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Novel mannosyl derivatives of peptidoglycan monomer: Synthesis and biological evaluation of immunomodulatory properties

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ABSTRACT

The aim of this work was to prepare mannosyl derivatives of peptidoglycan monomer (PGM, β-D-GlcNAc-(1→4)-D-MurNAc-L-Ala-D-*iso*Gln-*meso*DAP(εNH$_2$)-D-Ala-D-Ala) in order to study the effects of mannosylation on adjuvant (immunostimulating) activity. Novel Man-OCH$_2$CH(CH$_3$)CO–PGM isomers were substrates for *N*-acetylmuramyl-L-alanine amidase, like the parent PGM molecule. Adjuvant activity of Man-OCH$_2$CH(CH$_3$)CO–PGM was tested in the mouse model using ovalbumin as an antigen.

1. Introduction

Peptidoglycans, essential and unique components of bacterial cell walls, possess diverse biological activities that depend upon the size and composition of peptidoglycan fragments. Both high and low molecular mass peptidoglycan fragments, as well as synthetic peptidoglycans, affect the immune system of mammalian hosts.[1,2] Immunostimulating activity of a smaller size peptidoglycans with well defined structures, that are not immunogenic and are devoid of pyrogenicity and toxicity, has been studied extensively, since such compounds could be considered for the use as adjuvants for human and animal vaccines.[3,4]

Peptidoglycan monomer (PGM) studied in this work is a disaccharide pentapeptide β-D-GlcNAc-(1→4)-D-MurNAc-L-Ala-D-*iso*Gln-*meso*DAP(εNH$_2$)-D-Ala-D-Ala (Fig. 1) originating from *Brevibacterium divaricatum*. It was obtained after lysozyme hydrolysis of un-cross linked peptidoglycan polymer isolated from the culture fluid of penicillin treated bacteria.[5] Its structure was confirmed and described in several papers,[6,7] as well as its immunostimulating activity.[8,9] Two lipophilic derivatives of PGM were prepared and characterized and both derivatives exhibited immunostimulating properties in mice comparable to the parent molecule.[10,11]

Peptidoglycans in general are highly conserved structures unique to microorganisms that are not associated with human cells. These unique molecules are called pathogen-associated molecular patterns (PAMPs). Most body defense cells have pattern-recognition receptors (PRR) that recognize and bind PAMPs. The mechanism of action of peptidoglycans leading to immunostimulation in the host is not yet completely understood. However, several reports have demonstrated that peptidoglycans bind to Toll-like receptors[12,13] on the cell surface and that smaller peptidoglycan fragments bind intracellular NOD-receptors.[14,15] The recognition and binding of peptidoglycans leads to the activation of genes coding for pro-inflammatory cytokines. The interaction of PGM with the receptors has not been studied extensively and only the involvement of CD14 in the activation of human monocytes by PGM was reported.[16]

Mannose receptors (MR) present on the cell surface of macrophages and dendritic cells are also considered to be pattern-recognition receptors binding compounds comprising mannose (Man), *N*-acetylglucosamine (GlcNAc) and fucose. These receptors mediate endocytosis and phagocytosis but are also associated with signal transduction leading to cytokine release and production.[17,18] They are considered to be a link between innate and adaptive immunity. MR binds efficiently to GlcNAc, hence this receptor is a potential candidate to interact with PGM.

Furthermore, it was demonstrated that mannosylation of antigen leads to selective targeting and improved presentation in antigen presenting cells and, eventually, alters the potential for immunoregulation in vivo.[19,20]

This study was designed to investigate how mannosylation of PGM might affect the immunostimulating activity in mice. Direct linking of Man molecule to peptidoglycan fragments has not been reported so far, although the conjugates of mannosylated proteins with muramyl dipeptide (MDP) were described.[21,22] Such

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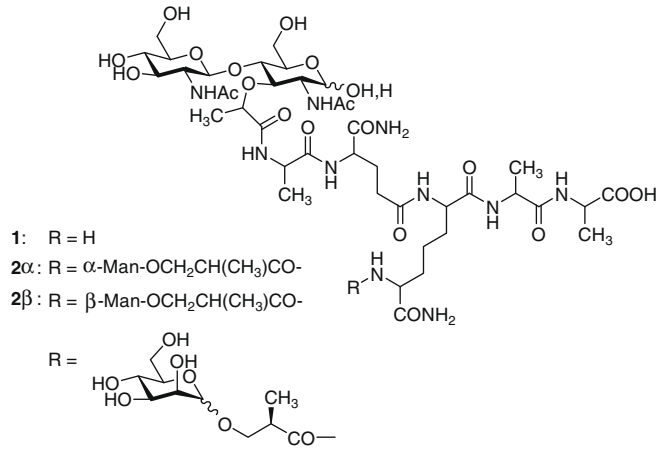


Figure 1. Structures of the peptidoglycan monomer **1** and mannosyl derivatives **2α** and **2β**.

conjugates exhibited enhanced immunostimulating and antitumor properties in comparison to conjugates without Man.

We now report the preparation of novel conjugates using Man linked to a chiral spacer and coupled to the unprotected peptidoglycan monomer through the free the amino group on diaminopimelic acid (Fig. 1). The effects of two newly formed diastereoisomers on immune reaction in mice and the susceptibility of these derivatives of PGM to the enzyme *N*-acetylmuramyl-L-alanine amidase were investigated.

2. Results

2.1. Chemistry

2.1.1. Preparation of methyl esters of *R*-(−)-(2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyloxy)-2-methylpropionic acid (3α, 3β)

Commercially available methyl (*R*)-3-hydroxy-2-methylpropionate was *O*-mannosylated using benzyl protected mannosyl trichloroacetimidate[23] as a good glycosyl donor. Glycosylation was promoted by the catalytic amount of trimethylsilyl trifluoromethanesulfonate (TMSOTf) and gave the anomeric mixture of **3α, 3β** (α/β ratio was found to be approximately 3:1). Pure anomers **3α** and **3β** were relatively easily separated from anomeric mixture by column chromatography on silica gel in diethyl ether/petroleum ether 1:1. Compounds were identified by TLC, ^{1}H NMR and ^{13}C NMR spectroscopies and elemental (C, H and N) analyses.

2.1.2. Preparation of *R*-(−)-(2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyloxy)-2-methylpropionic acids (4α, 4β)

The hydrolysis of the methyl ester of each anomer **3α** and **3β** was performed by saponification using 1 M sodium hydroxide in dioxane. Mixtures were acidified, extracted with diethyl ether and the residues were submitted to column chromatography on silica gel in $CHCl_3$/MeOH 9:1. Pure anomers of benzylated *O*-mannosyl carboxylic acids **4α** and **4β** were identified by TLC, ^{1}H NMR and ^{13}C NMR spectroscopies and elemental (C, H and N) analyses.

2.1.3. Preparation of *N*-hydroxysuccinimide esters of α- and β-anomers of *R*-(−)-3-(2,3,4,6-tetra-*O*-benzyl-D-mannopyranosyloxy)-2-methylpropionic acids (5α, 5β)

Activation of the carboxyl groups of α- and β-anomers of *R*-(−)-(2,3,4,6-tetra-*O*-benzyl-mannopyranosyloxy)-2-methylpropionic **4α** and **4β** was achieved by condensation of pure anomers of acids **4α** and **4β** with an excess of *N*-hydroxysuccinimide (HOSu) in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC × HCl). Water-soluble urea was removed by extraction with water and upon column chromatography on silica gel in $CHCl_3$/MeOH 9:1 **5α** or **5β** were obtained. Their purity was determined by thin layer chromatography (TLC) and the compounds were identified by ^{1}H NMR and ^{13}C NMR spectroscopies and elemental (C, H and N) analyses.

2.1.4. Preparation of benzylated Man-$OCH_2CH(CH_3)CO$–PGM derivatives (6α, 6β)

Benzylated Man-$OCH_2CH(CH_3)CO$–PGM derivatives **6α** and **6β** were prepared as pure anomers by condensation of unprotected PGM **1** with an excess of *N*-hydroxysuccinimide esters **5α** or **5β** in the presence of triethylamine in dimethylformamide (DMF). The course of the reaction was monitored by TLC. No side-products were detected, only traces of unreacted PGM. The reaction mixture was then directly applied on a column of Sephadex LH-20 which was eluted with the 20% solution of ethanol in water (Fig. 2). The fractions containing **6α** and **6β** were pooled, concentrated in vacuo and used in the following step without further purification (Scheme 1). Compounds were identified by TLC and mass spectrometry which in both compounds revealed the molecular ion $[M+H]^+$ at *m/z* 1617.6.

Derivatives **6α** and **6β** were also prepared directly from carboxylic acids **4α** and **4β**. Unprotected PGM reacted with acids **4α** and **4β** (1.2 equiv) in the presence of *N*-hydroxybenzotriazole (HOBt) (1.2 equiv), EDC × HCl (1.2 equiv) and triethylamine (1.2 equiv) in DMF. Products **6α** and **6β** were identified by TLC. But, side-products and unreacted PGM were also detected and it was impossible to isolate compounds **6α** and **6β** from this complex mixture.

