DAP*(D)-*NHNH-t-butyloxycarbonyl* (*IV*). *meso*-DAP-bis-(NHNH-BOC) (1.2 g, 2.86 mmoles; Dezélée and Bricas, 1967) was dissolved in 30 ml of water and hydrolyzed by calflens leucine aminopeptidase (3 ml of a 0.4-mg/ml solution) under the conditions previously described (Dezélée and Bricas, 1967). At the end of the reaction, the pH was adjusted to 9.0 and the temperature was lowered to about 0°. Benzyloxycarbonyl chloride (0.92 ml, 6.50 mmoles) was then added and the mixture was rigorously stirred at pH 9.0. After 40 min the rate of N acylation, as recorded by pH-Stat, decreased significantly. The mixture was extracted twice with 30 ml of ether (the precipitate of Z-NHNH-*t*-butyloxycarbonyl also formed during the acylation was thus extracted). The aqueous layer was acidified to pH 3.0 and extracted four times with 25 ml of ethyl acetate. The organic layers are pooled and dried over $MgSO_4$. The ethyl acetate was evaporated and the residue was crystallized from ether–hexane and recrystallized from ether: yield 0.62 g of crystals, 49%; mp 180–182° dec; $[\alpha]_D^{25}$ +3.6° (*c* 0.5, methanol). *Anal.* Calcd for $C_{20}H_{30}N_4O_7 \cdot 0.5\ H_2O$ (447.50): C, 53.7; H, 6.98; N, 12.5. Found: C, 53.3; H, 7.03; N, 12.2. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.82 and in solvent system 1-butanol–ethanol–ammonium hydroxide–water (80:20:10:20), R_F 0.56 (revealed with ninhydrin, with chlorine, and with Folin–Ciocalteu's reagent).

The stereochemical structure of the product thus obtained was controlled after the synthesis of DNP-(D)*meso*-DAP (Bricas *et al.*, 1967), oxidized by *C. adamanteus* L-amino acid oxidase as previously described (Dezélée and Bricas, 1968).

Chromatography of the aqueous layer before extractions shows a compound revealed with ninhydrin and with Folin–Ciocalteu's reagent with a slightly lower R_F than the derivative IV in the second solvent system mentioned above: 0.51 instead of 0.56. We assumed that this compound was Z-(D)-*meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl; its amount may be evaluated from the spot intensities to about 20 to 30% of the Z-(L)*meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl (IV). With the procedure of extractions described here this side product remains in the aqueous phase after acidification to pH 3.0 and extraction with ethyl acetate. Thus the monoacylation of *meso*-DAP(D)-NHNH-*t*-butyloxycarbonyl at pH 9.0 is not entirely stereoselective as previously assumed (Bricas *et al.*, 1967), but rather preferential.

Z-D-*Ala*-α-L-*GluOBzl*. α-L-GluOBzl (270 mg, 1.14 mmoles) (obtained by removal of the BOC-protecting group from BOC-α-L-Glu-OBzl prepared by the method of Nefkens and Nivard (1964)) was dissolved in 5 ml of water. To the stirred solution were added 0.159 ml (1.14 mmoles) of triethylamine and 370 mg (1.14 mmoles) of Z-D-AlaOSU (Anderson *et al.*, 1964), dissolved in 5 ml of dimethoxy-1-2-ethane; 4 hr later, the solvent was evaporated *in vacuo* and the residual aqueous solution was acidified to pH 2.5 with 2 N HCl. It was then extracted three times with 10 ml of ethyl acetate, the organic layers were pooled, dried over $MgSO_4$, and concentrated *in vacuo* to about 2 ml; hexane was added to crystallize the protected dipeptide: yield 400 mg, 80%; mp 132°; $[\alpha]_D^{25}$ −3.3° (*c* 1, methanol). *Anal.* Calcd for $C_{23}H_{26}N_2O_7$ (442.47): C, 62.4; H, 5.92; N, 6.33. Found: C, 62.5; H, 5.98; N, 6.49. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system 1-butanol–ethanol–ammonium hydroxide–water (80:20:10:20), R_F 0.54 (revealed with chlorine).

