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Occurrence of D-Alanyl-(D)-*meso*-diaminopimelic Acid and *meso*-Diaminopimelyl-*meso*-diaminopimelic Acid Interpeptide Linkages in the Peptidoglycan of *Mycobacteria*†

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ABSTRACT: Cross-linking between peptide units in the wall peptidoglycan of *Mycobacterium smegmatis* 21732 ATCC is mediated through D-alanyl-(D)-*meso*-diaminopimelic acid and *meso*-diaminopimelyl-*meso*-diaminopimelic acid linkages occurring in a ratio of about 2:1. The occurrence of D-alanyl-(D)-*meso*-diaminopimelic acid linkages was established by the action of the *Streptomyces albus* G DD-carboxypeptidase. The occurrence of *meso*-diaminopimelyl-*meso*-diaminopimelic acid linkages was established by isolation from partial acid hydrolysates and characterization by mass spectrometric analyses of the dipeptide *meso*-diaminopimelyl-*meso*-diaminopimelic acid and the tripeptide *meso*-diaminopimelyl-*meso*-diaminopimelyl-*meso*-diaminopimelic acid. *meso*-Diaminopimelyl-*meso*-diaminopimelic acid interpeptide linkages also occur in the wall peptidoglycan of *M. tuberculosis* BCG Pasteur strain.

The wall peptidoglycan presents a remarkable consistency of structure throughout the bacterial world (Ghuysen, 1968; Schleifer and Kandler, 1972). Basically, the glycan moiety consists of linear strands of alternating β-1,4-linked pyranoside *N*-acetylglucosamine and *N*-acetylmuramic acid residues. The carboxyl groups of the muramic acid residues are linked to peptide units having the general sequence L-Ala-D-Glu(L-R_3-D-Ala).[1] The peptide units of adjacent glycan strands are, in turn, cross-linked through bridges which extend from the C-terminal D-Ala of one tetrapeptide either to the ω-amino group of the L-R_3 residue or (through a Lys or Orn molecule) to the α-carboxyl group of D-glutamic acid of another peptide unit. The location of these bridges and their composition were used to divide bacterial species into four main chemotypes (Ghuysen 1968). From previous studies, the wall peptidoglycans of Mycobacteria appeared to be built up according to the same

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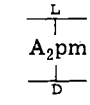
[1] Abbreviations used are: A_2pm, *meso*-diaminopimelic acid, the stereoisomery of which is represented as



R_3, third amino acid residue of a peptidoglycan (A_2pm, Lys, or Orn, etc.); N_2ph, dinitrophenyl.

framework (Lederer, 1971). *N*-Acetylmuramic acid, however, was found to be replaced by *N*-glycolylmuramic acid (Adam *et al.*, 1969; Azuma *et al.*, 1970), but this variation failed to significantly alter the basic structure of the polymer. The peptide moiety was found to be composed of L-Ala, D-Ala, D-Glu, and A_2pm and yielded, through enzymatic degradations, fragments among which the amidated tetrapeptide

L-Ala-D-Glu(NH$_2$)-A_2pm(NH$_2$)-D-Ala

and the amidated tripeptide

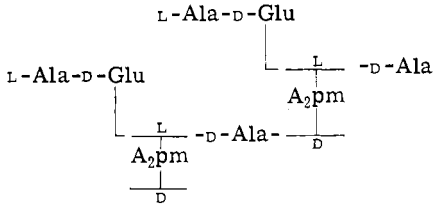
L-Ala-D-Glu(NH$_2$)-A_2pm(NH$_2$)

were isolated (Wietzerbin-Falszpan *et al.*, 1970; Kotani *et al.*, 1970). The absence of significant amounts of additional amino acids thus suggested that the interpeptide bridging was probably mediated through direct D-Ala(D)-A_2pm linkages (peptidoglycans of chemotype I; Ghuysen, 1968). Experiments devised in order to characterize these linkages led to the observation that part of the bridging in the wall peptidoglycans of Mycobacteria was mediated through a heretofore unknown type of interpeptide linkage extending from a free carboxyl group of the *meso*-diaminopimelic acid residue of one peptide unit to an amino group of the *meso*-diaminopimelic acid residue of another peptide unit.