2.1.5. Preparation of Man-$OCH_2CH(CH_3)CO$–PGM derivatives (2α, 2β)

Deprotected Man-$OCH_2CH(CH_3)CO$–PGM derivatives **2α** and **2β** were prepared by subjecting benzylated PGM conjugates **6α** and **6β** to hydrogen atmosphere in 50% ethanol and in the presence of catalytic quantities of palladium on charcoal. The catalyst was filtered off and the residues purified by column chromatographies on Biogel P-2 in water (Fig. 3). Fractions containing the desired products **2α** and **2β** were concentrated, passed through the 0.45 μm membrane and lyophilized to give pure **2α** in 87% yield and pure **2β** in 84% yield. The structures of both compounds were confirmed by ^{1}H NMR and ^{13}C NMR spectroscopies, mass spectrometry and total acid hydrolysis. Full assignment of ^{1}H-signals was not possible because of severe overlaps but partial assignments were possible by comparison with ^{1}H NMR spectra of PGM.[11] Mass spectra for **2α** and **2β** revealed the molecular ions $[M+H]^+$ at *m/z* 1257.5 and *m/z* 1257.4, respectively.

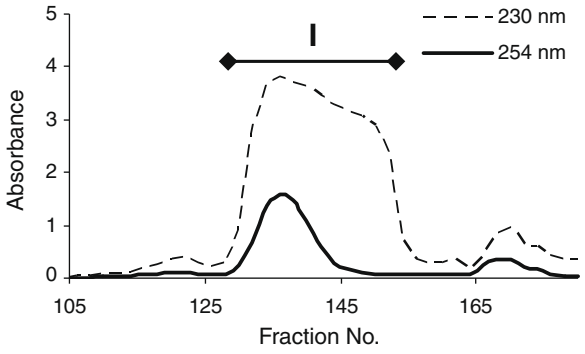
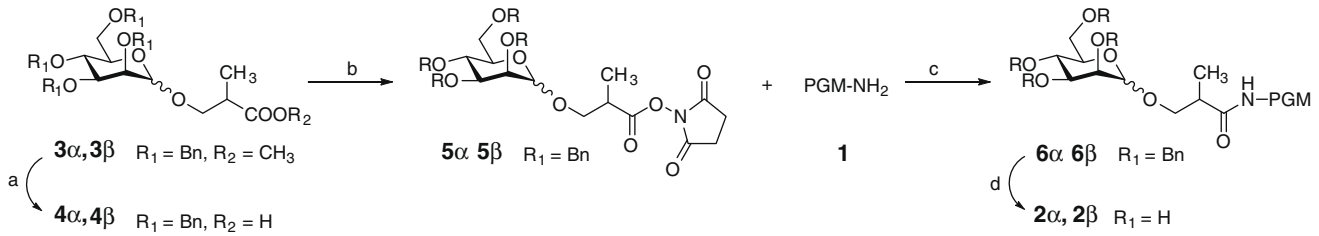


Figure 2. Separation of the benzyl protected Man-$OCH_2CH(CH_3)CO$–PGM **6α** and **6β** on a Sephadex LH-20 column (90 × 2.5 cm) in 20% EtOH. Peak I contains **6α** or **6β** with traces of HOSu.



Scheme 1. Synthesis of mannosyl derivatives of PGM **2α** and **2β**. Reagents: (a) 1 M NaOH, dioxan; (b) EDC × HCl, HOSu, dry DCM; (c) Et_3N, dry DMF; (d) H_2, 10% Pd/C, 50% EtOH.

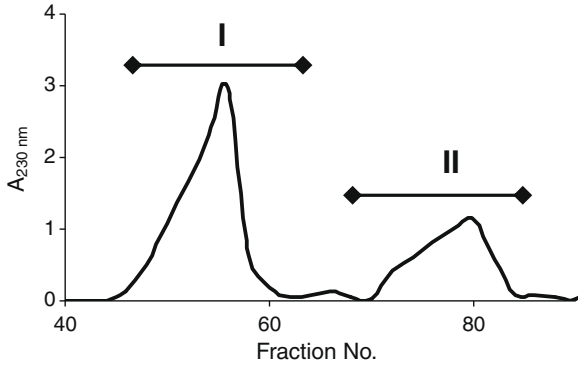


Figure 3. Purification of Man-$OCH_2CH(CH_3)CO$–PGM **2α** and **2β** on Biogel P-2 column (90 × 2.5 cm) in water. Peak I; glycoconjugate **2α** or **2β**, peak II: HOSu.

In order to unequivocally determine the structure of products **2α** and **2β** total acid hydrolysis of both compounds with 6 M hydrochloric acid was performed at 100 °C, for 16 h, followed by TLC to reveal the expected amino acid composition (Ala, Glu and diaminopimelic acid (DAP)). Acid hydrolysis in 2 M hydrochloric acid at 100 °C, for 6 h, followed by TLC revealed the expected monosaccharide composition (Man, $GlcNH_2$ and $MurNH_2$). Furthermore, three distinct peaks were obtained by RP-HPLC separation (Fig. 4), denoting the α- and β-anomeric configuration and the anhydro-form in the PGM part of the molecules.[24] The retention time for the compound **2α** was 15.25 min (31.8%), for the β-anomer, 16.41 min (61.8%) for the α-anomer and 19.01 min (3.5%) for the anhydro-PGM, respectively. Retention times for compound **2β** were 15.47 min (26.4%) and 16.60 min (59.2%) for β- and α-anomers and 19.17 min (3.2%) for the anhydro-PGM, respectively.

In order to unequivocally determine the site of binding of the α- and β-D-mannopyranosyloxy-2-methylpropionic acid residue to PGM, products **2α** and **2β** were submitted to enzymatic hydrolysis with *N*-acetylmuramyl-L-alanine amidase as described bellow.

2.1.6. Hydrolysis of Man-$OCH_2CH(CH_3)CO$–PGM derivatives (2α, 2β) with *N*-acetylmuramyl-L-alanine amidase

It was previously reported that PGM[25], as well as its (adamant-1-yl)-CH_2CO–PGM[10] and Boc-Tyr derivatives[26] can be enzymatically hydrolyzed with *N*-acetylmuramyl-L-alanine amidase from human serum as a catalyst. In all the cases reported regiospecific hydrolysis of the lactylamide bond connecting the disaccharide and pentapeptide or modified pentapeptide moieties occured. Thus, both **2α** and **2β** were treated with partially purified amidase which was isolated from human serum according to the

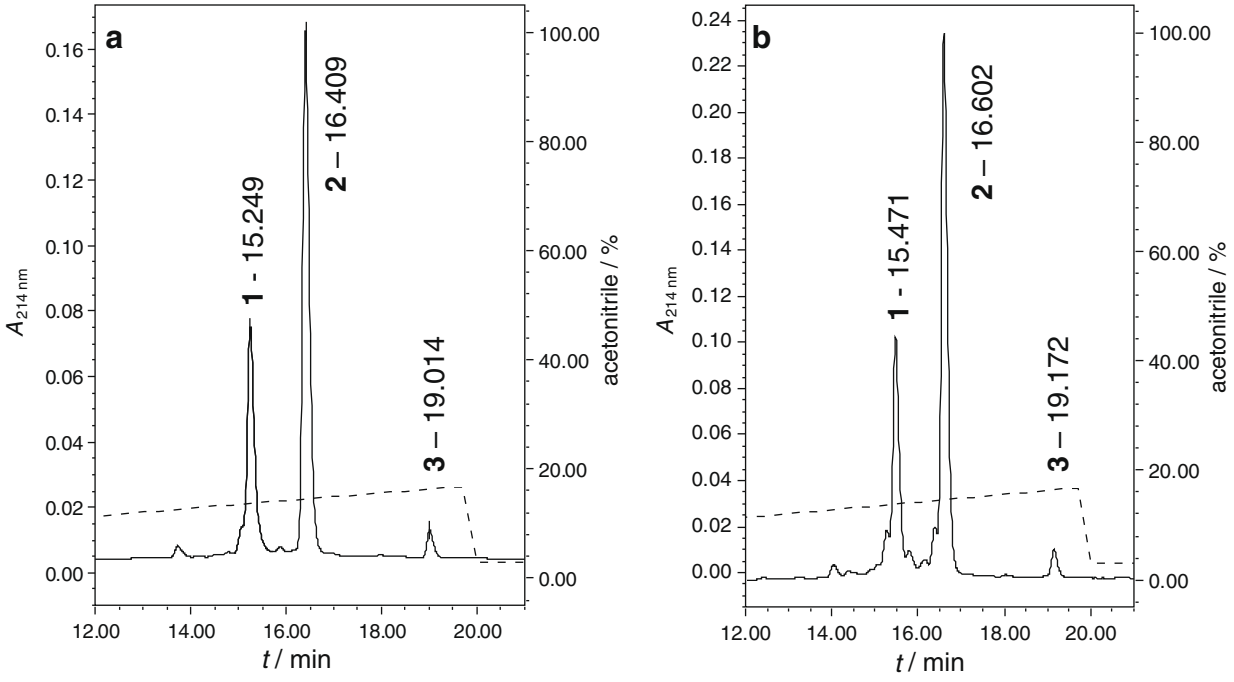
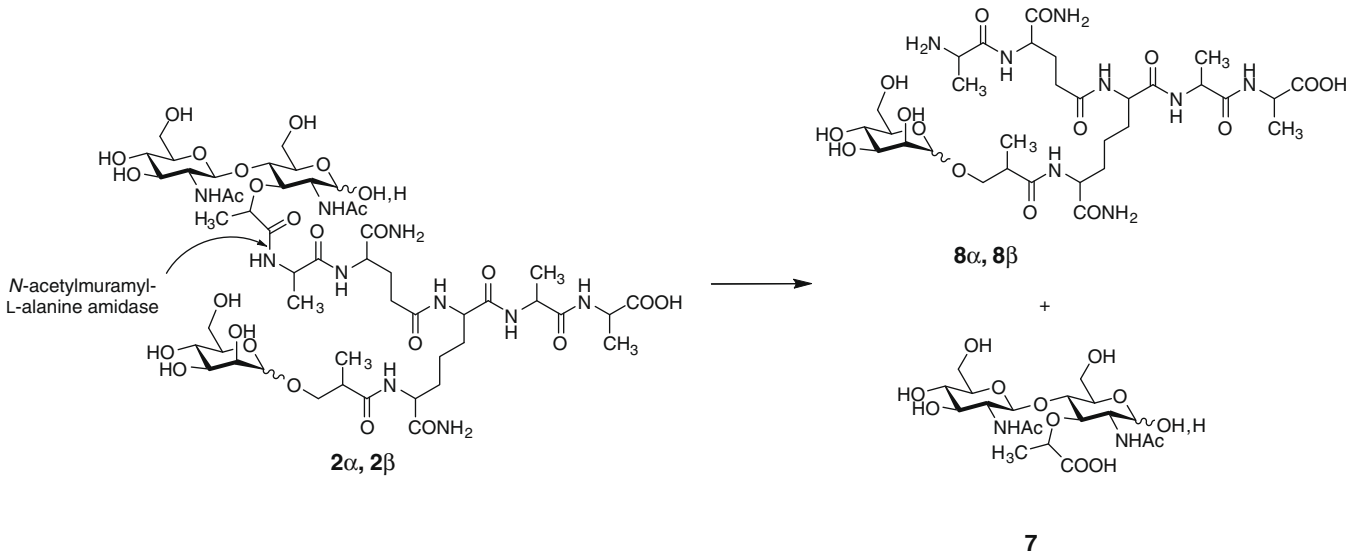


