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Authors	Rosana Ribić, Ranko Stojković, Lidija Milković, Mariastefania Antica, Marko Cigler and Srđanka Tomić
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ORCID® iDs	Rosana Ribić - https://orcid.org/0000-0001-7611-538X; Lidija Milković - https://orcid.org/0000-0002-4484-039X; Mariastefania Antica - https://orcid.org/0000-0001-8449-1078

Design, synthesis and biological evaluation of immunostimulating mannosylated desmuramyl peptides

Rosana Ribić*[1], Ranko Stojković[2], Lidija Milković[2], Mariastefania Antica[2], Marko Cigler[3] and Srđanka Tomić[4]

[1] University Center Varaždin, University North, Jurja Križanića 31b, HR-42000 Varaždin, Croatia

[2] Ruđer Bošković Institute, Bijenička cesta 54, HR-10000 Zagreb, Croatia

[3] Department of Chemistry, Technical University Munich, Lichtenbergstraße 4, D-85748 Garching, Germany

[4] Faculty of Science, University of Zagreb, Horvatovac 102a, HR-10000 Zagreb, Croatia

Email: Rosana Ribić - rosana.ribic@unin.hr

* Corresponding author

Abstract

Muramyl dipeptide is the minimal structure of peptidoglycan with adjuvant properties. Replacement of the *N*-acetylmuramyl moiety and increase of lipophilicity are important approaches in the preparation of muramyl dipeptide analogues with improved pharmacological properties. Mannose receptors present on immunocompetent cells are pattern-recognition receptors and by mannose ligands binding they affect the immune system. Here we present design, synthesis and biological evaluation of novel mannosylated desmuramyl peptide derivatives. Mannose was coupled to dipeptide containing lipophilic adamantane on N- or C-terminus through glycolyl or hydroxyisobutyryl linker. Adjuvant activities of synthesized compounds were investigated in the mouse model using ovalbumin as an antigen. Their activities were compared to the previously described mannosylated adamantane-containing desmuramyl peptide and peptidoglycan monomer. Tested compounds exhibited adjuvant activity and the strongest enhancement of IgG production was stimulated by the compound **21** (Man-OCH$_2$-D-(1-Ad)Gly-L-Ala-D-*iso*Gln).

Keywords

adamantane; adjuvant activity; desmuramyl peptide; mannose

Introduction

Dendritic cells capture and internalize invading pathogens. Pathogen-associated molecular patterns have been known for a long time to affect the immune system of mammalian hosts and therefore have been extensively studied as possible adjuvants for vaccines. Peptidoglycan is a polymeric component of Gram-positive and Gram-negative bacterial cell wall. Breakdown products of polymeric peptidoglycan are called muropeptides. Muropeptides act as agonists of pathogen recognition receptors (PRRs) and therefore stimulate immune response and induce T cell differentiation [1-3]. They activate innate immune responses and contribute to the development of adaptive immunity. Immune response is initiated by the activation of PRRs located on the immune cell surface, by cytosolic or endosomal PRRs. PRRs are classified into: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) [4]. Muramyl dipeptide (MDP, *N*-acetylmuramyl-L-alanyl-D-*iso*glutamine) is the smallest peptidoglycan fragment (Figure 1) capable of replacing whole *Mycobacterium* in complete Freund's adjuvant. MDP triggers an immune response by activating the mammalian NOD-like receptor, nucleotide binding oligomerization domain-containing protein 2 (NOD2). NOD2 is an intracellular protein that signals via the NF-κB pathway to proximally activate innate immunity through macrophage response as well as to more distally affect adaptive immunity through the production of antigen-specific T-cells [5]. MDP binding to NOD2 has been confirmed [6] as well as crystal structure of NOD2 in the inactive ADP-bound state [7]. MDP is the structural fragment of peptidoglycan monomer (PGM, Figure 1) which is used in this work. PGM is well-defined and characterized disaccharide pentapeptide, β-D-GlcNAc-(1→4)-D-MurNAc-L-Ala-D-*iso*Gln-*meso*DAP(εNH2)-D-Ala-D-Ala, originating from *Brevibacterium divaricatum* [8,9].