Materials and Methods

Wall Peptidoglycan of M. smegmatis Strain 21732 ATCC. Cells grown on Sauton's medium in Roux bottles for 9 days at 37° were disrupted with a French pressure cell. The walls were sequentially treated with trypsin and chymotrypsin, delipidated with acetone, alcohol–ether (1:1), and chloroform, and then treated with 0.1 N HCl (50 ml/g of walls) for 12 hr at 60°, delipidated with chloroform, and dried with acetone. For more details, see Wietzerbin-Falszpan *et al.* (1973).

Enzymes. Chalaropsis endo-N-acetylmuramidase hydrolyzes β-1,4-*N*-acetyl(or *N*-glycolyl)muramyl-*N*-acetylglucosamine linkages, hence producing fragments with *N*-acetyl(or glycolyl)muramic acid at the reducing end. This enzyme was a gift from Dr. J. H. Hash (1963). The DD-carboxypeptidase from *Streptomyces albus* G contained 0.9 unit/ml. One unit was defined in terms of micromoles of D-Ala liberated per minute from Ac_2-L-Lys-D-Ala-D-Ala when the tripeptide was incubated with the enzyme at 37° in 20 mM Tris-HCl buffer (pH 7.5) supplemented with 2 mM $MgCl_2$. This enzyme has a strict specificity for D-Ala-D-peptide bonds. It readily hydrolyzes the peptide dimer



into monomers by hydrolyzing the interpeptide D-alanyl-(D)-*meso*-diaminopimelic acid linkage. Amidation of the carboxyl group of *meso*-diaminopimelic acid causes a decreased effectiveness of the enzyme but does not abolish its activity (Leyh-Bouille *et al.*, 1970). Trypsin and chymotrypsin were kindly provided by Choay Laboratories, Paris.

Analytical Methods. Amino acids (after hydrolysis with 6 N HCl for 18 hr) and N-terminal groups were measured with the fluorodinitrobenzene technique (Ghuysen *et al.*, 1966) or with a Technicon amino acid analyzer (using a subtractive method for the N-terminal groups). D- and L-alanine were measured enzymatically and C-terminal groups were measured with the hydrazine technique (Ghuysen *et al.*, 1966). The stereoisomery of *meso*-diaminopimelic acid was established as described by Bricas *et al.* (1967). Amino sugars (after hydrolysis with 4 N HCl for 4 hr at 100°) were measured with the Elson-Morgan reaction (Ghuysen *et al.*, 1966) or with the help of a Technicon amino acid analyzer, and reducing groups were measured with a modified procedure of Park and Johnson (Ghuysen *et al.*, 1966). Muramic acid at the reducing end of glycan fragments was reduced with sodium borohydride (Ghuysen *et al.*, 1966); after hydrolysis with 4 N HCl at 100° for 4 hr, the remaining muramic acid was measured with a Technicon amino acid analyzer. Samples of *N*-acetylmuramic acid reduced and hydrolyzed under the same conditions were used as standards.

Paper Electrophoresis. Electrophoreses were carried out on Whatman No. 3MM paper (60 × 27 cm) under Varsol, using a Gilson electrophorator, at pH 3.5 (pyridine–acetic acid–water (1:10:90), v/v/v)) and 50 V/cm.

Chromatography. The following solvents were used: (I) 1-butanol–pyridine–acetic acid–water (30:20:6:24, v/v/v/v); (II) isobutyric acid–1 N ammonia (10:6, v/v). Thin-layer chromatography was carried out on silica gel or cellulose sheets (Eastman Kodak Co.,) and preparative chromatography on Whatman No. 3MM paper.

Partial acid hydrolyses were carried out in sealed tubes with 4 N HCl (0.1 ml/mg of compound), for 2 hr at 110°. The hydrolysates (filtered if necessary) were evaporated to dryness under vacuum.

Mass Spectrometric Analyses of Peptides. The peptides were acetylated and esterified as follows. The peptide (1 μmol) was dissolved in 50 μl of water and the solution was added under stirring with 200 μl of methanol and 80 μl of acetic anhydride. The mixture was allowed to stand at room temperature for 1 hr and then evaporated to dryness under vacuum. Disappearance of free amino groups was checked with ninhydrin reagent after chromatography of the acetylated peptide on a cellulose thin-layer plate with solvent I. The acetylated peptide was dissolved in 500 μl of a mixture of methanol containing 5% (w/w) dry HCl; the solution was allowed to stand at room temperature for 15 min and then evaporated to dryness under nitrogen. Mass spectra of the N-acetylated methyl ester derivatives of the peptides were obtained with an AEI MS 9 mass spectrometer operating at 70 eV and at a source temperature of 170–230°.

