# ANTITUMOUR GLYCOPEPTIDES FROM *LACTOBACILLUS BULGARICUS* CELL WALL

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## 1. Introduction

A crude preparation from *Lactobacillus bulgaricus* has been shown by one of us to show antitumour activity [1–4]. We have found this activity to be due to glycopeptides present in the preparation. The glycopeptides, polymer-homologs of molecular weight 1000, 2000 and approx. 10 000 designated as I, II and X, are apparently fragments of a peptidoglycan of the A4α type. Although adjuvant activity has been reported for a bacterial peptidoglycan and its fragments [5] this is the first time that a specific antitumour effect of bacterial cell wall glycopeptides has been observed. The highly promising biological properties of the glycopeptides I, II and X have served as impetus for their structural study, the results of which are presented in this paper.

## 2. Experimental

Antitumour activity was tested in mice inoculated subcutaneously with sarcoma S-180. Eight to ten days after inoculation, when the tumours were 10–12 mm in diameter, the animals were given intravenously a single dose of 4–6 mg of the substances under investigation.

The antitumour preparation from *L. bulgaricus* (strain *51*) was obtained by lysozyme and pepsin digestion of the bacterial cells according to Ref. [4]. *L. bulgaricus 51* cell walls were prepared by the method [6], and then digested by lysozyme. The neutral glycopeptides were separated from acidic and basic material by successive filtrations through Dowex 50 WX8 ($H^+$-form) and DEAE-cellulose and isolated by chromatography on Sephadex G-50 as shown in fig. 1.

Amino acids and amino sugars were analysed after hydrolysis of the glycopeptides in 5.7 N HCl (20 hr at 115°C) and in 4 N HCl (4 hr at 105°C), respectively, correction being made for the degradation of the

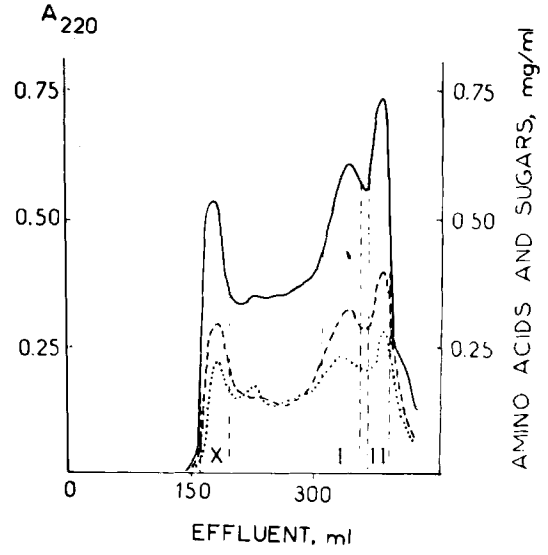



Fig. 1. Chromatography of neutral *L. bulgaricus* glycopeptides (200 mg) in aqueous solution on a Sephadex G-50 column (2.5 × 80 cm). Fractions X, I and II weighed 30, 58 and 42 mg, respectively. The optical density (at 220 nm) is shown by a solid line, the amino acid content (measured with Folin's reagent) by a dotted line, the sugar content (determined by the anthrone method) by a dashed line.

amino sugars under these conditions (approx. 20% for the 4 hr period). Volatile acids (acetic and lactic, identified as the *p*-bromophenacyl esters by mass-spectrometry) were liberated by heating with 4 N $H_2SO_4$ (3 hr at 100°C) and estimated in the distillate by titration with 0.01 N NaOH. For the enzymic hydrolysis, 1% glycopeptide solutions were incubated for 72 hr at 37°C with 0.1% pronase at pH 8.5. N-terminal amino acids were determined by the dansyl method. Molecular weights were determined in aqueous solutions osmometrically and by the sedimentation equilibrium method at 70 000 *g*. Electrophoresis was run at 12 V/cm on FN-2 paper at pH 3.5, 6.5 and 8.6 $^1$H- and $^{13}$CNMR spectra were measured in $D_2O$ at 100 and 25.16 MHz, respectively, CD-spectra in 0.1 N HCl.

## 3. Results and discussion

As can be seen from fig.1, the glycopeptide fraction of the cell wall digest comprised three major substances, I, II and X. The antitumour effect of these substances and of the crude *L. bulgaricus* preparation turned out to be most marked on sarcoma S-180, which was therefore used for the tests. Already 4–8 hr after administration of the preparation the effect could be detected as microscopic degenerations in the tumour cells. This progressed to profound necrosis within 24–72 hr, no relapses being observed in a one year period in 30–40% of the cases; the cured animals acquired immunity to replantation of the same tumour.

The three glycopeptides I, II and X are neutral as shown by their electrophoretic immobility and by their failure to adsorb onto cation and anion exchangers. They all appear to associate in aqueous solution, the apparent molecular weight, as measured osmometrically, varying with concentration (even for the smallest glycopeptide I it changed from 1100 to 2000 as the concentration was raised from 0.5% to 10%). The molecular weights estimated (with an accuracy of 10%) by sedimentation equilibrium distribution at concentrations below 0.1 mg/ml were found to be 1000, 2000 and 10 000 for glycopeptides I, II and X, respectively.