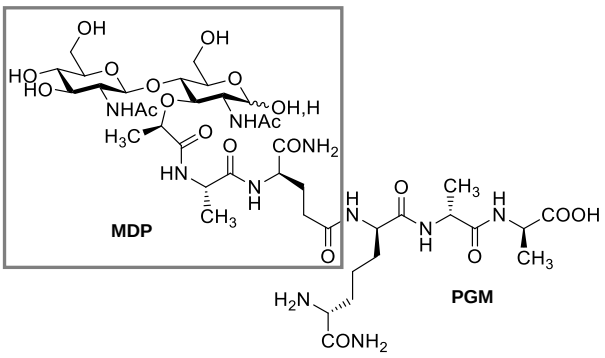


Figure 1: Peptidoglycan fragments with immunostimulating properties.

Peptidoglycan activates macrophages via TLR2 receptor, whereas MDP lacks TLR2-agonistic activity [10]. PGM and MDP have similar immunostimulating activity and they are reduced in comparison to the potent complete Freud's adjuvant which is used as golden standard for adjuvant activity [11]. However, strong toxicity of complete Freud's adjuvant disables its clinical application. MDP is too pyrogenic for clinical application as well and suffers from rapid elimination. Therefore, numerous MDP analogues and derivatives were synthesized, in order to improve the properties of the parent molecule [12-16]. Replacement of the *N*-acetylmuramyl moiety with various acyl groups represents an important approach in the design of new immunologically active MDP analogues [17]. MDP analogues lacking the sugar moiety are called desmuramyl peptides. Structure-activity studies of the MDP derivatives and analogues suggest that L-Ala-D-*iso*Gln pharmacophore is essential for the immunostimulatory properties but the introduction of lipophilic substituent into MDP analogues can increase its adjuvant activity [12,18]. Up to now, our research was directed towards isomeric desmuramyl peptides containing lipophilic unnatural amino acid, adamantylglycine (AdGly), bound to the N-terminus of L-Ala-D-*iso*Gln dipeptide part as well as their mannosylated derivatives. Different isomers of mannosylated adamantyl tripeptides, regarding the chiral centers introduced at adamantlyglycine and spacer which connect the sugar part to the

adamantyltripeptide were synthesized and biologically evaluated [19-21]. The best adjuvant activity in experiments in vivo showed the ManAdTP derivative (Figure 2) which has a D-configuration at the (adamant-1yl)glycine moiety and (*R*)-configuration on hydroxyisobutyryl linker. Its activity was higher than PGM that was used as reference compound.

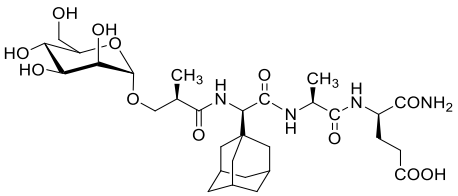


Figure 2: Immunostimulating mannosylated desmuramyl peptide (ManAdTP).

Results indicate that introduction of mannose plays a significant role in stimulation of the immune response and the possibility of effecting the immune response by mannose receptors family, group I CLRs, present on immunocompetent cells (such as macrophages and dendritic cells) [22,23]. It has been shown that uptake of liposomes displaying mannose ligands attached to the surface enhanced the uptake in human monocyte derived dendritic cells [24].

Here we describe structure-activity relationship (SAR) study on novel mannosylated desmuramyl peptides derivatives in which two series of compounds were prepared: (i) derivatives containing glycolyl linker between mannose and dipeptide, and (ii) derivatives containing parent (*R*)-hydroxyisobutyryl linker. In both series, positions of adamantane binding (to N- or C-terminus) were altered in comparison with derivatives lacking adamantane moiety. Immunostimulating properties of synthesized derivatives were assessed *in vivo* using ovalbumin as an antigen.