Results

Degradation of the Wall Peptidoglycan of M. smegmatis with Chalaropsis endo-N-Acetylmuramidase. The peptidoglycan preparation (100 mg, *i.e.* 40 μequiv of disaccharide peptide unit) in which muramic acid, glucosamine, A_2pm, Glu, and Ala occurred in a molar ratio of 1:1:1:1:1.40 was incubated with the *Chalaropsis* enzyme (3 mg in 50 ml, final volume, of 0.01 N sodium acetate buffer (pH 5.0)) for 18 hr at 37 °. The reaction mixture was centrifuged and the supernatant fraction, which contained 95% of the original peptidoglycan in the form of disaccharide units substituting the intact peptide moiety, was lyophilized, placed on two columns of Sephadex G50 and Sephadex G25 ($V_0 + V_i$ = 760 ml) connected in series and eluted with 0.1 M LiCl. Resolution of the degradation products into particular, well-separated fractions was not observed. The eluted material was divided into two main samples with elution volumes ranging from 360 to 468 ml and from 468 to 585 ml, respectively. Each sample was lyophilized and chromatographed separately in 0.1 N acetic acid on a column of Sephadex G25 ($V_0 + V_i$ = 415 ml). The first sample gave rise to a

TABLE I: Chemical Composition of the Wall Peptidoglycan of *M. smegmatis* and of Fractions Obtained after Sequential Degradations with *Chalaropsis endo-N*-Acetylmuramidase and *Streptomyces albus* G DD-Carboxypeptidase.

Starting Materials	Enzymatic Treatment	Fraction Obtained	Total A_2pm (μmol/100 mg of Peptidoglycan)	Amino Acid Composition (Molar Ratio)					Mono-NH_2-A_2pm per A_2pm Residue
				A_2-pm	Total Ala	L-Ala	D-Ala	D-Glu	
Peptidoglycan	None		40	1	1.40			1	0.20–0.25
Peptidoglycan	*Chalaropsis endo-N*-acetyl-muramidase	Fraction K_D = 0	15	1	1.40	0.93	0.47	0.95	0.16–(0.63)[a]
		Fraction K_D = 0.12	18	1	1.60	1.0	0.60	1	0.32–(0.68)[a]
		Fraction K_D = 0.34	1	1	1.60			1	0.48–(0.72)[a]
		Fraction K_D = 0.42	1	1	1.30			1	0.63–(0.76)[a]
		Fraction K_D = 0.51	0.5	1	1.70			1	1.00–(1.00)[a]
			35.5						
Pooled K_D = 0 and K_D = 0.12 fractions	DD-Carboxy-peptidase	Monomer		1	1.77			1	1.00
		Dimer		1	1.74			1	0.45
		Trimer		1	1.21			0.89	0.35

[a] After incubation with the DD-carboxypeptidase; all the fractions contained one disaccharide (β-1,4-*N*-acetylglucosaminyl-*N*-glycolylmuramic acid) per A_2pm residue.

fraction with a K_D value = 0. The second sample gave four fractions with K_D values of 0.12, 0.34, 0.42, and 0.51, respectively. Amino acid analyses and recoveries are given in Table I. The total amount of peptidoglycan recovered (35.5 μequiv) compared fairly well with the nominal amount of peptidoglycan hydrolyzed (40 μequiv). Strikingly, more than 90% of the degraded peptidoglycan was recovered in fraction K_D = 0 and K_D = 0.12 altogether. Reduction of the material contained in each of these fractions with sodium borohydride caused the total disappearance of the muramic acid residues, thus demonstrating that the glycan chains had been quantitatively degraded into disaccharide units and that the polymeric structure of both fractions was probably attributable to a high extent of peptide cross-linking. Estimation of the terminal amino groups of *meso*-diaminopimelic acid suggested that, on the average, fraction K_D = 0 was a hexamer of the disaccharide peptide and that fraction K_D = 0.12 was a trimer of the disaccharide peptide (Table I).