Z-(L),*Z*-D-*Ala*-γ-L-*Glu*(α*OBzl*)-(D)*meso-DAP*-(D)-*NHNH-t-butyloxycarbonyl* (*Dicyclohexylammonium Salt*) (*XIV*). The γ-*N*-hydroxysuccinimide ester XIII of the protected dipeptide could not be crystallized and was directly used for coupling with IV.

Z-D-*Ala*-α-L-*GluOBzl* (150 mg, 0.34 mmole) was dissolved in 10 ml of dimethoxy-1-2-ethane. *N*-Hydroxysuccinimide (40 mg, 0.34 mmole) and dicyclohexylcarbodiimide (70 mg, 0.34 mmole) were then added. The mixture was stirred overnight at room temperature. The precipitate of dicyclohexylurea was filtered off and the solution was concentrated *in vacuo* to dryness.

The residue was dissolved in 15 ml of dimethoxy-1-2-ethane and added to a solution of 150 mg (0.34 mmole) of IV and 0.037 ml (0.34 mmole) of *N*-methylmorpholine in 15 ml of water. The mixture was stirred for 6 hr at room temperature. The solvent was then removed *in vacuo*. The aqueous solution was acidified to pH 3.0 and extracted with ethyl acetate; the pooled organic layers were dried over $MgSO_4$ and the solvent was evaporated to dryness. The residual oil was dissolved in 3 ml of acetone; 0.07 ml of dicyclohexylamine was added to the solution and the dicyclohexylammonium salt of the tripeptide thus obtained was crystallized from acetone–hexane: yield 265 mg, 73%; mp 125–127°; $[\alpha]_D^{25}$ +13.0° (*c* 0.3, methanol). *Anal.* Calcd for $C_{55}H_{77}N_7O_{13} \cdot H_2O$ (1062.29): C, 62.2; H, 7.49; N, 9.23. Found: C, 61.7; H, 7.26; N, 9.19. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.88 (revealed with chlorine and with Folin–Ciocalteu's reagent).

Z-(L),*Z*-D-*Ala*-γ-L-*Glu*(α-*OBzl*)-(D)*meso-DAP*-(L)-L-*AlaO-Bzl*-(D)-*NHNH-t-butyloxycarbonyl* (*XV*). A solution of 2.65 mg (0.25 mmole) of dicyclohexylammonium salt of the protected tripeptide XIV in 5 ml of methanol was acidified to pH 3.0 with HCl in tetrahydrofuran. The solvent was evaporated *in vacuo* and the residue was triturated with 2 ml of tetrahydrofuran; the precipitate was filtered off and washed with 2 ml of tetrahydrofuran. The solution was cooled to −10°, and 0.033 ml (0.25 mmole) of isobutyl chlorocarbonate and 0.035 ml (0.25 mmole) of triethylamine were added. After 5-min stirring at −10° a solution of 105 mg (0.30 mmole) of L-AlaOBzl-*p*-toluenesulfonate (Zervas *et al.*, 1957) and 0.041 ml (0.30 mmole) of triethylamine in 4 ml of tetrahydrofuran was added to the mixture. The mixture was then allowed to rise to room temperature and then was stirred overnight. Tetrahydrofuran was removed *in vacuo* and the residue was taken up in 20 ml of ethyl acetate and 5 ml of water. The ethyl acetate solution was washed four times with a 10% citric acid solution, twice with water, six times with 1 M $NaHCO_3$, and twice with water. It was then dried over $MgSO_4$ and concentrated *in vacuo* to dryness. The oily residue was crystallized in ethanol–ether: yield 147 mg, 57%; mp 159–160°; $[\alpha]_D^{25}$ −4.0° (*c* 0.2, methanol). *Anal.* Calcd for $C_{53}H_{65}N_7O_{14}$ (1026.15): C, 62.0; H, 6.39; N, 9.56. Found: C, 62.1; H, 6.53; N, 9.30. The compound travels as a single spot on silica gel thin-layer chromatography in solvent system A, R_F 0.98 (revealed with chlorine and with Folin–Ciocalteu's reagent).