Figure 4. Chromatogram of (a) α-Man-$OCH_2CH(CH_3)CO$–PGM **2α**; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-component; (b) β-Man-$OCH_2CH(CH_3)CO$–PGM **2β**; 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-component.



Scheme 2. Products of the enzymatic hydrolysis of Man-$OCH_2CH(CH_3)CO$–PGM **2α** and **2β**, respectively, with *N*-acetylmuramyl-L-alanine amidase.

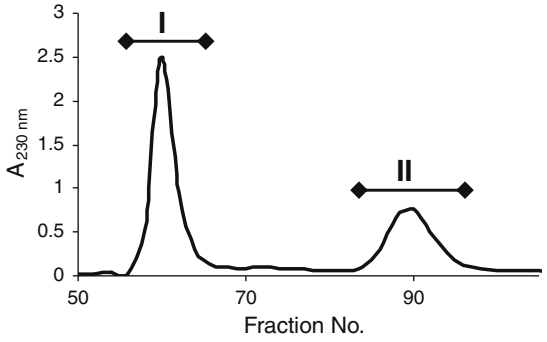


Figure 5. Separation of products of the enzymatic hydrolysis of Man-$OCH_2CH(CH_3)CO$–PGM **2α**, **2β** on Sephadex G-25 fine column (90 × 2.5 cm) in water. Peak I, disaccharide **7**; peak II, Man-$OCH_2CH(CH_3)CO$–pentapeptide **8α** or **8β**.

previously reported procedure in TRIS–HCl buffer, pH 7.9.[25] The reaction was monitored by thin layer chromatography. The products of hydrolysis (Scheme 2) were isolated by column chromatography on Sephadex G-25 fine in water (Fig. 5). Eluted first in both cases (hydrolysis of **2α** and **2β**) was the disaccharide **7**, followed by the α- or β-anomer of Man-$OCH_2CH(CH_3)CO$–pentapeptide, **8α** and **8β**, respectively. These results proved that enzymatic hydrolysis of **2α** and **2β** occurs at the same site as in the previously reported cases leading to the regiospecific cleavage of the lactylamide bond.

The structure of the obtained disaccharide from both hydrolysis reactions co-migrated on TLC with the authentic sample of GlcNAc-MurNAc formed by the hydrolysis of PGM catalyzed by *N*-acetylmuramyl-L-alanine amidase.[6] Its structure was further determined by ^1H NMR and ^{13}C NMR spectroscopies.

The structures of α- and β-Man-$OCH_2CH(CH_3)CO$–pentapeptides **8α** and **8β** obtained by enzymatic hydrolysis were confirmed by ^1H NMR and ^{13}C NMR spectroscopies and mass spectroscopy which in both cases revealed the molecular ion $[M+H]^+$ at *m/z* 779.7.

2.2. Testing of adjuvant activity

Adjuvant activity of Man-$OCH_2CH(CH_3)CO$–PGM **2α** and **2β** was estimated by their immunostimulatory effect on secondary humoral immune response to ovalbumin in mice. Anti-OVA IgG was determined in the sera by ELISA after the second booster (Fig. 6a). PGM tested in parallel induced significantly higher levels of anti-OVA IgG in comparison to values induced by OVA alone.

Man-$OCH_2CH(CH_3)CO$–PGM **2α** and the mixture of **2α** and **2β** had no effect on anti-OVA IgG level in comparison to the group treated only with OVA. Man-$OCH_2CH(CH_3)CO$–PGM **2ß** caused the decrease in antibody level that was significantly lower than in groups treated only with OVA, or with OVA and PGM. Therefore, it could be concluded that the novel compounds have no adjuvant activity. However, the modulation of immune response was observed following analyses of IgG subtypes, IgG1 and IgG2a, as indicators of Th2 or Th1 immune response. The effects on IgG1 induction were basically the same as those observed for total specific IgG and in the levels of IgG2a there were no significant differences, although PGM-treated group had higher values.

When IgG1/IgG2a ratios were calculated (Table 1) it was evident that the group treated with OVA alone had the lowest ratio, and all groups treated with PGM or mannosylated derivatives had higher values, indicating the switch toward Th2 type of response. The highest value, significantly different from the control group treated with OVA, was obtained for group treated with Man-$OCH_2CH(CH_3)CO$–PGM **2α**.

3. Discussion

Natural peptidoglycan molecules exhibit various biological activities, and the most often investigated were their immunomodulating characteristics.[2,27–29] Smaller synthetic molecules, such as muramyl dipeptides and tripeptides were investigated as well. Up to now a number of structure–activity studies were reported. These investigations include synthetic modifications in the first step, possibilities of which are numerous since they can be affected in both, the saccharidic and peptide parts of the molecule. Synthetic modifications described so far were mostly carried out on smaller, synthetic molecules since the protection and deprotection steps needed in the synthesis of multifunctional compounds get more complicated in more complex compounds such as the bacterial PGM monomer which is a disaccharide pentapeptide. Thus, several synthetically modified MDP, syntheses of which were patented, have already been included in clinical trials.[30] Furthermore, formulations in liposomes of these compounds were found to be of

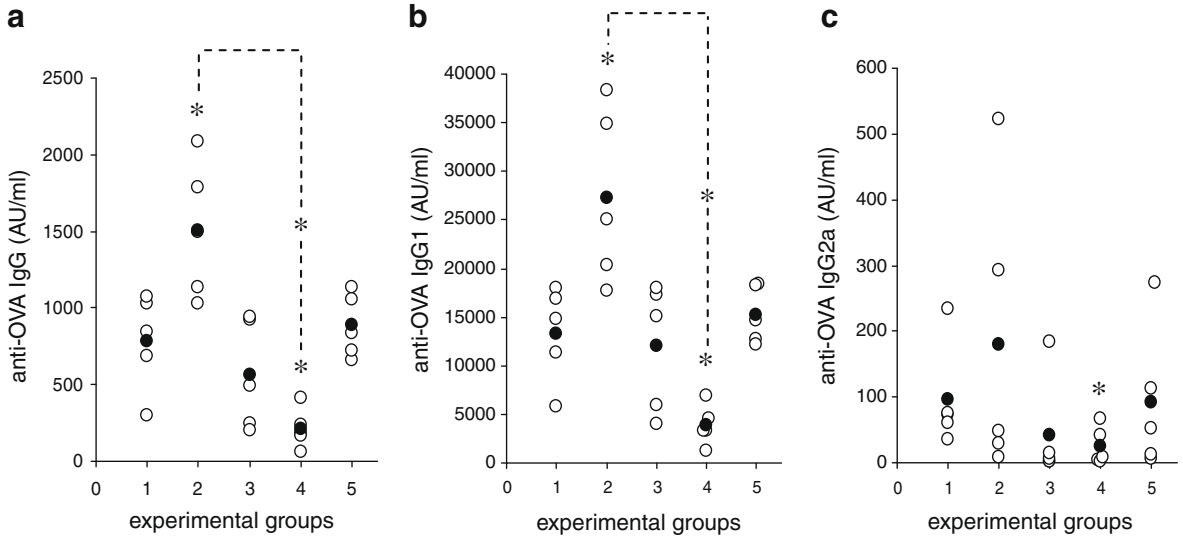


Figure 6. The effect of PGM **1** and its mannosyl derivates **2α** and **2β** on production of anti-OVA IgG (a) and its subtypes, anti-OVA IgG1 (b) and anti-OVA IgG2a (c) respectively, in NIH/OlaHsd mice immunized with OVA as an antigen. Sera were analyzed after the second booster. Experimental group: 1—OVA alone, 2—**1**, 3—**2α**, 4—**2 β**, 5—mixture of **2α** and **2β**. ● denotes group mean value, ○ denotes each serum separately. The difference between two experimental groups was statistically evaluated by Mann–Whitney U-nonparametric test (Statistica 6.0 for Windows, StatSoft Inc.) with probability value less then 0.05 ($p < 0.05$) considered as significant. * $p < 0.05$ in comparison to antigen alone-treated group or in comparison to the group connected with dashed line.