Hydrolysis of the D-Alanyl-(D)-meso-diaminopimelic Acid Interpeptide Linkages with the DD-Carboxypeptidase from Streptomyces albus G. Treatment of fraction K_D = 0 and of fraction K_D = 0.12 (see preceding section) with *Streptomyces albus* G enzyme resulted in the parallel exposure of (mono) terminal amino groups of *meso*-diaminopimelic acid and of terminal carboxyl groups of alanine. Chromatography on silica gel thin-layer plates with solvent II of both fractions K_D = 0 and K_D = 0.12, before and after treatment with the enzyme, showed that the hydrolysis of the sensitive bonds caused an increased mobility of the products. These results together with the known specificity of the DD-carboxypeptidase (see Materials and Methods) demonstrated the occurrence of D-alanyl-(D)-*meso*-diaminopimelic acid interpeptide linkages in the peptide moiety of the wall peptidoglycan. However, data of Table I also showed that, at completion of the reaction, about 30–40% of the *meso*-diaminopimelic acid residues of fractions K_D = 0 and K_D = 0.12 remained with both amino groups unexposed. This observation suggested that other interpeptide linkages that would involve *meso*-diaminopimelic acid but would not be sensitive to the DD-carboxypeptidase might occur as minor constituents of these fractions. Fractions K_D = 0 and K_D = 0.12 were pooled and an aliquot of the mixture (15 μequiv of peptidoglycan unit) was incubated with the DD-carboxypeptidase (1.35 units, in 6.5 ml, final volume, of 20 mM Tris-HCl buffer (pH 7.5)–2 mM $MgCl_2$) for 18 hr at 37°. The reaction mixture was chromatographed in 0.1 N acetic acid, on a column of Sephadex G25 ($V_0 + V_i$ = 330 ml), and yielded three fractions which, on the basis of their K_D values and chemical compositions (Table I), were characterized as disaccharide peptide monomer, bis(disaccharide) peptide dimer, and tris(disaccharide) peptide trimer, respectively. Strikingly, the total (L + D) alanine content of the tris(disaccharide) peptide trimer was very low, thus excluding the possibility that three peptide units, L-Ala-D-Glu(A_2pm-D-Ala), were interlinked through two D-alanyl-(D)-*meso*-diaminopimelic acid linkages (in which case the ratio Ala:A_2pm should be 2:1 or at least 1.67:1 if the peptide unit at the C terminal of the trimer lacked the D-alanine residue).

Partial Acid Hydrolysis of the Cell Wall Peptidoglycan and Isolation of the Dipeptide A_2pm-A_2pm and the Tripeptide A_2pm-A_2pm-A_2pm. The tris(disaccharide) peptide trimer isolated after sequential degradation of the wall peptidoglycan with *Chalaropsis* enzyme and *Streptomyces albus* G DD-carboxypeptidase (see preceding section) was submitted to partial acid hydrolysis (Materials and Methods) and the hydrolysate was in turn submitted to preparative paper electrophoresis at pH 3.5 (Materials and Methods) (Figure 1). Compounds 1–7 were eluted from the paper and analyzed (except compound 6 which was present only in trace amounts). Both compounds 5 and 7 were found to be only composed of diaminopimelic acid, which was found to be the meso isomer (Materials and Methods). By paper chromatography with solvent I, they migrate as single peptides and exhibited R_F values (relative to alanine) of 0.2 for peptide 5 and 0.1 for peptide 7 (Figure 2). Acid hydrolysis of the dinitrophenylated derivative of peptide 5 yielded equivalent amounts of di-N_2ph-A_2pm and mono-N_2ph-A_2pm, hence suggesting the dimeric structure A_2pm-A_2pm. Acid hydrolysis of the dinitrophenylated peptide 7 yielded a mixture of di-N_2ph-A_2pm and mono-N_2ph-A_2pm, suggesting a trimeric