Results and Discussion

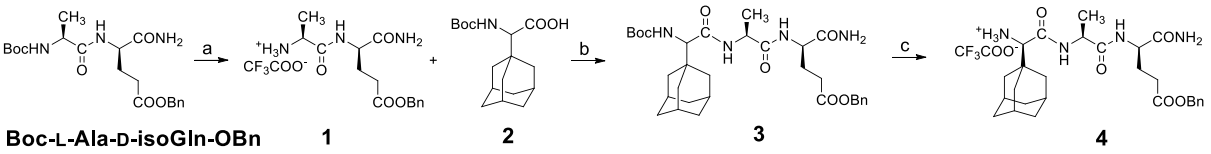
Design

Desmuramyl peptides enter into the cell by passive absorption and this process depends on lipophilicity [25]. Numerous analogues and derivatives which incorporated different lipophilic groups have shown improved activity [14,18,21,26]. To enable the systematic investigation of influence of lipophilic adamantane on immunostimulating activity, derivatives with altered position of adamantane moiety (at N- or C-terminus of the dipeptide), as well as derivatives lacking adamantane moiety, were prepared. Furthermore, herein we describe a strategy for the preparation of two series of mannose MDP analogues based on ManAdTP hit compound. Two series of mannoconjugates were prepared: (i) derivatives with glycolyl linker and (ii) the ones with (*R*)-hydroxyisobutyryl linker which is present in the parent ManAdTP. Glycolic linker was introduced due to the fact that *N*-glycolyl muramyl peptides induce significantly higher activation of NOD2 than MDP [27,28]. Mycobacteria present in complete Freuds adjuvant, and related Actinomycetes, produce *N*-glycolyl MDP by the hydroxylase action on MDP (*N*-acetyl muramic acid within the peptidoglycan). Influence of structural modifications on immunomodulating properties was estimated by the immunostimulatory effect on secondary humoral response to ovalbumin (antigen) in BALB/c mice.

Chemistry

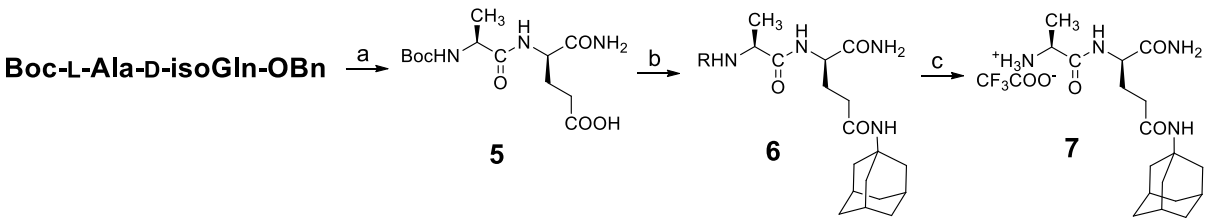
Peptide building blocks were prepared starting from fully protected desmuramyl peptide, Boc-L-Ala-D-*iso*GlnOBn. For the synthesis of derivatives lacking the adamantane moiety, dipeptide **1** with benzyl protection on C-terminus was obtained

after Boc-deprotection as previously reported (Scheme 1) [29]. Peptide **1** was also used for the synthesis of adamantly-containing desmuramyl tripeptide **3**.



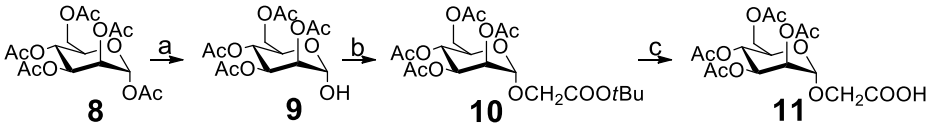
Scheme 1: Synthesis of desmuramyl peptides modified at N-terminus. Reagents and conditions: a) TFA/DCM = 1:2, rt, 1h, quantitative; b) EDC×HCl, HOBt×H_2O, Et_3N, DCM/dioxane = 1:1, 0°C → rt, 48h, 82%; c) TFA/DCM = 1:2, rt, 1h followed by chromatographic separation of isomers, 43%.

Compound **1** was coupled with previously prepared racemic Boc-protected (adamant-1-yl)glycine [30] **2** using the carbodiimide EDC/HOBt method [21]. Obtained mixture of BocAdGly-L-Ala-D-*iso*GlnOBn diastereoisomers was treated with trifluoroacetic acid in order to remove the Boc protecting group while the diastereoisomer **4** with D-L-D amino acid sequence was separated from the isomer mixture using silica gel column chromatography and $CHCl_3$/MeOH 1:1 as eluent. The spectral data of the isolated isomer **4** were compared to the published D-AdGly-L-Ala-D-*iso*Gln prepared from tripeptide *tert*-butyl ester [21]. Desmuramyl peptides **1** and **4** were further used for condensation reactions with hydroxyisobutyryl and glycolyl mannosides. Synthesis of desmuramyl peptide **7** with adamantane moiety bound at C-terminus is presented in Scheme 2.