Table 1
The ratio of anti-OVA IgG1 and anti-OVA IgG2a (IgG1/IgG2a) antibodies developed in mice after immunisation and two boosters with PGM **1** and its mannosyl derivates **2α** and **2β**

Experimental groups	Average of $\log_{10}$ IgG1/IgG2a ± SD
1. OVA	2.2 ± 0.3
2. OVA + **1**	2.6 ± 0.7
3. OVA + **2α**	3.0 ± 0.6*
4. OVA + **2β**	2.5 ± 0.5
5. OVA + mixture of **2α** and **2β**	2.5 ± 0.6

$\log_{10}$ of IgG1/IgG2a value was calculated for each mouse serum; results for each experimental group ($n = 5$) are reported as average ± standard deviation (SD).
* $p < 0.05$ in comparison to OVA-treated group.

interest. Therefore, modifications with lipophilic substituents have been the mainstream of research since lipophilic moieties in parent compounds makes them better constituents for incorporation in liposomes. Several successful attempts to synthetically modify PGM monomer as a parent compound were made in spite of its complex structure which contains several groups prone to chemical modifications such as hydroxyls in the disaccharide and the free carboxyl and amino groups in the pentapeptide part of the molecule. However, the preferential conformation of PGM in aqueous and dimethylsulfoxide solutions was determined, and it was shown that the amino group of *meso*-diaminopimelic acid (*meso*-DAP) is exposed and therefore readily available for some reactions without the interference of other reactive groups.[11] In our previous paper on the synthesis of Boc-Tyr-PGM[31] and (adamant-1-yl)-CH_2CO–PGM[10] it was demonstrated that the PGM monomer can be modified in its pentapeptide part without any protection when reagents such as *N*-hydroxysuccinimide esters and anhydrides are used. Electrophilic carbonyl on anhydrides and *N*-hydroxysuccinimide esters react much faster with the free amino group of *meso*-DAP than with free hydroxyls in the carbohydrate moiety due to the greater nucleophilicity of the amino group when compared to hydroxyls.

In the present work we have shown that the free amino group on *meso*-DAP could be substituted with completely different, hydrophilic substituents such as monosaccharides, as well. Due to our long-standing interest in monosaccharide conjugates,[32,33] their synthesis[34,35] and enzymatic transformations[36–40] modification of an unprotected, complex and immunomodulating molecule such as PGM was a special challenge. Substitution with saccharides on the other hand may contribute to the recognition of the biologically active molecule by lectins, cell proteins which specifically and reversibly bind sugar containing structures.[41] Therefore, we chose Man, as one of monosaccharides that specific lectins recognize, to be incorporated as a substituent in the PGM structure. PGM was connected to Man through an *O*-glycosidically bonded linker ((*R*)-2-methylpropionate) (Fig. 1). We decided to use a chiral linker of *R*-configuration to study the regioselectivity of *N*-acetylmuramyl-L-alanine amidase since the absolute configuration of the linker was the same as in the lactyl part on MurNAc. Furthermore, spacer arms are useful for possible increased accessibility of the ligand to its receptor, since we expected that Man might direct the novel mannosylated PGM to MR.

The desired mannosyl derivatives of PGM (**2α** and **2β**) were synthesized by a sequence of steps (Scheme 1), the first of which was the preparation of benzylated mannosyl esters **3α** and **3β** (in α/β ratio of approximately 3:1) containing a chiral linker of *R*-configuration. Saponification of chromatographically separated anomers led to acids **4α** and **4β** which were activated by transforming them to *N*-hydroxysuccinimide esters **5α** and **5β**. The formation of the amide bond with the free amino group on PGM was performed in the next step to give **6α** and **6β** followed by the removal of the benzyl protection of hydroxyls on a Man moiety. This final step led to the formation of the desired final diastereoisomers **2α** and **2β**.

It was also shown that the method involving isolation of activated *N*-hydroxysuccinimide esters followed by the formation of the amide bond with the free amino group on PGM is much better than the strategy of in situ activation of the carboxyl group by coupling reagents EDC × HCl/HOBt.

The two diastereoisomers **2α** and **2β** are the first PGM derivatives comprising carbohydrate substituents. They are also the first mannose substituted peptidoglycan fragments of lower molecular mass synthesized so far. To the best of our knowledge, Man was not linked to either peptide or saccharide part of peptidoglycans.

In one report several carbohydrate analogues of MDP were prepared and their immunoadjuvant activity tested; D-gluco, D-manno and D-galacto-analogues showed strong activity comparable to that of MDP, whereas numerous other positional, configurational and optical analogues had no activity.[42] It should be noted that in the described D-manno analogue Man was not linked to MDP, but GlcNAc moiety was substituted by the mannopyranose ring. Several reports concern the linking of Man to polymers comprising covalently linked MDP. MDP bound to poly-L-lysine substituted with Man[21] and MDP bound to serum albumin substituted with Man[22,43] were more efficient in activation of macrophages than MDP alone or conjugated to protein without mannose. In another report, the preparation of macromolecular conjugate comprising Man, D-glucose analogue of MDP and carboxymethyl dextran was described and the immunological activities investigated.[44] The mannosylated conjugate had increased activity in comparison to the conjugate without Man.

The properties and biological activity of our mannosylated PGM **2α** and **2β** were studied in two models. One aspect was the study of susceptibility to hydrolysis with *N*-acetylmuramyl-L-alanine amidase (Scheme 2), since both lipophilic derivatives of PGM described earlier[10] were good substrates for this enzyme. We demonstrated that Man did not affect the susceptibility of lactylamide bond to hydrolysis with *N*-acetylmuramyl-L-alanine amidase in both isomers. Further, hydrolysis was regiospecific; only lactylamide bond was cleaved, despite the structural similarity with the amide bond connecting amino group of *meso*-DAP and the chiral linker.

The second aspect was testing of immunomodulating properties in vivo in a well defined mouse model. In this first and preliminary testing, the levels of specific anti-OVA IgG were determined. Anti-OVA IgG level reflects the properties of a potential adjuvant to increase the humoral immune response. We found that mannosyl derivatives of PGM **2α** and **2ß** did not stimulate the immune response specific for OVA in comparison to PGM **1**. Moreover, **2ß** even exhibited immunosuppressive properties. Analysis of IgG1 and IgG2a, respectively, indicated that **2α** further improved the switch toward Th2 direction in comparison to PGM **1**.

The results clearly showed that mannosylation changed the biological activity of PGM. The possible reasons could be:

1. The drastic change in the tertiary structure and conformation caused by the hydrophilic substituent—the molecule with different conformation might hinder the binding to receptors relevant for PGM. In any case, both diastereoisomers irrespective of changes in conformation were still good substrates for the enzyme *N*-acetylmuramyl-L-alanine amidase, so probably only the peptide portion distant from lactylamide bond was changed.
2. Binding to MR—this could result in induction of different cytokine program than binding to Toll or Nod receptors. In several reports it was suggested that binding to MR was associated with decreased immune response.[45,46] We have no evidence so far, that PGM **1** or the novel mannosylated PGM **2α**, **2ß** bind to MR, or that such receptors are in any way implicated in the mechanism of action of these compounds, and further research on this topic is required.

4. Conclusion

The data obtained in this study indicate that mannosylation of PGM alters its effects on the immune system. The assay of total specific IgG showed that the immunostimulating activity of two novel Man-$OCH_2CH(CH_3)CO$–PGM isomers was decreased in comparison to the parent molecule. The assay of IgG subtypes also showed that the isomers switched the type of immune reaction toward Th2. Although these results are not sufficient to document fully the effects on immune response, they indicate that the properties of novel Man-$OCH_2CH(CH_3)CO$–PGM isomers merit further research. Several aspects should be covered: (a) the study of conformation of both isomers in order to investigate the differences in these diastereoisomers, but also the differences in conformation of novel derivatives and more lipophilic Boc-Tyr-PGM and (adamant-1-yl)-CH_2CO–PGM described earlier, (b) direct investigation of possible binding to MR on antigen presenting cells and (c) a detailed study of effects on cellular immunity, that is, cytokine profile induced by novel derivatives in comparison to PGM. Each of these aspects requires a separate, comprehensive study. It could be expected, that the data on conformation and biological activity of Man-$OCH_2CH(CH_3)CO$–PGM isomers might contribute to the knowledge of molecular requirements of sugar binding to cell surface receptors and to structure–activity relationship.

5. Experimental

5.1. Materials and methods

Peptidoglycan monomer was prepared in PLIVA, Chemical and Pharmaceutical Works (Zagreb, Croatia), according to the previously described procedure.[5] Bovine serum albumin (BSA), Tween 20, monoclonal anti-chicken egg albumin (clone OVA-14, mouse IgG1 isotype), avidin-peroxidase and *o*-phenylenediamine dihydrochloride (OPD) were from Sigma (USA). Ovalbumin (OVA) was purchased from Serva, Germany. Horseradish peroxidase conjugated goat anti-mouse IgG (HRP-anti-mouse IgG) was from Bio-Rad Laboratories, USA. Biotin-conjugated rat anti-mouse IgG1 and anti-mouse IgG2a monoclonal antibodies and streptavidin-peroxidase were purchased from PharMingen, Becton Dickinson (USA). Chemicals for buffers and solutions were from Kemika, Croatia, unless stated otherwise.

Starting compound, 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate, was prepared as previously described.[23] Most of the chemical reagents used in syntheses were obtained from Fluka and Aldrich Corp. All organic solvents were purified using standard procedures.