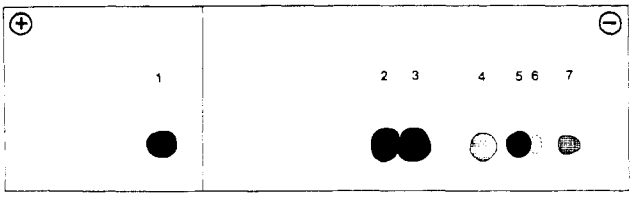


FIGURE 1: Paper electrophoresis at pH 3.5 of a partial acid hydrolysate of the trimer of the disaccharide peptide (Table I) isolated after sequential degradation of the wall peptidoglycan with *Chalaropsis* enzyme and *Streptomyces albus* G DD-carboxypeptidase. Electrophoresis was carried out on Whatman No. 3MM paper, at pH 3.5 and 50 V/cm for 105 min: (1) glutamic acid; (2) *meso*-diaminopimelic acid; (3) alanine; (4) a peptide containing alanine and glutamic acid; (5) dipeptide $(A_2pm)_2$; (6) not identified; (7) tripeptide $(A_2pm)_3$.

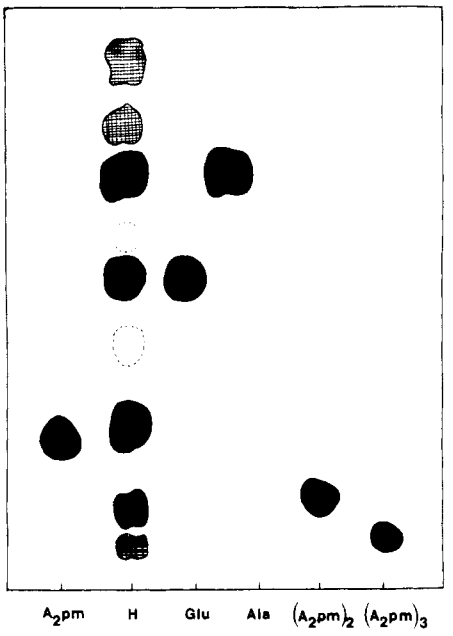
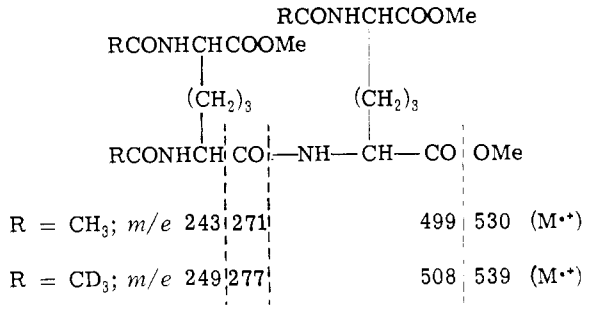


FIGURE 2: Paper chromatography of a partial acid hydrolysate of the trimer of the disaccharide peptide (H) (see Figure 1; Table I). Chromatography was carried out on Whatman No. 3MM paper with solvent I.

structure A_2pm-A_2pm-A_2pm. The structures were proved by mass spectrometry (see below). Subsequently, larger amounts of dipeptide A_2pm-A_2pm and tripeptide A_2pm-A_2pm-A_2pm were isolated directly from partial acid hydrolysates of intact peptidoglycan or even of crude, delipidated walls, by combined preparative paper chromatography in solvent I and paper electrophoresis at pH 3.5.

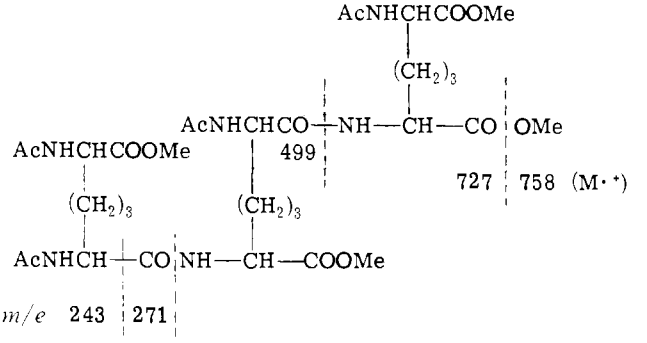
Isolation of the Dipeptide A_2pm-A_2pm and the Tripeptide A_2pm-A_2pm-A_2pm from the Wall Peptidoglycan of M. tuberculosis BCG Pasteur Strain. The peptide oligomer fractions obtained after degradation of the wall peptidoglycan of *M. tuberculosis* BCG with the *Myxobacter* ALI enzyme (Wietzerbin-Falszpan *et al.*, 1973) were submitted to partial acid hydrolysis. The hydrolysates were in turn submitted to paper chromatography and paper electrophoresis under the same conditions as above and yielded the previously described dipeptide A_2pm-A_2pm and the tripeptide A_2pm-A_2pm-A_2pm. The same peptides were also obtained from BCG cell walls.