Scheme 2: Synthesis of C-modified desmuramyl peptides. Reagents and conditions: a) H_2, 10% Pd/C, MeOH, 38 PSI, rt, 24h, 96%; b) 1-adamantamine hydrochloride, EDC×HCl, HOBt×H_2O, Et_3N, DCM/dioxane = 1:1, 0°C → rt, 48h, 60%; c) TFA/DCM = 1:2, rt, 1h, quantitative.

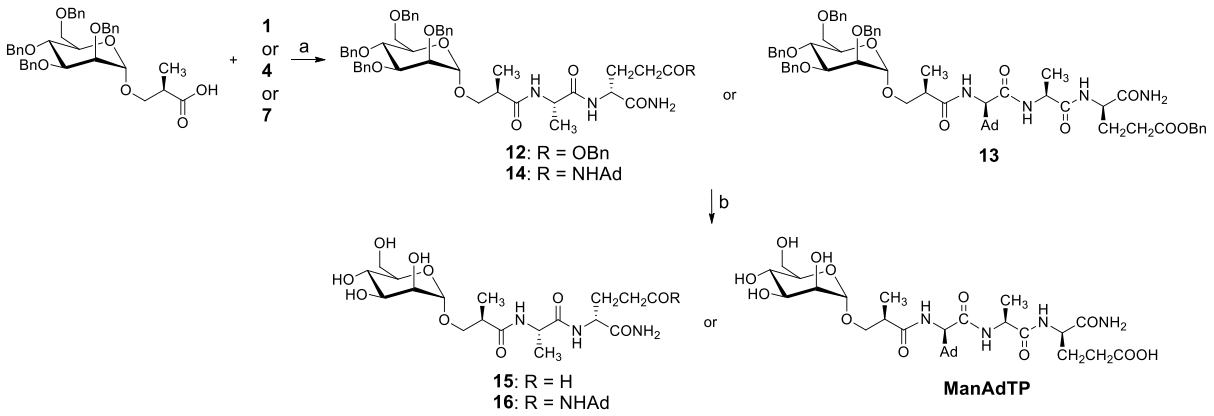
After hydrogenolysis of the starting dipeptide, condensation of free carboxyl group with 1-adamantamine hydrochloride was performed. Boc deprotection of obtained compound **6** gave the trifluoroacetic salt of peptide **7** which was used in the synthesis of mannoconjugates. Mannose precursor containing the glycolyl linker **11** was prepared in a three-step procedure shown in Scheme 3.



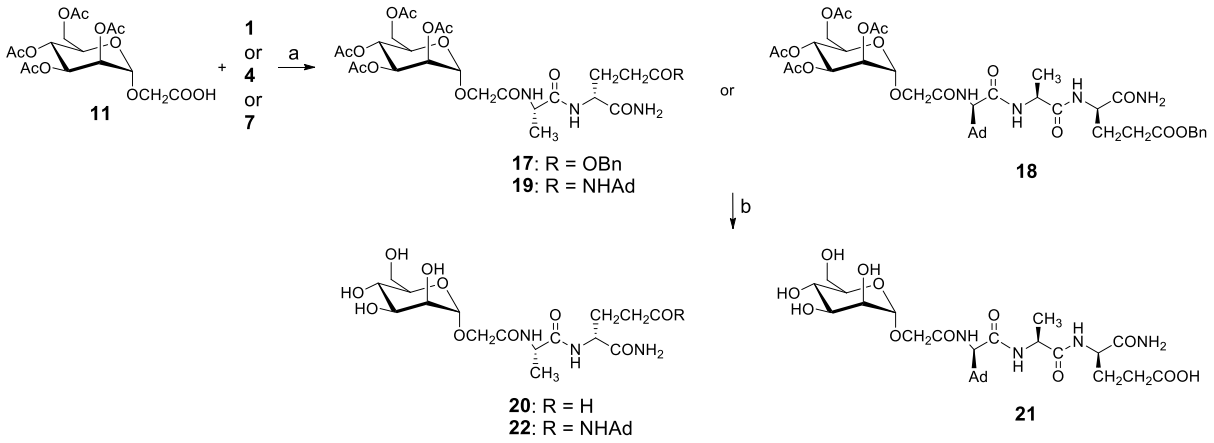
Scheme 3: Synthesis of mannose precursor. Reagents and conditions: a) $Zn(OAc)_2$×H_2O, abs. MeOH, rt, 20h, 60%; b) $BrCH_2COOC(CH_3)_3$, K_2CO_3, dry DMF, rt, 2h, 81%; c) TFA, dry DCM, rt, 1,5h, 72%.