D-*Ala*-γ-L-*Glu*-(D)*meso-diaminopimelic Acid*-(L)-L-*Ala* (*V*). XV (50 mg, 0.049 mmole) was hydrogenated for 18 hr in 3 ml of acetic acid with 20 mg of palladium black. The filtered

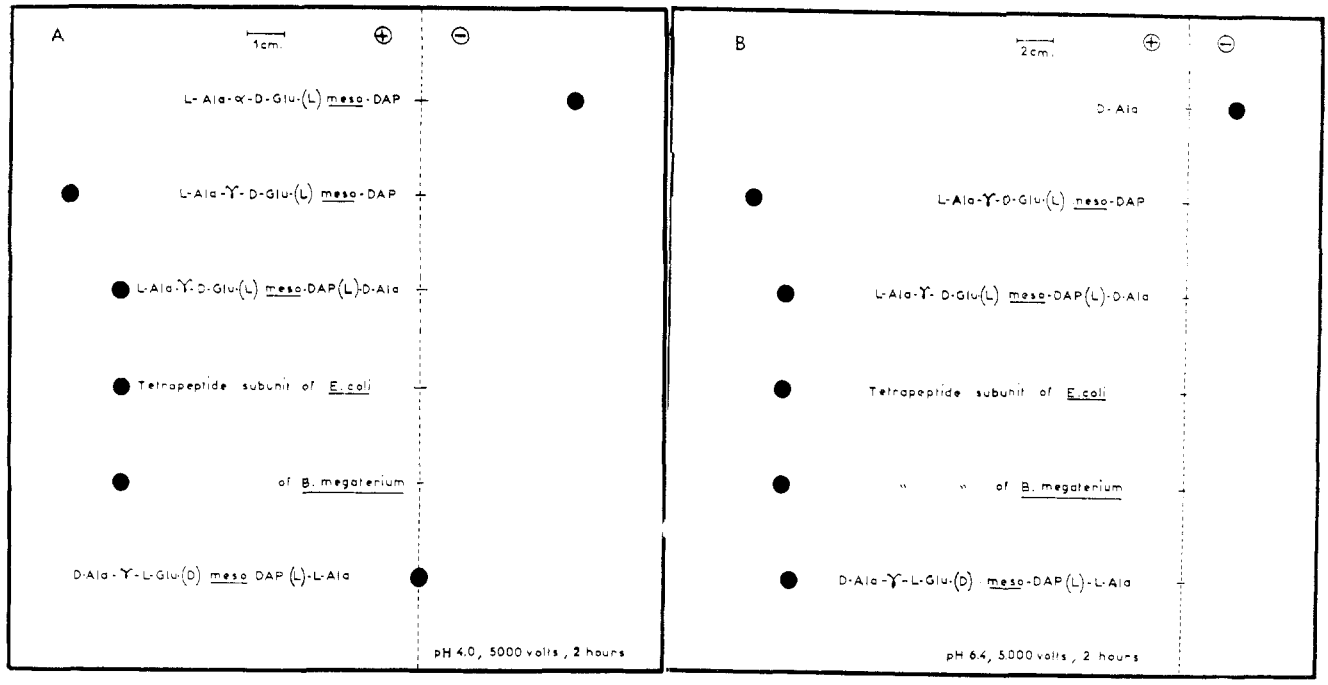

FIGURE 6: High-voltage paper electropherograms at pH 4.0 (A) and at pH 6.4 (B) of the isomeric synthetic tri- and tetrapeptides and the corresponding natural tetrapeptide subunits of the cell wall peptidoglycans of *E. coli* B and *B. megaterium* KM.

solution was concentrated to dryness *in vacuo*. The residue travels as single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.31 (revealed with ninhydrin, with chlorine, and with Folin–Ciocalteu's reagent). It was dissolved in 2 ml of trifluoroacetic acid and stirred for 15 min at room temperature. The trifluoroacetic acid was then evaporated and the residue was washed twice by trituration with ether. The compound travels as single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.50 (revealed with ninhydrin, with chlorine, and with Folin–Ciocalteu's reagent).