Mass Spectrometric Analyses of Dipeptide A_2pm-A_2pm and Tripeptide A_2pm-A_2pm-A_2pm. The molecular ion at *m/e* 530 and the peaks at *m/e* 499, 271, and 243 observed in the mass spectrum of the *N*-acetyl methyl ester derivative of the dipeptide (Figure 3) and the molecular ion at *m/e* 539 and the peaks at *m/e* 508, 277, and 249 in the mass spectrum of its *N*-trideuterioacetyl methyl ester derivative are consistent with structure I.



I

Similarly, the molecular ion at *m/e* 758 and the peaks at *m/e* 727, 499, 271, and 243 in the mass spectrum of the *N*-acetyl methyl ester derivative of the tripeptide are consistent with structure II and they are shifted to appropriate masses in the mass spectrum of the *N*-trideuterioacetyl methyl ester derivative.



II

The mass spectrum of the *N*-acetyl methyl esters of both dipeptide and tripeptide, as well as the mass spectrum of the *N,N*-diacetyl dimethyl ester of A_2pm ($M^{\cdot+}$ 302), exhibited intense peaks at *m/e* 184 and 142. These are shifted to *m/e* 187 and 143, respectively, in the mass spectra of the *N*-trideuter-

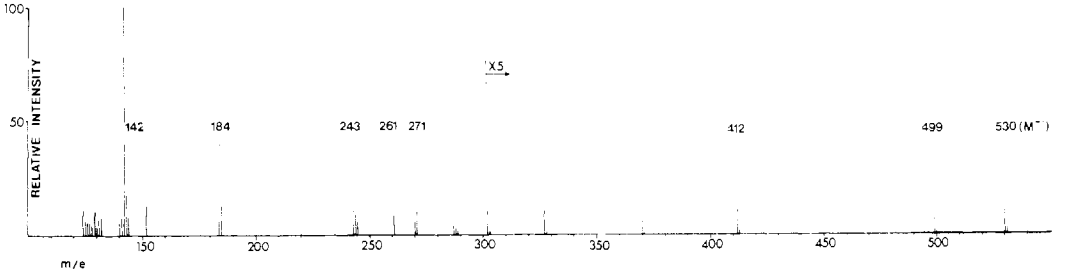
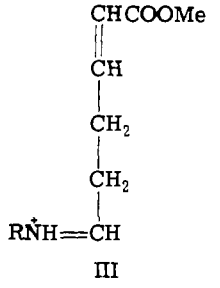


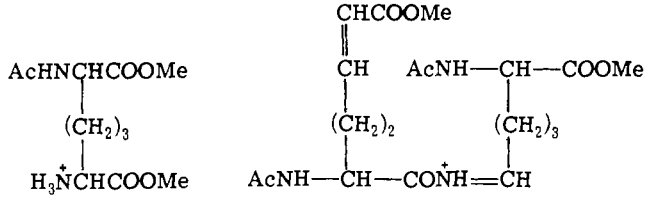
FIGURE 3: Mass spectrum of the *N*-acetyl methyl ester derivative of A_2pm-A_2pm.

ioacetyl methyl esters of both A_2pm and A_2pm-A_2pm, suggesting that the ions corresponding to these peaks originated from the N-terminal ends and could be explained by structure III, the transition *m/e* 184 → 142 being due to loss of elements of ketene from the *N*-acetyl groups. In addition, the mass spectra of the *N*-acetyl methyl esters of both the dipeptide and the tripeptide exhibited two other peaks at *m/e* 261 and 412 which are shifted to *m/e* 264 and 418, respectively, in the mass spectra of their *N*-trideuterioacetyl methyl esters.