Column chromatography (solvents and proportions are given in the text) was performed on Merck Silica Gel 60 (size 70–230 mesh ASTM), Sephadex LH-20 (Pharmacia, Sweden) and Biogel P-2 (Bio-rad Laboratories, USA). For TLC monitoring Fluka Silica Gel (60 F 254) plates (0.25 mm) were used. Visualization was effected by use of UV light, iodine and by charring with H_2SO_4 and ninhydrin.

Optical rotations were measured at room temperature using the Schmidt + Haensch Polartronic NH8. NMR spectra were recorded using Bruker Avance (300 MHz) spectrometer. C, H and N analyses were provided by the Analytical Services Laboratory of Ruđer Bošković Institute, Zagreb. Mass spectra were recorded with Waters MS—Quattro micro instrument. Absorbance was measured on a Perkin–Elmer Lambda 3 UV–vis spectrometer.

Chromatographic separations were carried using Waters HPLC System: Waters 600 System Controller, Waters 600 Pump, Waters In-Line Degasser AF, 2996 photodiode array detection (DAD) system, Empower Software (Milford, MA, USA). A Lichrosorb RP-18 column, 244 mm × 4 mm, 5 μm (Merck, Germany) was used. The samples were applied using a Rheodyne 7725i injector with an effective loop volume of 20 μL. Analyses were performed at a flow rate of 1.0 mL/min at room temperature and the eluate was monitored at 214 nm. The gradient solvent system used was made of acetonitrile containing 0.035% TFA and water containing 0.05% TFA. The percentage of acetonitrile at 0, 20 and 21 min was 3, 17 and 3, respectively and a running time was 25 min.

5.2. Mannosylation of methyl (*R*)-3-hydroxy-2-methylpropionate

Methyl (*R*)-3-hydroxy-2-methylpropionate (193.4 μL, 1.75 mmol, 1.2 equiv) was suspended in dry DCM (5 mL) at 0 °C under N_2 and TMSOTf was added (7.9 μL, 0.04 mmol, 0.03 equiv). 2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyl trichloroacetimidate (1000 mg, 1.46 mmol) suspended in dry DCM (10 mL) was then added dropwise within 15 min after which the reaction mixture was stirred for the additional 2 h and monitored by TLC (diethyl ether/petroleum ether 1:1). Reaction mixture was than diluted with DCM (20 mL) and extracted with 1 M $NaHCO_3$ (20 mL). Organic layer was washed with water (20 mL) and dried over Na_2SO_4. After filtration, the organic layer was concentrated in vacuo and the residue was purified by flash chromatography on silica gel (diethyl ether/petroleum ether 1:1). The product was isolated as anomeric pale yellow oil mixture (**3α, 3β**) (767 mg, 82%; Anal. Calcd for $C_{39}H_{44}O_8$: C, 73.10; H, 6.92. Found: C, 72.91; H, 6.84.

Pure anomers **3α** and **3β** were separated from anomeric mixture using column chromatography on silica gel (diethyl ether/petroleum ether 1:1) and α/β ratio was approximately 3:1.

5.2.1. Methyl (*R*)-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyloxy)-2-methylpropionate (3α)

R_f = 0.38 (diethyl ether/petroleum ether 1:1); $[\alpha]_D$ +17.3 (*c* 0.62, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.37–7.16 (m, 20 H, H–Ar), 4.86 (d, 1H, J_{gem} = 10.38 Hz, CH_2Ph), 4.85 (s, 1 H, H-1), 4.74–4.53 (m, 6H, $3CH_2Ph$), 4.50 (d, 1H, J_{gem} = 10.79 Hz, CH_2Ph), 3.97 (app t, 1H, *J* = 9.38 Hz, *J* = 9.41 Hz, H-4), 3.84 (dd, 1H, $J_{2,3}$ = 2.96 Hz, $J_{3,4}$ = 9.33 Hz, H-3), 3.77 (dd, 1H, $J_{5,6a}$ = 5.50 Hz, $J_{6a,6b}$ = 11.14 Hz, H-6a), 3.73–3.68 (m, 4H, OCH_2, H-2, H-6b), 3.64 (s, H, OCH_3), 3.59–3.56 (m, 1H, H-5), 2.72–2.66 (m, 1H, CH), 1.12 (d, *J* = 7.15 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ 174.85 (C=O), 138.52, 138.52, 138.47, 138.39 (4C–Ar), 128.40–127.31 (CH–Ar), 98.36 (C1), 80.08, 74.92, 74.84, 72.16 (C2–C5), 75.03, 73.36, 72.61, 72.22 ($4CH_2Ph$), 69.35 (C6), 69.33 (OCH_2), 51.63 (OCH_3), 39.96 (CH), 13.97 (CH_3).

5.2.2. Methyl (*R*)-(2,3,4,6-tetra-*O*-benzyl-β-D-mannopyranosyloxy)-2-methylpropionate (3β)

R_f = 0.28 (diethyl ether/petroleum ether 1:1); $[\alpha]_D$ −39.3 (*c* 0.51, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.45–7.18 (m, 20 H, H–Ar), 4.95 (d, 1H, J_{gem} = 12.49 Hz, CH_2Ph), 4.90 (d, 1H, J_{gem} = 10.74 Hz, CH_2Ph), 4.81 (d, 1H, J_{gem} = 12.43 Hz, CH_2Ph), 4.53 (d, 1H, J_{gem} = 10.65 Hz, CH_2Ph), 4.65–4.42 (m, 4H, $2CH_2Ph$), 4.39 (s, 1H, H-1), 4.16–4.14 (m, 1H, H-4), 3.88–3.79 (m, 2H, H-2, H-3), 3.76–3.71 (m, 2H, OCH_2), 3.66 (s, 3 H, OCH_3), 3.59 3.56 (m, 1H, H-6a), 3.50–3.48 (m, 1H, H-6b), 3.45–3.43 (m, 1H, H-5), 2.84–2.80 (m, 1H, CH), 1.24 (d, 3H, *J* = 6.95 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 174.72 (C=O), 138.77, 138.51, 138.38, 138.19 (4C–Ar), 128.40–127.31 (CH–Ar), 101.85 (C1), 82.27, 76.09, 74.89, 73.62 (C2–C5), 75.13, 73.70, 73.47, 71.44 ($4CH_2Ph$), 71.37 (C6), 69.62 (OCH_2), 51.70 (OCH_3), 40.09 (CH), 13.93 (CH_3).

5.3. General procedure for methyl ester hydrolysis

To a solution of pure anomer of each ester derivative **3α** or **3β** (200 mg, 0.31 mmol) in dioxan (3 mL) 10 equiv of 1 M NaOH (3.13 mL) was added and the reaction mixture was stirred for 24 h at room temperature and monitored by TLC (diethyl ether/petroleum ether 1:1). Reaction mixtures were then neutralized with 0.5 M HCl. Mixtures were extracted with diethyl ether (10 mL) and organic layers dried over Na_2SO_4. After filtration, the organic layers were concentrated in vacuo and the residues purified by column chromatography on silica gel ($CHCl_3$/MeOH 9:1). Product **4α** (182.8 mg, 94%) or **4β** (162.3 mg, 83%) were afforded as pale yellow oil.

5.3.1. (*R*)-(2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyloxy)-2-methylpropionic acid (4α)

R_f = 0.61 ($CHCl_3$/MeOH 9:1); $[\alpha]_D$ +13.1 (*c* 0.21, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.36–7.13 (m, 20H, H–Ar), 4.85 (d, 1H, $J_{1,2}$ = 1.77 Hz, H-1), 4.84 (d, 1H, J_{gem} = 10.69 Hz, CH_2Ph), 4.74–4.51 (m, 6H, $3CH_2Ph$), 4.49 (d, 1H, J_{gem} = 10.80 Hz, CH_2Ph), 3.96 (app t, 1H, *J* = 9.29 Hz, *J* = 9.36 Hz, H-4), 3.84 (dd, 1H, $J_{2,3}$ = 3.00 Hz, $J_{3,4}$ = 9.21 Hz, H-3), 3.77–3.69 (m, 5H, OCH_2, H-2, H-6a, H-6b), 3.60–3.55 (m, 1H, H-5), 2.76–2.64 (m, 1H, CH), 1.13 (d, 3H, *J* = 7.12 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 179.51 (C=O), 138.39, 138.33, 138.26, 138.26 (4C–Ar), 128.31 127.46 (CH–Ar), 98.41 (C1), 79.93, 74.81, 74.81, 72.04 (C2–C5), 75.06, 73.33, 72.63, 72.22 ($4CH_2Ph$), 69.17 (C6), 69.17 (OCH_2), 39.83 (CH), 13.85 (CH_3). Anal. Calcd for $C_{38}H_{42}O_8$: C, 72.82; H, 6.75. Found: C, 72.75; H, 6.66.