Stereoselective α-anomeric deacetylation of peracetylated mannose 8 was followed by the S_N2 substitution of bromine from *tert*-butyl bromoacetate in the presence of potassium carbonate. Removal of ester group from compound **10** resulted with *O*-mannoside **11** with a free carboxyl group available for coupling of peptide moieties. Synthesis of benzyl protected α-mannoside containing (*R*)-hydroxyisobutyryl linker was previously described [31]. Condensations of peptides **1**, **4** and **7** with carboxy-

functionalized mannosides containing (*R*)-hydroxyisobutyryl and glycolyl linker are shown in Scheme 4 and 5, respectively.



Scheme 4: Synthesis of mannosylated peptides with hydroxyisobutyryl linker. Reagents and conditions: a) EDC×HCl, HOBt×H_2O, Et_3N, DCM/dioxane = 1:1, 0°C → rt, 48h, 52-90%; (b) H_2, 10 % Pd/C, 48 h, rt, 83-92%.



Scheme 5: Synthesis of mannosylated peptides with glycolyl linker. Reagents and conditions: a) EDC×HCl, HOBt×H_2O, Et_3N, DCM/DMF = 1:1, 0°C → rt, 72h, 26-67%; b) NaOMe/MeOH, rt, 1h, 59-89%.

For the amide bond formation between mannose and peptide part, an optimized EDC/HOBt method was used in each case. Synthesized compounds represent a small series of mannosylated desmuramyl peptides designed in a way that the introduced structural differences could answer two key questions: (i) Will the glycolyl linker in this class of compounds amplify immunostimulatory activity similar to the N-glycolyl derivative of MDP?, (ii) Which relative position of the adamantane group in the mannosylated desmuramyl peptides causes the greatest increase of the adjuvant activity?

Testing of immunostimulating activity

Adjuvant activity was estimated by the immunostimulatory effect on secondary humoral response to well-established model antigen ovalbumin (OVA) in BALB/c mice according to previously described in vivo studies [11,32.] Anti-OVA IgG, anti-OVA IgG1 and anti-OVA IgG2a were determined in the mice sera after supplementing the mice with the second booster. The comparison of induced anti-OVA IgG levels was carried out quantitatively and the subclasses of IgG, IgG1 and IgG2a, as indicators of Th1 or Th2 type of immune response, were also determined. The adjuvant activity of synthesized compounds was evaluated in comparison to the mannosylated adamantyl tripeptide ManAdTP and PGM. PGM was used as reference adjuvant in previously published studies since PGM and MDP have similar immunological properties: both stimulate the Th2-biased immune response specific for OVA antigen [11,32].

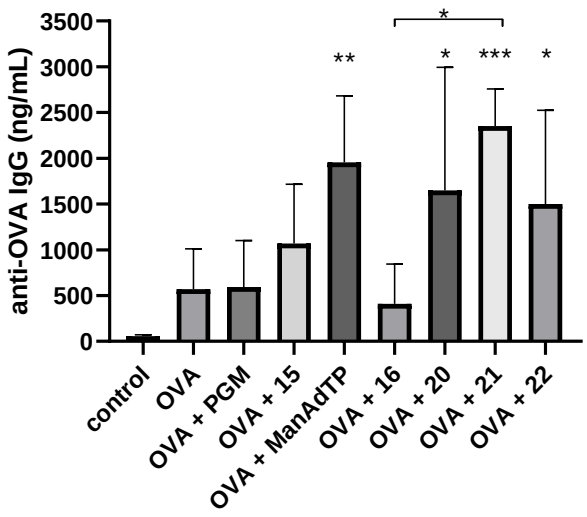


Figure 3: The effect of mannosyl desmuramyl peptides on production of anti-OVA IgG in BALB/c mice immunized with OVA as an antigen. Bar graphs represent average values from individual mice from each group (n=5). *p<0.05, **p<0.01 and ***p<0.001 denote statistical significance in comparison to the control group or groups connected with dashed line.