III

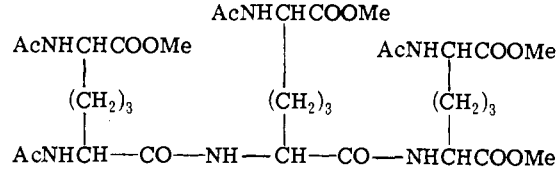
R	*m/e*
CH_3CO	184
H	142
CD_3CO	187
D	143



IV, *m/e* 261 V, *m/e* 412

The peak at *m/e* 261 may be attributed to ions represented by structure IV originating from the C-terminal ends after rearrangement of two hydrogen atoms, while the peak at *m/e* 412 may be assigned a structure V originating from the N-terminal ends.

The above results amply confirm the isolation of the dipeptide A_2pm-A_2pm and the tripeptide A_2pm-A_2pm-A_2pm. Based on our knowledge of the mycobacterial peptidoglycan (Wietzerbin-Falszpan, *et al.*, 1970; Lederer, 1971), the linkage between the second and the third A_2pm of the tripeptide should most probably be as represented in structure II; mass spectrometric analyses, however, did not allow us to exclude the possible isomeric formula VI.



VI

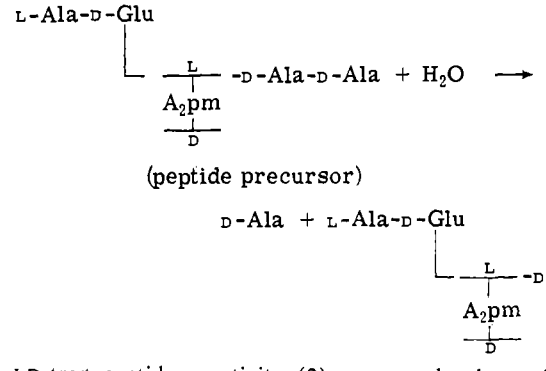
Discussion

The wall peptidoglycan of *M. smegmatis* is a highly cross-linked network in which 70–80% of the *meso*-diaminopimelic acid residues are involved in peptide cross-linkages (Cunto *et al.*, 1969, and this paper). A similar highly cross-linked structure was found for the peptidoglycan of *M. tuberculosis* $H_{37}R_v$ (Kotani *et al.*, 1970) and *M. tuberculosis* BCG (Wietzerbin-Falszpan *et al.*, 1973, and unpublished results). The hydrolytic action of the *Streptomyces albus* G DD-carboxypeptidase on the peptide moiety of the wall peptidoglycan of *M. smegmatis* showed that at least part of the bridging between L-Ala-D-Glu(A_2pm-D-Ala) units (containing two amide groups) was mediated through D-alanyl-(D)-*meso*-diaminopimelic acid linkages. Similarly, the hydrolytic action of the *Streptomyces* L3 D-Ala-A_2pm endopeptidase on the wall peptidoglycan of *M. tuberculosis* $H_{37}R_v$ (Kotani *et al.*, 1970) was consistent with the presence of the same type of interpeptide bond in this latter species. D-Alanyl-(D)-*meso*-diaminopimelic interpeptide linkages are characteristic of peptidoglycans of chemotype I (Ghuysen, 1968).

The low alanine content of the wall peptidoglycans of Mycobacteria *M. smegmatis* (Table I), *M. tuberculosis* BCG (Wietzerbin-Falszpan *et al.*, 1973), and *M. tuberculosis* $H_{37}R_v$ (Kotani *et al.*, 1970), however, was a first indication that interpeptide linkages involving *meso*-diaminopimelic acid but not D-alanine might be present in addition to D-alanyl-(D)-*meso*-diaminopimelic acid linkages. The isolation and mass spectrometric characterization of the dipeptide A_2pm-A_2pm and the tripeptide A_2pm-A_2pm-A_2pm in the wall peptidoglycan of *M. smegmatis* provided experimental support for this idea and established that part of the peptide cross-linkages extended between *meso*-diaminopimelic acid residues. From the present analyses, D-Ala-(D)-A_2pm and A_2pm-A_2pm linkages occur in a ratio of about 2:1. The dipeptide A_2pm-A_2pm was also isolated from partial acid hydrolysates of the wall peptidoglycan of *M. tuberculosis* strain Brévannes (unpublished results) and both dipeptide A_2pm-A_2pm and tripeptide A_2pm-A_2pm-A_2pm were isolated from the wall peptidoglycan of *M. tuberculosis* BCG. At present, the pattern of distribution of both D-Ala-(D)-A_2pm and A_2pm-A_2pm interpeptide linkages within these wall peptiodoglycans is not known.