5.3.2. (*R*)-(2,3,4,6-Tetra-*O*-benzyl-β-D-mannopyranosyloxy)-2-methylpropionic acid (4β)

R_f = 0.50 ($CHCl_3$/MeOH 9:1); $[\alpha]_D$ −61.2 (*c* 0.44, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.57–7.28 (m, 20 H, H–Ar), 5.06 (d, 1H, J_{gem} = 12.34 Hz, CH_2Ph), 5.01 (d, 1H, J_{gem} = 10.96 Hz, CH_2Ph), 4.94 (d, 1H, J_{gem} = 12.59 Hz, CH_2Ph), 4.64 (d, 1H, J_{gem} = 10.71 Hz, CH_2Ph), 4.77–4.56 (m, 4H, $2CH_2Ph$), 4.53 (s, 1H, H-1), 4.29–4.24 (m, 1H, H-4), 4.03–3.94 (m, 2H, H-2, H-3), 3.91–3.86 (m, 2H, OCH_2), 3.76–3.71 (m, 1H, H-6a), 3.64–3.55 (m, 2H, H-5, H-6b), 2.98–2.91 (m, 1H, CH), 1.37 (d, *J* = 7.06 Hz, 3H, CH_3). ^{13}C NMR ($CDCl_3$): δ 177.43 (C=O), 138.75, 138.39, 138.39, 138.19 (4C–Ar), 128.37–127.32 (CH–Ar), 101.74 (C1), 82.20, 75.99, 74.91, 73.46 (C2–C5), 75.04, 73.95, 73.86, 71.63 ($4CH_2Ph$), 71.22 (C6), 69.77 (OCH_2), 39.88 (CH), 13.71 (CH_3). Anal. Calcd for $C_{38}H_{42}O_8$: C, 72.82; H, 6.75. Found: C, 72.67; H, 6.62.

5.4. General procedure for synthesis of *N*-hydroxysuccinimide esters

To solution of pure anomer of each carboxylic acid **4α** or **4β** (150 mg, 0.24 mmol) in dry DCM (5 mL) at 0 °C EDC × HCl (68.9 mg, 0.36 mmol, 1.5 equiv) and HOSu (41.7 mg, 0.36 mmol, 1.5 equiv) were added. The mixture was stirred for 1 h at 0 °C and than overnight at room temperature. The solution was then diluted with DCM (10 mL) and extracted twice with water (10 mL). Organic layer was dried over Na_2SO_4 and after filtration, evaporated. The residue was purified by column chromatography on silica gel ($CHCl_3$/MeOH 9:1) to afford product **5α** or **5β** (172.6 mg or 173.7 mg, 78%) as pale yellow oil.

5.4.1. (*R*)-3-(2,3,4,6-Tetra-*O*-benzyl-α-D-mannopyranosyloxy)-2-methylpropionic acid *N*-hydroxysuccinimide ester (5α)

R_f = 0.85 ($CHCl_3$/MeOH 9:1); $[\alpha]_D$ +8.5 (*c* 0.47, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.38–7.15 (m, 20H, H–Ar), 4.90 (d, 1H, $J_{1,2}$ = 1.51 Hz, H-1), 4.87 (d, 1H, J_{gem} = 10.90 Hz, CH_2Ph), 4.78–4.60 (m, 5H, CH_2Ph), 4.54 (d, 1H, J_{gem} = 12.42 Hz, CH_2Ph), 4.49 (d, 1H, J_{gem} = 10.40 Hz, CH_2Ph), 4.00–3.60 (m, 8H, OCH_2, H-2, H-3, H-4, H-5, H-6a, H-6b), 3.08–2.97 (m, 1H, CH), 2.78 (s, 4H, $2CH_2$), 1.26 (d, 3H, *J* = 3.52 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 169.98, 168.86 (C=O), 138.6, 138.52, 138.34 (4C–Ar), 128.24–127.41 (CH–Ar), 98.82 (C1), 79.93, 74.81, 74.66, 72.22 (C2–C5), 74.96, 73.33, 72.77, 71.92 (4 CH_2Ph), 69.28 (C6), 68.93 (OCH_2), 38.18 (CH), 25.55 (CH_2), 13.65 (CH_3). Anal. Calcd for $C_{42}H_{45}NO_{10}$: C, 69.69; H, 6.27; N, 1.94. Found: C, 69.93; H, 6.17; N, 2.00.

5.4.2. (*R*)-3-(2,3,4,6-Tetra-*O*-benzyl-β-D-mannopyranosyloxy)-2-methylpropionic acid *N*-hydroxysuccinimide ester (5β)

R_f = 0.83 ($CHCl_3$/MeOH 9:1); $[\alpha]_D$ −77.8 (*c* 0.27, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 7.46–7.15 (m, 20H, H–Ar), 4.96 (d, 1H, J_{gem} = 12.56 Hz, CH_2Ph), 4.89 (d, 1H, J_{gem} = 10.81 Hz, CH_2Ph), 4.79 (d, 1H, J_{gem} = 12.53 Hz, CH_2Ph), 4.65–4.40 (m, 5H, CH_2Ph), 4.44 (s, 1H,

H-1), 4.21 (dd, 1H, $J_{6a,6b}$ = 10.04 Hz, $J_{6a,5}$ = 4.06 Hz, H-6a), 4.05 (d, 1H, $J_{2,3}$ = 2.84 Hz, H-2), 3.90–3.71 (m, 4H, OCH_2, H-4, H-6b), 3.55 (dd, 1H, $J_{2,3}$ = 2.94 Hz, $J_{3,4}$ = 9.37 Hz, H-3), 3.49–3.44 (m, 1H, H-5), 3.15–3.05 (m, 1H, CH), 2.80 (s, 4H, $2CH_2$), 1.40 (d, 3H, J = 7.05 Hz, CH_3). ^{13}C NMR ($CDCl_3$): δ 169.31, 168.87 (C=O), 138.96, 138.37, 138.35, 138.25 (4C–Ar), 128.27–127.19 (CH–Ar), 102.23 (C1), 82.12, 75.91, 74.67, 73.78 (C2–C5), 75.07, 74.07, 73.43, 71.11 ($4CH_2Ph$), 70.81 (C6), 68.59 (OCH_2), 38.12 (CH), 25.56 ($2CH_2$), 13.52 (CH_3). Anal. Calcd for $C_{42}H_{45}NO_{10}$: C, 69.69; H, 6.27; N, 1.94. Found: C, 69.71; H, 6.13; N, 2.15.

5.5. General procedure for preparation of benzylated Man-OCH_2 $CH(CH_3)CO$–PGM

To a solution of *N*-hydroxysuccinimide esters **5α** or **5β** (43.4 mg, 0.06 mmol, 1.2 equiv) in dry DMF (2 mL), peptidoglycan monomer **1** (50 mg, 0.05 mmol) was added, followed by triethylamine (13.9 μL, 1 mmol, 2 equiv). The reaction was carried out at 0 °C for 1 h and then at room temperature over 48 h. The reaction was monitored by TLC in solvent system 2-propanol/ammonia 7:3. After 48 h the reaction mixture was directly applied on a Sephadex LH-20 column (90 × 2.5 cm) and eluted with 20% EtOH in water. Absorbance of fractions (3 mL) was measured at 230 nm and 254 nm. Those corresponding to benzyl protected Man-$OCH_2CH(CH_3)CO$–PGM **6α** and **6β** were pooled, evaporated and used in the following step. R_f values (2-propanol/ammonia 7:3) of **6α** and **6β** were 0.40 and 0.37, respectively. ESI-MS: calcd for $C_{78}H_{108}N_{10}O_{27}$: 1616.7; found $[M+H]^+$ at *m/z* 1617.6.

5.6. General procedure for preparation of Man-OCH_2CH-$(CH_3)CO$–PGM

To a solution of PGM conjugate **6α** or **6β** in 50% EtOH in water (20 mL) 40 mg of 10% Pd/C was added. The mixture was subjected to hydrogen atmosphere under 2.5 bars at room temperature and stirred for 24 h. The catalyst was filtrated and the filtrate concentrated in vacuo. The residue was purified by column chromatography on Biogel P-2 in water. Absorbance of fractions (3 mL) was measured at 230 nm and those containing Man-$OCH_2CH(CH_3)$-CO–PGM **2α** or **2β** was combined, concentrated, passed through 0.45 μm membrane and lyophilized, to give the pure **2α** (55 mg, 87%) or **2β** (53 mg, 84%).

5.6.1. *N*-Acetyl-β-D-glucosaminyl-(1→4)-*N*-acetylmuramyl-L-alanyl-D-*iso*-glutaminyl-{N^{ε}-[(*R*)-3-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyloxy)-2-methylpropanoyl]}-*meso*-2,6-diaminopimelyl-D-alanyl-D-alanine (2α)

R_f = 0.55 (2-propanol/ethanol/ammonia/H_2O 5:3:2:2); ^{1}H NMR (D_2O): δ 5.22 (d, <1H, $J_{1,2}$ = 3.41 Hz, H-1; α-MurNAc), 4.77 (d, 1H, $J_{1,2}$ = 1.73 Hz, H-1; α-Man), 4.62 (d, 1H, $J_{1,2}$ = 8.34 Hz, H-1; β-GlcNAc), 4.56–4.51 (m, 1H), 4.36–4.23 (m, 5H), 4.09 (q, 1H, J = 7.20 Hz, CH-α; Lac), 3.94–3.39 (m, 21H), 2.81–2.75 (m, 1H, CH), 2.40–2.37 (m, 2H), 2.23–1.45 (m, 10H), 2.03 (s, 3H, CH_3; MurNAc), 1.95 (s, 3H, CH_3; GlcNAc), 1.42 (d, 3H, J = 7.23 Hz, CH_3; Ala), 1.38 (d, 3H, J = 6.75 Hz, CH_3; Lac), 1.35 (d, 3H, J = 7.15 Hz, CH_3; Ala), 1.31 (d, 3H, J = 7.25 Hz, CH_3; Ala), 1.07 (d, 3H, J = 7.01 Hz; CH_3). ^{13}C NMR (D_2O, dioxane): δ 179.66 (COOH), 177.75, 176.51, 175.76, 175.58, 174.97, 174.85, 174.54, 174.09, 173.60, 173.34 (5CONH, $2CONH_2$, $2CH_3CO$, lactyl CO), 100.36 (C1; β-GlcNAc), 100.12 (C1; α-Man), 90.18 (C1; α-MurNAc), 77.42, 76.19, 76.05, 75.37, 73.50, 72.74, 71.00, 70.45, 70.20, 69.86, 66.66 (C2–C5; α-Man, C3, C4, C5; β-GlcNAc, C3, C4, C5; α-MurNAc, CH-α; Lac), 69.49 (C6; α-Man), 61.06, 61.05, 59.69 (C6; α-MurNAc, C6; β-GlcNAc, OCH_2), 55.96 (CH-ε; DAP), 53.77, 53.59, 53.12, 52.70, 50.96, 49.89, 49.34 (3CH-α; 3Ala, CH-α; *iso*Gln, CH-α; DAP, C2; α-MurNAc, C2; β-GlcNAc), 40.52 (CH), 31.46, 30.45, 30.36 (CH_2-δ; DAP, CH_2-β; DAP, CH_2-γ; *iso*Gln), 27.07 (CH_2-β; *iso*Gln), 22.07, 22.03, ($2CH_3$; α-MurNAc, β-GlcNAc), 21.50 (CH_2-γ; DAP), 18.13 (CH_3; Lac), 17.39, 16.65, 16.63 ($3CH_3$; 3Ala), 13.19 (CH_3). ESI-MS: calcd for $C_{50}H_{84}N_{10}O_{27}$: 1256.6; found $[M+H]^+$ at *m/z* 1257.5.