On acid hydrolysis, glycopeptide I yielded 2 moles each of ammonia and acetic acid, and also muramic, aspartic and glutamic acids, glucosamine, alanine and lysine in the ratio 1:1:1:1:2:1. By the sign of the Cotton effect at 210 nm, lysine was proved to have the L-configuration, whereas aspartic and glutamic acids had the D-configuration. Alanine was found to be optically inactive and therefore its two residues in glycopeptide I must have opposite configurations.

The same amino acids and amino sugars in the same proportions were found in glycopeptides II and X.

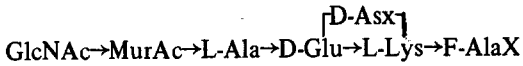
The NMR spectra of glycopeptide I showed that none of its components had been missed in the acid hydrolysis. Thus, the $^{13}$CNMR spectrum indicated the presence of 10 C=O (180–185 ppm), 11 $CH_3$ and $CH_2$ attached to carbon (20–50 ppm) and 19 CH and $CH_2$ attached to oxygen or nitrogen (55–115 ppm) of a total of 40 C-atoms. The $^1$HNMR spectrum in $D_2O$ displayed the signals of 3 (C)$CH_3$ (1.45–1.60 ppm), 2 (CO)$CH_3$ (2.05–2.15 ppm), 6 (C)$CH_2$ (2.0–3.4 ppm) and 22 H in the form of CH or $CH_2$ attached to O or N (3.5–5.2 ppm), whereas the spectrum in $CF_3CO_2H$ displayed signals from 61 H of which 12 were amide hydrogens resonating at 6.5–7.8 ppm.

Acid hydrolysis of the $NaBH_4$-reduced glycopeptide I yielded glucosamine and muramitol, muramic acid being completely absent from the hydrolysate. Hence, in the parent substance the muramic aldehyde group is free, whereas glucosamine is glycosylated.

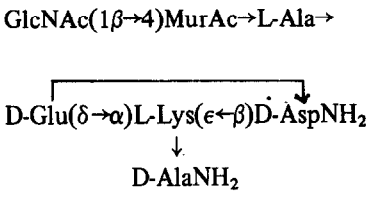
On partial hydrolysis of I with 0.03 N HCl (12 hr at 105°C) two glycopeptides were isolated by paper chromatography. One of them consisted of Mur, Ala, Glu, Lys, and Asp in a 1:2:1:1:1 ratio, while the other contained one mole each of $GlcNH_2$, Mur, Ala, Glu, and Lys, the absence of either glucosamine or of a second mole of Ala thereby demonstrating their terminal position in the parent molecule.

The amino acid sequence of I was elucidated by hydrolysis with pronase under forced conditions since the glycopeptide resisted the action of pepsin and trypsin. Two ninhydrin negative products were isolated from the hydrolysate; namely, the disaccharide GlcNAc–MurAc, obtained earlier from the *Mycobacterium smegmatis* cell walls [7], and a glycopeptide containing equimolecular amounts of $GlcNH_2$, Mur, Ala, and Glu. In turn the ninhydrin positive products revealed the tetrapeptide Ala, Asp, Glu, Lys, the first amino acid being N-terminal and of L-configuration.

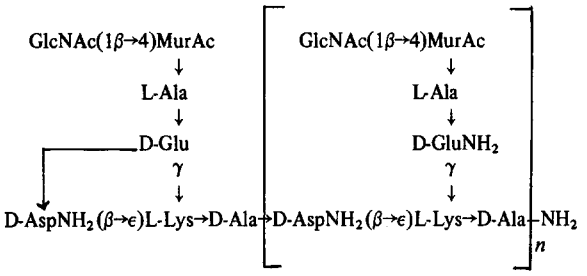
The following sequence was therefore inferred for glycopeptide I:




The structure of glycopeptide I indicated its origin from a peptidoglycan related to the A4α group, a similar sequence GlcNAc(1β→4)MurAc→L-Ala→D-Glu-(δ→α)L-Lys(ε←β)D-Asp being encountered in these biopolymers [8]. The similarity between the sequences is however limited by the presence of a ring in the peptide moiety of glycopeptide I and by amidation of its D-alanine residue and of the exocyclic carboxyl of the D-aspartic residue, as shown by the neutral properties of the molecule and the formation of 2 moles of $NH_3$ on acid hydrolysis. Consequently, in glycopeptide I *N*-acetyl glucosamine is apparently linked to muramic acid by a 1β→4 bond, lysine is acylated at the α-amino group by the δ-carboxyl of glutamic acid and at the ε-amino group by the β-carboxyl of aspartic acid, which in turn is α-amidated:




Glycopeptides II and X, like I, yielded glucosamine, muramitol, and no muramic acid on treatment with $NaBH_4$ followed by acid hydrolysis. Therefore, no unbroken oligosaccharide chain is present in these two glycopeptides, their GlcNAc–MurAc disaccharide residues being interconnected by peptide cross-links. As fragments of the A4α type of peptidoglycans these compounds obviously consist of repeated peptide units linked to each other by the D-isoasparagine amino group and the D-alanine carboxyl. Since both glycopeptides are neutral, their α-carboxyls of the glutamic and aspartic residues and the terminal alanine residue are apparently amidated; moreover their peptide moiety should contain a ring which very likely is the same as that in glycopeptide I. Hence, glycopeptides II and X can be ascribed the general formula




where $n$ = 1 or 8–10.

## References

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