# ISOLATION OF UDP-N-GLYCOLYLMURAMYL-(ALA, GLU, DAP) FROM MYCOBACTERIUM PHLEI

J.-F. PETIT, A. ADAM and J. WIETZERBIN-FALSZPAN
*Institut de Biochimie, Faculté des Sciences, 91 Orsay, France*

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## 1. Introduction

In the cell wall of various species of *Mycobateria*, muramic acid is present as its *N*-glycolyl derivative and glucosamine as its *N*-acetyl derivative [1–3].

Studies on the biosynthesis of *N*-acetylmuramic acid [4–6] have shown that this compound is synthesized as UDP-*N*-acetylmuramic acid by condensation of enolpyruvate with UDP-*N*-acetylglucosamine and subsequent reduction.

In a previous paper we suggested [2] that *N*-glycolylmuramic acid might be synthetized by the following pathway.

UDP-*N*-acetylglucosamine → UDP-*N*-acetylmuramic acid → UDP-*N*-glycolylmuramic acid.

This pathway is in accordance with the one proposed by Schoop et al. [7] for the biosynthesis of *N*-glycolylneuraminic acid from *N*-acetylneuraminic acid.

To test this hypothesis we wished to isolate from a *Mycobateria* a muramic acid containing precursor of the peptidoglycan and to identify its *N*-acyl substituent.

To obtain such a precursor we used D-cycloserine, an antibiotic which inhibits the biosynthesis of D-alanyl-D-alanine, and thus leads to the accumulation of UDP-*N*-acylmuramyl (Ala, Glu, DAP) [8–10] in the cells of sensitive bacteria.

## 2. Material and methods

*Mycobacterium phlei*, strain no. 356 A.T.C.C., known to contain *N*-glyclolylmuramic acid in its peptidoglycan [3], was grown in Brodie's medium [11] in a Giratory shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) at 37°C in 2 l erlenmeyer flasks containing 800 ml of medium. The growth of the culture was followed by measuring its optical density at 600 nm.

Chromatography was performed on Whatman no. 1 paper in the following solvents:

1. Isobutyric acid-N ammonia (10:6);
2. Ethanol-M ammonium acetate pH 7.5 (7.5:3);
3. Butanol-pyridine-water (5:3:2);
4. Butanol-acetic acid-water (5:1:2).

Uridine was identified by its absorption spectrum at pH 7 ($\lambda_{max}$ = 262 nm), the absence of modification of the spectrum by acidification at pH 2 and the spectral shift between pH 7 and 11 [12].

Muramic acid was identified by its elution volume in a Technicon amino acid analyser** and its absorption spectrum in the Elson-Morgan reaction after total hydrolysis (HCl 4 N, 6 hr 100°C). It was determined quantitatively by the latter reaction.

Alanine, glutamic acid and $\alpha,\alpha'$-diaminopimelic acid (DAP) were determined with a Technicon amino acid analyser**.

*N*-glycolylmuramic acid was a synthetic product prepared by Dr. P.Sinaÿ [2,13]. *N*-acetylmuramic acid was prepared from cell walls of *Micrococcus lysodeikticus* as described in [2].

* 117th communication on the constituents of *Mycobateria*. 116th communication see: C. Amar-Nacasch and E.Vilkas, Bull. Soc. Chim. Biol., in press.

** We wish to thank Dr Cl.Gros and Mrs B.Charetteur for performing the analyses.

## 3. Results

D-cycloserine, kindly given by "Produits Roche S.A." (10 Rue Crillon, Paris 4e), was added at a concentration of 10 μg/ml to the culture at two thirds of the exponential phase. The culture was then shaken for three additional hours and the cells harvested in a refrigerated centrifuge (15 min, 27,500 *g*).

From 4 l of culture, 17.5 g of cells (wet weight) were obtained. These cells, which have a definite tendency to autolysis, were resuspended into 87.5 ml of cold water and sonicated for 25 min (30 ml of suspension at a time) in a 10 kc Raytheon sonic oscillator. The resulting suspension was centrifuged for 45 min at 27,500 *g*. A 20% trichloracetic acid (TCA) solution was added in the cold to the supernatant to obtain a final concentration of 5% TCA. The material precipitated by TCA was removed by a centrifugation of 10 min at 27,500 *g*. The resulting supernatant was extracted five times with an equal volume of ether in the cold in order to remove TCA, concentrated to 5 ml in a rotating evaporator and filtered through two connected columns of Sephadex G-25 (h = 91 cm; $\phi$ = 2.5 cm) and G-15 (h = 84 cm; $\phi$ = 2.5 cm) in 0.02 M ammonium acetate [14].