One possible mechanism for the synthesis of the interpeptide bonds A_2pm-A_2pm could be the sequential involvement of a DD-carboxypeptidase and of a LD-transpeptidase, according to the following reactions:

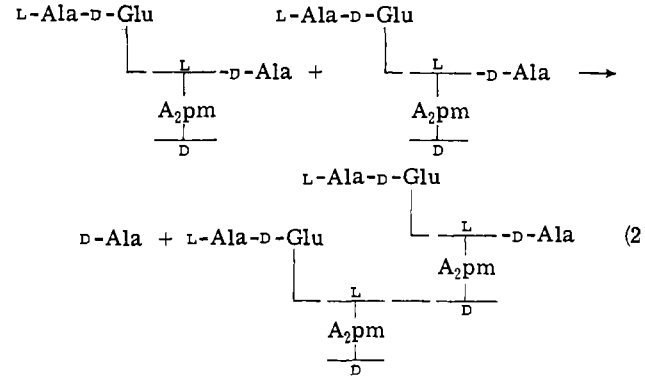
DD-carboxypeptidase activity (1)



(peptide precursor)

(1)

LD-transpeptidase activity (2) as recently shown in isolated membranes of *S. faecalis* (Coyette *et al.*, 1974)



(2)

According to this scheme, the peptide bonds in the isolated dipeptide A_2pm-A_2pm and tripeptide A_2pm-A_2pm-A_2pm should extend from an L carbon to a D carbon. This problem is currently under investigation.

Atypical N-ω-(L-R_3)-L-R_3 interpeptide linkages between L-Ala-D-Glu(L-R_3-D-Ala) peptide units—like the A_2pm-A_2pm peptide bonds in Mycobacteria—might occur as minor components of many bacterial peptidoglycans and might have escaped detection during previous studies.

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Isolation and Characterization of a Novel Lipid, 1(3),2-Diacylglyceryl-(3)-*O*-4′-(*N,N,N*-trimethyl)homoserine, from *Ochromonas danica*

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ABSTRACT: The phytoflagellate *Ochromonas danica* is unique in containing high concentrations of the detergent-like chlorosulfolipids, about 0.2 μmol/mg of total cell protein; in contrast, the organism contains less than 0.01 μmol of phospholipids/mg of protein. A second novel polar lipid has now been isolated from *O. danica* and characterized as 1(3),2-diacylglycerol-3(1)-*O*-4′-(*N,N,N*-trimethyl)homoserine, by chemical and mass spectrometric analysis. Its concentration in *O. danica* is 0.14 μmol/mg of protein, and it is suggested that this lipid, rather than the detergent-like chlorosulfolipids, largely substitutes for the usual phospholipids in membrane bilayer structures in *O. danica*.

The occurrence of unusual lipids is fairly common in bacteria and protozoa (see reviews by Thompson and Nozawa, 1972; Kates, 1964). Recently, a novel class of lipids has been isolated and characterized in the phytoflagellate, *Ochromonas danica* (Elovson and Vagelos, 1969). These compounds are derivatives of docosane 1,14-disulfate (Mayers and Haines, 1967), where up to six chlorine atoms are substituted for hydrogens on the hydrocarbon backbone (Elovson and Vagelos, 1969, 1970).

The presence of the sulfate groups in these unique lipids make them potent anionic detergents which in fact inhibit a number of enzymes *in vitro* (Elovson and Vagelos, 1969). Since preliminary results suggest that the chlorosulfolipids are enriched in cellular membranes, the present work was undertaken to characterize the other polar lipids of *O. danica*. This paper reports on the isolation and characterization of yet another novel lipid from *O. danica*, which consists of a *N,N,N*-trimethylhomoserine group linked to a 1,2-digylceride moiety through an oxygen-ether bond. This zwitterionic lipid constitutes over 50% of the total "normal" polar lipids in *O. danica*.

Experimental Section

Methods

Culture. Ochromonas danica was obtained from the American Type Culture Collection, ATCC 30004. Growth medium

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