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Note

N.m.r. titration of a natural immunoadjuvant, disaccharide-pentapeptide, peptidoglycan monomer and related compounds

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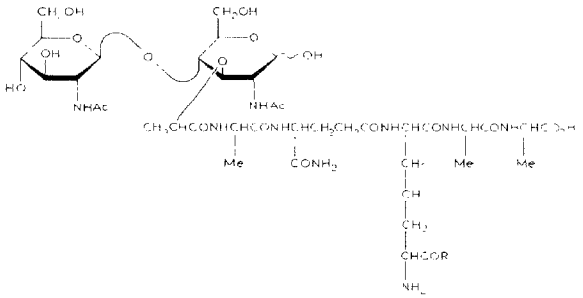
Peptidoglycans, which are common constituents of the bacterial cell-wall, exhibit various biological activities[1]. The natural biopolymer and synthetic analogues of low molecular weight show marked immunostimulating properties[2–5].

The peptidoglycan monomer (PGM, **1**) used in this study was previously characterised[6–8] as [2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-3-*O*-(D-ethyl-1-carbonyl)-D-glucopyranose]-L-alanyl-D-isoglutaminyl-[(L)-*meso*-diaminopimeloyl-(L)-D-alanyl-D-alanine] (**1a**). In most instances, it has been found that PGM's from different sources contain one free amino and one free carboxyl group at the *meso*-diaminopimelic acid residue, and a second carboxyl group at the C-terminal alanine[9] (Ala-5).



1 R = NH
1a R = OH

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By using potentiometric titration, it has now been established that **1** consumes equivalent amounts of acid and base. Accordingly, **1** should contain an equal number of free carboxyl and amino groups.

In order to determine unambiguously the positions of the free carboxyl and

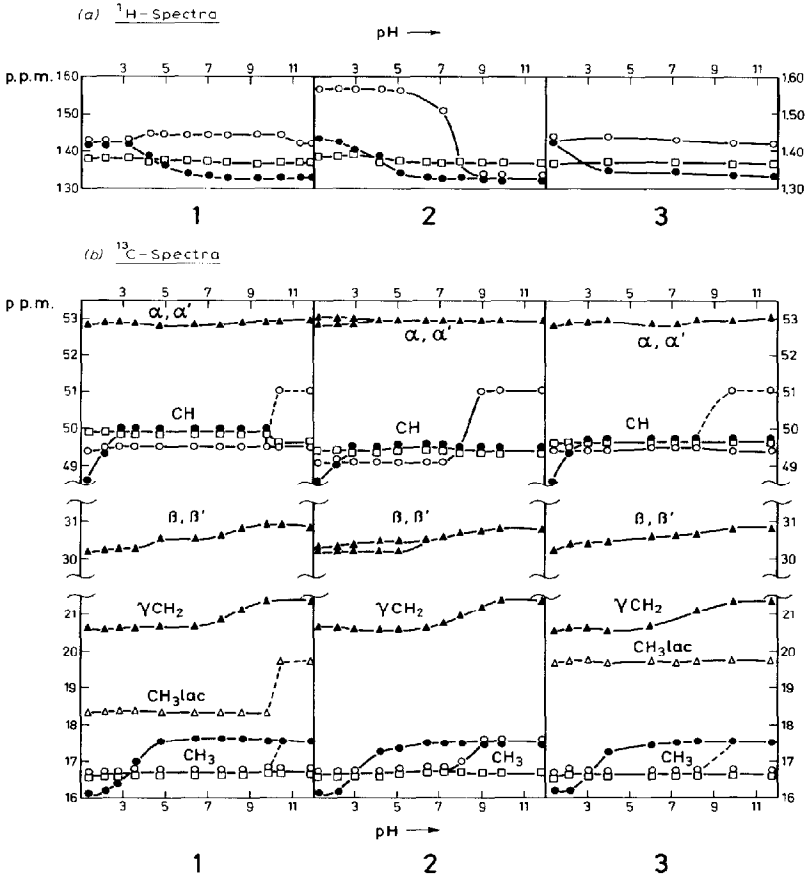

Fig. 1. N.m.r. titration (*a*, ^{1}H; *b*, ^{13}C) of peptidoglycan monomer (PGM, **1**), pentapeptide (**2**), and D-lactoylpentapeptide (**3**). Designation of signals: Ala-1 (—○—), Ala-4 (—□—), Ala-5 (—●—), *meso*-diaminopimelic acid residue (—▲—), D-lactoyl residue (—△—)

amino groups in **1**. ^{1}H- and ^{13}C-n.m.r. pH-titrations of **1** and compounds related to portions of **1**, namely, the pentapeptide[10] **2** and the lactoyl-pentapeptide[6] **3**, have been carried out. This approach is useful for determining the amino acid sequence of small peptides[11–14], and it has been applied to synthetic peptidoglycan pentapeptides[15]. Delbarre *et al.*[16], using this method, showed that tetra- and pentapeptides obtained from *Streptomyces stimulosus* cells contain one free carboxyl group at the LL-diaminopimelic acid residue.