5.6.2. *N*-Acetyl-β-D-glucosaminyl-(1→4)-*N*-acetylmuramyl-L-alanyl-D-*iso*-glutaminyl-{N^{ε}-[(*R*)-3-(2,3,4,6-tetra-*O*-benzyl-β-D-mannopyranosyloxy)-2-methylpropanoyl]}-*meso*-2,6-diaminopimelyl-D-alanyl-D-alanine (2β)

R_f = 0.52 (2-propanol/ethanol/ammonia/H_2O 5:3:2:2); ^{1}H NMR (D_2O): δ 5.22 (d, <1H, J = 3.19 Hz, H-1; α-MurNAc), 4.82 (d, 1H, $J_{1,2}$ = 0.59 Hz, H-1; β-Man), 4.61 (d, 1H, J = 7.73 Hz, H-1; β-GlcNAc), 4.60–4.50 (m, 1H), 4.42–4.21 (m, 5H), 4.08 (q, 1H, J = 7.22 Hz, CH-α; Lac), 3.95–3.29 (m, 21 H), 2.85–2.77 (m, 1H, CH), 2.40–2.35 (m, 2H), 2.18–1.46 (m, 10H), 2.03 (s, 3H, CH_3; MurNAc), 1.94 (s, 3H, CH_3; GlcNAc), 1.42 (d, 3H, J = 7.22 Hz, CH_3; Ala), 1.36 (pt, 3H, J = 7.42 Hz, J = 7.34 Hz, CH_3; Lac, CH_3; Ala), 1.31 (d, 3H, J = 7.27 Hz, CH_3; Ala), 1.06 (d, 3H, J = 6.91 Hz, CH_3). ^{13}C NMR (D_2O, dioxane): δ 179.71 (COOH), 177.83, 176.66, 175.78, 175.57, 174.97, 174.88, 174.57, 174.03, 173.64, 173.38 (5CONH, $2CONH_2$, $2CH_3CO$, lactyl CO), 100.36 (C1; β-GlcNAc), 99.59 (C1; β-Man), 90.17 (C1; α-MurNAc), 77.43, 76.17, 76.04, 75.14, 73.49, 72.80, 70.99, 70.38, 70.20, 66.79 (C2–C5; α-Man, C3, C4, C5; β-GlcNAc, C3, C4, C5; α-MurNAc, CH-α; Lac), 71.10 (C6; β-Man), 61.01, 61.01, 59.69 (C6; α-MurNAc, C6; β-GlcNAc, OCH_2), 55.96, (CH-ε; DAP), 53.80, 53.58, 53.22, 52.70, 50.97, 49.90, 49.35 (3CH-α; 3Ala, CH-α; *iso*Gln, CH-α; DAP, C2; α-MurNAc, C2; β-GlcNAc), 40.28 (CH), 31.45, 30.42, 30.31 (CH_2-δ; DAP, CH_2-β; DAP, CH_2-γ; *iso*Gln), 27.06 (CH_2-β; *iso*Gln), 22.06, 22.03 ($2CH_3$; α-MurNAc, β-GlcNAc), 21.49 (CH_2-γ; DAP), 18.12 (CH_3; Lac), 17.36, 16.64, 16.61 ($3CH_3$, 3Ala), 12.90 (CH_3). ESI-MS: calcd for $C_{50}H_{84}N_{10}O_{27}$: 1256.6; found $[M+H]^+$ at *m/z* 1257.4.

Total acid hydrolysis of products **2α** and **2β** in 6 M hydrochloric acid at 100 °C, for 16 h, followed by TLC in solvent system *n*-butanol/acetic acid/H_2O 6:1.5:2.5 revealed the expected amino acid composition (Ala, Glu and DAP), and acid hydrolysis in 2 M HCl at 100 °C for 6 h, followed by TLC in solvent system acetonitrile/H_2O 5:1 revealed the expected monosaccharide composition (Man, $GlcNH_2$ and $MurNH_2$).

For testing of biological activity, special batches of endotoxin-free **2α** and **2β** were prepared by passing the aqueous solution of **2α** and **2β** through Detoxy gel column (Pierce, The Netherlands) followed by lyophilization.

5.7. General procedure for hydrolysis of Man-$OCH_2CH(CH_3)CO$–PGM with *N*-acetylmuramyl-L-alanine amidase

A solution of Man-$OCH_2CH(CH_3)CO$–PGM **2α** or **2β** (50 mg, 0.04 mmol) in TRIS–HCl buffer (0.05 M, pH 7.9) containing 0.02 M $MgCl_2$ was incubated with *N*-acetylmuramyl-L-alanine amidase from human serum[25] (500 μL, 0.816 i.u./mL) at 37 °C, for 24 h. The reaction was monitored by TLC in solvent system 2-propanol/ammonia 7:3. The reaction mixture was directly applied to a Sephadex G-25 fine column (90 × 2.5 cm) in water. Absorbance of fractions (3 mL) was measured at 230 nm and those containing Man-$OCH_2CH(CH_3)CO$–pentapeptide **8α** or **8β** and disaccharide **7** were pooled and evaporated in vacuo. Obtained products were: Man-$OCH_2CH(CH_3)CO$–pentapeptide **8α** (28.5 mg, 91%) or **8β** (28.3 mg, 91%) and disaccharide **7** (20.7 mg, from hydrolysis of compound **2α**; 20.9 mg, from hydrolysis of compound **2β**).

5.7.1. *N*-Acetyl-β-D-glucosaminyl-(1→4)-*N*-acetylmuramyl acid (7)

R_f = 0.40 (2-propanol/ethanol/ammonia/H_2O 5:3:2:2); ^{1}H NMR (DMSO): δ 10.43 (br s, 1H, COOH), 7.82 (d, 1H, J = 8.30 Hz, NH),

6.25 (s, 1H, NH), 5.34 (d, 1H, J = 2.78 Hz, H-1; α-MurNAc), 4.36 (d, 1H, $J_{1,2}$ = 7.66 Hz, H-1; β-GlcNAc), 4.32–4.24 (m, 1H, H-5; MurNAc), 4.03 (q, 1H, J = 7.12 Hz, CH-α; Lac), 3.76–3.36 (m, 11H), 1.80 (s, 3H, CH_3; MurNAc), 1.78 (s, 3H, CH_3; GlcNAc), 1.21 (d, 3H, J = 6.77 Hz, CH_3; Lac). ^{13}C NMR (CD_3OD): δ 181.94 (COOH), 173.78, 173.66 ($2CH_3CO$), 101.65 (C1; GlcNAc), 91.38 (C1; MurNAc), 79.89, 78.73, 77.62, 75.97, 75.39, 72.97 (C3, C4, C5; GlcNAc, C3, C4, C5; MurNAc, CH-α; Lac), 63.49 (C6; MurNAc), 61.26 (C6; GlcNAc), 58.54 (C2; GlcNAc), 56.72 (C2; MurNAc), 23.05, 22.79 ($2CH_3$; MurNAc, GlcNAc), 19.80 (CH_3; Lac).