The effluent shows four well separated peaks of absorption at 260 nm. The first of them, which has a $K_D$ of 0.11, is the only one to contain muramic acid, as determined by its absorption spectrum in the Elson-Morgan reaction after hydrolysis (HCl 4 N, 6 hr, 100°).

The material from this peak was lyophylised and the ultra-violet absorbing material was purified by preparative chromatography in solvent (1) ($R_{UDP\text{-}GlcNac}$ = 0.63) and in solvent (2) ($R_{UDP\text{-}GlcNac}$ = 0.23) (GlcNac = *N*-acetylglucosamine).

The resulting nucleotide contains equimolar ratios of uridine, muramic acid, alanine, glutamic acid and DAP.

By hydrolysis in 0.023 N HCl at 100°C, it gives rise to a Morgan-Elson reactive product; as judged by this reaction, hydrolysis is complete within 20 min. The products of the hydrolysis were identified by chromatography in solvent (2) as UDP and UMP ($R_{UDP\text{-}GlcNac}$ = 0.29 and 0.5) and a ninhydrin reactive compound ($R_{UDP\text{-}GlcNac}$ = 0.70) containing equimolar amounts of muramic acid, alanine, glutamic acid and DAP.

The latter compound was used to identify the *N*-acyl substituent of muramic acid in the nucleotide precursor. After acid hydrolysis (2 hr 2 N $H_2SO_4$, 100°C) the hydrolysate was extracted 10 times with ether; the ether was evaporated and glycolic acid was identified with the Eegriwe reagent [2, 15, 16].

Two enzymes known to split the linkage between *N*-acylmuramic acid and alanine in soluble fragments of peptidoglycan, namely the *N*-acylmuramyl-L-alanine amidase from *Streptomyces albus* G (kindly given by Professor Ghuysen) [17] and the bacteriolytic enzyme from *Myxobacter* $AL_1$ [18] were unable to release *N*-acylmuramic acid from the *N*-acylmuramyl-tripeptide.

It is known from the work of Perkins [19], Ghuysen et al. [20] and Tipper [21] that alkali treatment of muramic acid or derivatives whose reducing group are free leads to quantitative elimination of the lactate moiety; muramic acid is converted to an insaturated glucosamine derivative, probably $\Delta$-2, 3-glucosamineen. When muramic acid is *N*-acylated, the product obtained, presumably $\Delta$-2, 3-*N*-acylglucosamineen, is a chromogen able to react at room temperature on paper chromatograms with the Ehrlich reagent, giving a violet product [20, 21].

In preliminary experiments, *N*-glucolylmuramic acid and *N*-acetylmuramic acid were treated with 0.02 M phosphate buffer, pH 12.5 at 37° for two hours, chromatographed in solvents (3) and (4) and the chromatograms were sprayed with the Ehrlich reagent. As shown in table 1, the chromogens arising from *N*-glycolylmuramic acid and *N*-acetylmuramic acid can be differentiated by their chromatographic mobilities.

We therefore treated the *N*-acylmuramylpeptide in the same way and we obtained a single chromogen having the same $R_{GlcNac}$ as the chromogen arising from *N*-glycolylmuramic acid (table 1).

Table 1

| Origin of the chromogen | Solvent 3 | Solvent 4 |
|---|---|---|
| *N*-acetylmuramic acid | 1.60 | 1.60 |
| *N*-glycolylmuramic acid | 1.44 | 1.25 |
| *N*-acylmuramyl-(Ala,Glu,DAP) from D-cycloserine treated cells of *M. phlei* | 1.44 | 1.25 |

$R_{GlcNac}$ of the chromogens formed by treating *N*-subtituted muramic acid derivatives at pH 12.5 for 2 hr at 37°C.

## Conclusion

The *N*-substituent of muramic acid in the UDP-*N*-acylmuramyl-tripeptide which accumulates in cells of *M. phlei* incubated in the presence of D-cycloserine is a glycolyl group; thus, in this microorganism, muramic acid is *N*-glycolytated prior to its incorporation into the peptidoglycan. This is in favour of the existence of a specific enzyme oxidizing UDP-*N*-acetylmuramic acid to UDP-*N*-glycolylmuramic acid.

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