The pH-dependence of methyl signals of alanine residues in **1–3** was clearly observable by ^{1}H-n.m.r. spectroscopy (Fig. 1a). Deprotonation of the *C*-terminal carboxyl groups produced an upfield shift of 0.095, 0.104, and 0.097 p.p.m. for **1**, **2**, and **3**, respectively, whereas protonation of the *N*-terminal amino group of alanine (Ala-1) in **2** caused a shift of 0.233 p.p.m. in the same direction. These values accord with literature data[11,15,16]. The methyl signals (1.38 p.p.m.) of the D-lactoyl residue and D-alanine at position 4 (Ala-4) in **1** and **2** coincide. The spectra of **1** and **3** contain the Ala-1 methyl signal at 1.43 p.p.m.; both of them are pH-independent.

The interpretation of ^{1}H-n.m.r. results was confirmed by the ^{13}C-n.m.r. experiments (Fig. 1b). On deprotonation of the *C*-terminal alanine (Ala-5) carboxyl group in **1–3**, the methyl and methine signals of Ala-5 residues shifted downfield by 1.37 and 1.04 p.p.m., respectively. Protonation of the *N*-terminal amino group of Ala-1 in **2** caused a downfield shift of the methyl and methine signals of 0.84 and 1.92 p.p.m., respectively.

The pH-independent methyl and methine signals of Ala-4 in all three compounds appeared at 16.65 and 49.45 p.p.m., respectively. The methyl signals of the D-lactoyl residue in **1** and **3** were at 18.35 and 19.65 p.p.m., respectively, and the former revealed a virtual pH-dependence at pH >9 (dashed portion of the curve labelled as CH_3lac). This behaviour can only be explained as a consequence of alkaline degradation of **1** into **3**; a longer exposure to such alkaline conditions (~10 days, room temperature) led to a partial degradation of **1** and **3** into **2** (dashed curves of the alanine signals). Additional evidence of degradation of **1** into **3** was a shift of the methine signal of the D-lactoyl residue to higher field (from 77.45 p.p.m.[8] to 67.6 p.p.m.).

The pH change from 7 to 10 shifted the γ-methylene signal of the *meso*-diaminopimelic acid residue downfield by 0.68 p.p.m. in **1** and **2**, and 0.79 p.p.m. in **3**; the β'-methylene signals in **1–3** were also shifted downfield by 0.56 p.p.m. The β-methylene and α,α'-methine signals (except α'-methine in **2**) were shown to be pH-independent. The somewhat unexpected shift of γ-methylene signals might be related to the similar behaviour of the δ-carbon atom of lysylglycine, which has been attributed[14] to a combination of electrostatic and inductive effects. However, in the present case, the pH effect is ~6 times smaller, probably because the γ-carbon atom in the *meso*-diaminopimelic acid residue is more remote from the titrateable amino group than in lysylglycine.

From the data shown in Fig. 1, the approximate pK values of the dissociable

groups in **1**–**3** have been estimated. Thus, the p*K* of the *C*-terminal alanine residues in **1** and **2** is 3.5, and that in **3** is 3.0, whereas the p*K* of the amino group of the *meso*-diaminopimelic acid residues in **2** and **3** is 8.5, and in **1** is 8.2. The p*K* of the amino group of the *N*-terminal alanine in **2** is 7.6.

These results show that **1** contains only two dissociable groups, namely, the carboxyl group of the *C*-terminal alanine, and the amino group of the *meso*-diaminopimelic acid residue at the D-chiral centre. Contrary to the previous assumption[8], the carboxyl group at the (D)-*meso*-diaminopimelic acid residue should be substituted. The nitrogen analysis of **1** points to the presence of one amide group, and the only reasonable suggestion seems to be an amido group at the D-chiral centre of the *meso*-diaminopimelic acid residue.

EXPERIMENTAL

PGM (**1**) was obtained by lysozyme-digestion of linear, non-cross-linked, peptidoglycan polymer-chains isolated from culture fluids of penicillin-treated *Brevibacterium divaricatum*[6,7]

Anal. Calc. for $C_{40}H_{67}N_9O_{21}$: N, 12.48. Calc. for $C_{40}H_{68}N_{10}O_{20}$: N, 13.88. Found: N, 14.03.

Pentapeptide **2** was obtained from **1** by hydrolysis with *N*-acetylmuramoyl-L-alanine amidase[10]. The lactoylpentapeptide **3** was obtained from **1** by reaction in aqueous ammonia[6].

Potentiometric titration was carried out with a PHM 82 standard pH-meter (Radiometer, Copenhagen) equipped with a TTT 80 titrator, ABU 80 autoburette, and REÇ 80 servograph. PGM (~5 mg) was titrated with 0.01M HCl or 0.01M NaOH, and, in the former case, retitrated with 0.01M NaOH.

N.m.r. spectra were recorded at room temperature, for 0.1M solutions in 99.75% D_2O in 5-mm o.d. tubes, with a JEOL FX 90 Q Fourier-transform spectrometer operating at 89.55 (^1H) and 22.5 MHz (^{13}C), respectively. The sweep width used for ^1H spectra was 1000 Hz, the pulse width was 14 or 29 μs, the acquisition time was 2.1 s, and the digital resolution was 0.0027 p.p.m. The sweep width used for ^{13}C spectra was 5200 Hz, the pulse width was 5 μs, the acquisition time was 2 s, and the digital resolution was 0.056 p.p.m. Chemical shifts were measured relative to internal 1,4-dioxane, set at 3.65 (^1H) and 66.6 p.p.m. (^{13}C) downfield of Me_4Si.

The pH of the solution for n.m.r. spectroscopy was adjusted with 6M HCl or 6M NaOH, without correction for pD.

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