5.7.2. L-Alanyl-D-*iso*-glutaminyl-{N^{ε}-[(*R*)-3-(2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranosyloxy)-2-methylpropanoyl]}-*meso*-2,6-diaminopimelyl-D-alanyl-D-alanine (8α)

R_f = 0.29 (2-propanol/ethanol/ammonia/H_2O 5:3:2:2); $[\alpha]_D$ +23.5 (*c* 0.17, DMSO/H_2O 1:1); ^{1}H NMR (DMSO-d_6): δ 8.73 (br s, 1H, NH), 8.26 (d, 1H, J = 7.31 Hz, NH), 7.98 (d, 1H, J = 7.82 Hz, NH), 7.95 (d, 1H, J = 8.25 Hz, NH), 7.62 (d, 1H, J = 6.31 Hz, NH), 7.47 (s, 1H, NH), 7.25 (s, 1H, NH), 7.07 (s, 1H, NH), 6.98 (s, 1H, NH), 4.56 (s, 1H, H-1), 4.24 (q, 1H, J = 7.30 Hz, CH-α; Ala), 4.19 (t, 1H, J = 7.34 Hz, CH-α; DAP), 4.17–4.12 (m, 2H, 2CH-α; Ala, *iso*Gln), 3.91–3.86 (m, 1H, CH-ε; DAP), 3.72 (q, 1H, J = 6.63 Hz, CH-α; Ala), 3.65 (pd, 1H, J = 10.28 Hz, H-6a), 3.55 (br s, 1H, H-2), 3.49–3.42 (m, 4H, OCH_2, H-3, H-6b), 3.36 (app t, 1H, J = 9.77 Hz, J = 9.15 Hz, H-4), 3.32–3.28 (m, 1H, H-5), 2.68–2.62 (m, 1H, CH), 2.20–2.12 (m, 3H, CH_2; γ,γ′-*iso*Gln, CH; β-*iso*Gln), 2.02–1.96 (m, 1H, CH; β′-*iso*Gln), 1.77–1.71 (m, 2H, 2CH; δ-DAP, β-DAP), 1.67–1.57 (m, 2H, 2CH; δ′-DAP, β′-DAP), 1.49–1.44 (m, 2H, CH_2; γ,γ′-DAP), 1.30 (d, 3H, J = 6.84 Hz, CH_3, Ala), 1.20 (app t, 6H, J = 6.57 Hz, J = 6.65 Hz, $2CH_3$; 2Ala), 0.97 (d, 3H, J = 6.85 Hz, CH_3).^{13}C NMR (DMSO-d_6): δ 173.42, 173.30, 172.72, 171.53, 171.08 (5CONH, $2CONH_2$, COOH), 99. 84 (C1), 73.63, 70.74, 69.91, 66.95 (C2–C5), 68.80 (C6), 61.15 (OCH_2), 52.39 (CH-ε; DAP), 51.95 (CH-α; DAP), 51.89 (*C*H-α; *iso*Gln), 49.14, 48.29, 48.00 (CH-α; 3Ala), 39.58 (CH), 31.34 (CH_2-δ; DAP), 31.10 (CH_2-γ; *iso*Gln, CH_2-β; DAP), 27.38 (CH_2-β; *iso*Gln), 21.48 (CH_2-γ; DAP), 18.19, 17.83, 17.51 (CH_3, 3Ala), 14.23 (CH_3). ESI-MS: calcd for $C_{31}H_{54}N_8O_{15}$: 778.4; found $[M+H]^+$ at *m/z* 779.7.

5.7.3. L-Alanyl-D-*iso*-glutaminyl-{N^{ε}-[(*R*)-3-(2,3,4,6-tetra-*O*-benzyl-β-D-mannopyranosyloxy)-2-methylpropanoyl]}-*meso*-2,6-diaminopimelyl-D-alanyl-D-alanine (8β)

R_f = 0.26 (2-propanol/ethanol/ammonia/H_2O 5:3:2:2); $[\alpha]_D$ +11.8 (*c* 0.17, DMSO/H_2O 1:1); ^{1}H NMR (DMSO-d_6): δ 8.71 (br s, 1H, NH), 8.24 (d, 1H, J = 7.26 Hz, NH), 7.99 (d, 1H, J = 7.75 Hz, NH), 7.96 (d, 1H, J = 8.10 Hz, NH), 7.63 (d, 1H, J = 6.53 Hz, NH), 7.49 (s, 1H, NH), 7.25 (s, 1H, NH), 7.07 (s, 1H, NH), 6.98 (s, 1H, NH), 4.55 (s, 1H, H-1), 4.25–4.11 (m, 4H, 4CH-α; 2Ala, DAP, *iso*Gln), 3.89–3.87 (m, 1H, CH-ε; DAP), 3.74 (q, 1H, J = 6.66 Hz, CH-α; Ala), 3.64 (dd, 1H, $J_{6a,6b}$ = 11.24 Hz, $J_{6a,5}$ = 1.12 Hz, H-6a), 3.55 (d, 1H, J = 1.52 Hz, H-2), 3.48–3.41 (m, 4H, OCH_2, H-3, H-6b), 3.36 (app t, 1H, J = 9.54 Hz, J = 9.14 Hz, H-4), 3.32–3.28 (m, 1H, H-5), 2.68–2.62 (m, 1H, CH), 2.22–2.12 (m, 3H, CH_2; γ,γ′-*iso*Gln, CH; β-*iso*Gln), 2.02–1.96 (m, 1H, CH; β′-*iso*Gln), 1.77–1.71 (m, 2H, 2CH; δ-DAP, β-DAP), 1.67–1.57 (m, 2H, 2CH; δ′-DAP, β′-DAP), 1.52–1.44 (m, 2H, CH_2; γ,γ′-DAP), 1.30 (d, 3H, J = 6.90 Hz, CH_3, Ala), 1.20 (app t, 6H, J = 6.15 Hz, J = 6.62 Hz, $2CH_3$; 2Ala), 0.97 (d, 3H, J = 6.86 Hz, CH_3). ^{13}C NMR (DMSO-d_6): δ 173.69, 173.52, 173.44, 173.37, 172.90, 171.63, 171.29, 170.93 (5CONH, $2CONH_2$, COOH), 99.91 (C1), 73.87, 70.82, 70.05, 66.89 (C2–C5), 68.83 (C6), 61.18 (OCH_2), 52.43 (CH-ε; DAP), 52.19 (CH-α; DAP), 52.00 (*C*H-α; *iso*Gln), 49.02, 48.89, 48.18 (CH-α; 3Ala), 39.64 (CH), 31.55 (CH_2-δ; DAP), 31.34 (CH_2-β; DAP), 31.18 (CH_2-γ; *iso*Gln), 27.41 (CH_2-β; *iso*Gln), 21.72 (CH_2-γ; DAP), 18.34, 18.28, 17.82 (CH_3, 3Ala), 14.57 (CH_3). ESI-MS: calcd for $C_{31}H_{54}N_8O_{15}$: 778.4; found $[M+H]^+$ at *m/z* 779.7.

5.8. Experiments in vivo

Experiments in vivo were performed on NIH/OlaHsd (H-2^q) inbred mice strains. All mice used were females from 2 to 2.5 months old. During the experimental period animals were housed in the animal facility of the Institute of Immunology. Commercial food and water were provided *ad libitum*. All animal work was performed according to Croatian Law on Animal Welfare (1999).

Experimental groups of five mice were immunized and boosted two times subcutaneously (*s.c.*) into the tail base at 21-days intervals. Mice were anaesthetised prior to blood collection on 7th day after each booster. Sera were prepared, decomplemented at 56 °C for 30 min and stored at −20 °C until tested.

The dose of antigen OVA was 10 μg per mouse. The dose of PGM and its derivates was 200 μg per mouse. The injection volume in all experimental groups was 0.1 mL per mouse.

5.9. Enzyme immunoassays for qualitative and quantitative determination of immunoglobulin G specific for OVA (anti-OVA IgG) in mice sera

Enzyme immunoassays (ELISA) were performed on flat-bottomed high binding microtitar plates (Costar, USA) according to previously described procedure.[47] Briefly, plates were coated with 15 μg/mL OVA solution in carbonate buffer, pH 9.6, and left overnight at room temperature (rt). Non-specific antibody binding was blocked by incubation with 0.5% (w/v) BSA in PBS-T (0.05% (v/v) Tween 20 in PBS) buffer (200 μL/well) for 2 h at 37 °C. Mice sera to be tested (100 μL/well) and standard preparation were added in five binary dilutions in duplicates and incubated overnight at rt. Plates were washed and 4000 × diluted HRP-anti-mouse IgG was added (100 μL/well) and incubated for 2 h at 37 °C. After washing, the OPD solution (0.6 mg/mL in citrate–phosphate buffer pH 5.0) was added (100 μL/well) and incubated for 30 min at rt in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 (50 μL/well) and absorbance at 492 nm (A492) was measured using a microplate reader (Thermo Scientific Multiscan Spectrum, Finland). All washings (three times after each step) were done with PBS-T buffer in microplate washer (Multiwash Labsystems, Finland). The relative quantities of anti-OVA IgG were determined by parallel line assay comparing each serum to monoclonal anti-chicken egg albumin, declared as standard preparation, to which 20,000 arbitrary units per milliliters (AU/mL) were voluntarily assigned.[9]

5.10. Enzyme immunoassays for qualitative and quantitative determination of immunoglobulin G subtypes specific for OVA, anti-OVA IgG1 and anti-OVA IgG2a respectively, in mice sera

ELISAs for determination of anti-OVA IgG1 and anti-OVA IgG2a were performed as follows: after sera incubation on microplates coated with OVA, biotinylated rat anti-mouse IgG1 at 0.05 μg/mL or biotinylated rat anti-mouse IgG2a at 0.5 μg/mL was added, respectively, 100 μL to each well and incubated for 2 h at 37 °C. After washing, streptavidin-peroxidase (100,000×) was added for determination of IgG1, while avidin-peroxidase (50,000×) was used for IgG2a determination, (100 μL/well) and incubated for another 2 h at 37 °C. Plates were washed and the substrate solution of 100 μL per well was added and incubated for 30 min at rt in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 (50 μL/well) and A492 was measured using a microplate reader.

The relative quantities of antibody subtypes were determined by parallel line assay using appropriate standard preparations. A monoclonal anti-OVA IgG1 was a standard for relative quantification of anti-OVA IgG1 to which was assigned 400,000 AU/mL, while

polyclonal mouse serum containing high levels of anti-OVA IgG2a was used as a standard for relative quantification of IgG2a specific antibodies with voluntarily assigned 20,000 AU/mL.

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