This residue was dissolved in 3 ml of aqueous acetic acid (60% v/v), and 15 mg of active MnO_2 was added. After 30-min stirring at room temperature, the MnO_2 was filtered off and washed with aqueous acetic acid. The filtrate and washings were concentrated to dryness *in vacuo*. The residue was dissolved in 10 ml of ethanol–water (50% v/v) and after cooling of the solution at $-10°$ the pH is adjusted to 9.0 with ammonium hydroxide. The solution was kept 6 hr at $-12°$; the precipitate of manganite formed was centrifuged off and washed twice with ethanol–water. The pH of the supernatant was then adjusted to 6.0 with acetic acid and concentrated to dryness *in vacuo*. Any contaminating ammonium acetate was evaporated under high vacuum until the weight of the residue was constant (yield from XV, 15 mg, 65%). The free tetrapeptide so obtained travels as a single spot on silica gel thin-layer chromatography in solvent system B, R_F 0.08 (revealed with ninhydrin); $[\alpha]_D^{25}$ $-1.5°$ (*c* 0.7, methanol).

The results of the amino acid analysis of a sample after hydrolysis in 6 N HCl for 6 hr at 110° were: glutamic acid = 1.00, alanine = 1.99, *meso*-diaminopimelic acid = 0.97.

Results and Discussion

Tetrapeptides I and V were submitted to paper electrophoresis at pH 4.0 and 6.4. As expected, the two peptides exhibited a significant difference in their migration at pH 4 (Figure 6). This can be explained by the difference of the p*K* of the carboxyl groups in the two peptides. In peptide I a carboxyl group is in α of a free amino group (p*K* between 2.1 and 2.4) and the two others are in α of a peptide bond (p*K* between 3.1 and 3.5), whereas in peptide V the three carboxyl groups are all in α of a peptide bond (p*K* between 3.1 and 3.5) (see p*K* values of amino acids and peptides in Greenstein and Winitz, 1961). Thus at pH 4.0, peptide I is expected to be more anionic than peptide V. This was actually observed. At pH 6.4 the two peptides had nearly the same mobility, the difference of p*K* of the carboxyl groups having no more effects on their ionization at this pH.

The tetrapeptide subunits of the cell wall peptidoglycans of *E. coli* B and *B. megaterium* KM have the same electrophoretic mobility as the tetrapeptide I at pH 4.0. The structure proposed, L-Ala-γ-D-Glu-(L)*meso*-DAP(L)-D-Ala, is therefore confirmed and especially these results clearly demonstrated that the C-terminal D-alanine is linked to the carboxyl group of the *meso*-diaminopimelic acid which is adjacent to the L-asymmetric carbon of this amino acid.

The synthesis of the peptides I and V represents the first application of a general method of stereospecific preparation of unsymmetrical peptides of *meso*-diaminopimelic acid. The main problem concerning the synthesis of such peptides is how to introduce selectively a substitution either on a functional group adjacent to the L-asymmetric carbon of the *meso*-diaminopimelic acid or on one adjacent to the D-asymmetric carbon of this amino acid.

Methods associating both enzymatic and chemical reactions were developed (Bricas *et al.*, 1962, Nicot and Bricas 1963a; Bricas and Nicot, 1966). Further progress on this route was the demonstration of the stereospecific hydrolysis by leucine aminopeptidase of the *N'*-α-L-aminoacyl-*N*-acylhydrazine derivatives (Nicot and Bricas, 1963b). Thus the key compound for these synthesis was obtained by the use of a symmetrical *meso*-diaminopimelic acid derivative, *meso*-DAP-bis(NHNH-t-butyloxycarbonyl) or *meso*-DAP-bis(NHNHZ) and by the enzymatic stereoselective splitting of the hydrazide bond in the α position of a free amino group belonging to the L moiety of the *meso*-diaminopimelic acid (Bricas and Nicot, 1966; Dezélée and Bricas, 1967). The next step was the stereospecific substitution of the amino groups of the *meso*-diaminopimelic residue by preferential N monoacylation of the *meso*-DAP(D)-NHNH-t-butyloxycarbonyl derivative (Dezélée and Bricas, 1967; Bricas *et al.*, 1967), or finally by the stereoselective method described in this paper.

With these methods it is now possible to synthesize any peptide or derivative of *meso*-diaminopimelic acid. Thus stereochemically defined compounds can be obtained and used as reference substances to solve special structural problems concerning the natural peptides of the *meso*-diaminopimelic acid and to study the particular stereospecificity of the bacterial peptidases that split peptide bonds present in the *meso*-diaminopimelic acid containing peptidoglycans.

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