In general, when compared to the group treated with no adjuvant (OVA alone), enhancement in total anti-OVA IgG antibody production was observed in all groups except in group that received **16** (Figure 3). High levels of IgG antibody present even in OVA treated group, led to relatively weak stimulation of total antibody production in PGM-injected group. ManAdTP elicited better immune response than OVA alone and PGM-injected group. These results are in very good agreement with previous research where stimulation of anti-OVA IgG antibody production by ManAdTP and parent non-mannosylated AdTP were investigated in the NIH/OlaHsd mouse model in comparison to PGM [33]. In BALB/c mice used in this study, enhancement in total anti-OVA IgG antibody production by ManAdTP is statistically significant (p<0.01). A statistically significant boost in antibody responses to OVA antigen was observed in all groups immunized with compounds containing glycolyl linker (p<0.05 and and

p<0.001) in comparison to the control group. Immunization with compound **21** which has an adamantly tripeptide moiety attached to mannose through the glycolyl linker led to the highest and statistically the most significant increase in the specific IgG response (*p*<0.001). Additionally, amplification in total IgG antibody production was observed in all groups immunized with mannosylated desmuramyl peptides containing glycolyl linker **20**-**22** relatively to analogues **15**, ManAdTP and **16**. Results directly indicate that the introduction of the glycolyl linker plays a significant role in stimulation of the immune response in this class of adjuvants. Similarly, *N*-glycolyl muramyl peptides obtained by the oxidation of *N*-acetyl group of MDP, induce significantly higher activation of NOD2 than MDP [27,28]. Glycopeptide **21** was identified as the most potent adjuvant in this experiment and in this class of adjuvants, so far. Introduction of lipophilic moiety positively influences the adjuvant properties of MDP derivatives and analogues [12]. In both, hydroxyisobutyryl and glycolyl derivatives, introduction of bulky and lipophilic adamantane showed to be suitable for immunostimulatory activity. Adjuvant activity changes in respect to the position of adamant-1-yl moiety in peptide part. Anti-OVA IgG antibody stimulation was higher in the groups immunized with ManAdTP and **21** in respect to groups treated with **16** and **22**, respectively. This leads to the conclusion that the most suitable position of adamantane in this class of compounds is at peptide N-terminus. Adamantane can act as membrane anchor for mannose structures and thus be exposed on liposome surfaces and as such used in targeted drug delivery [34]. It can be also incorporated into β-cyclodextrine cavity, a powerful supramolecular nanoparticle carrier for targeted drug-delivery [35]. Previous research suggested a design of mannosylated desmuramyl peptides with adamantane at the C-terminus in order to facilitate the incorporation into hydrophobic layer of cavity because of the

minor steric hindrance of mannose and peptide part during the inclusion process of the adamantane [34,36].

It is well known that vaccine adjuvants can enhance or modulate the Th1/Th2-bias of induced immune response. Interferon-γ (as a Th1 cytokine) and IL-4 (as a Th2 cytokine) induce isotype switching to IgG2a and IgG1, respectively. Therefore, isotype profile of antigen specific anti-OVA IgG antibodies, IgG1 and IgG2a, is usually measured as a marker of Th1 and Th2 type immune response bias [37,38]. In this study, the type of generated immune response was indirectly estimated by quantification of OVA-specific IgG1 (for activation of Th2 type) and IgG2a (for activation of Th1 type) and calculation of the respective IgG1/IgG2a ratio. When the amount of anti-OVA IgG1 antibodies was measured (Figure 4a), it was observed that in all groups high levels of IgG1 antibody was present and the highest response, which was also statistically significant (p<0.001), was given by compound **21**. A slight suppression was noticed only in the production of anti-OVA IgG1 antibodies when compound **16** was administered.

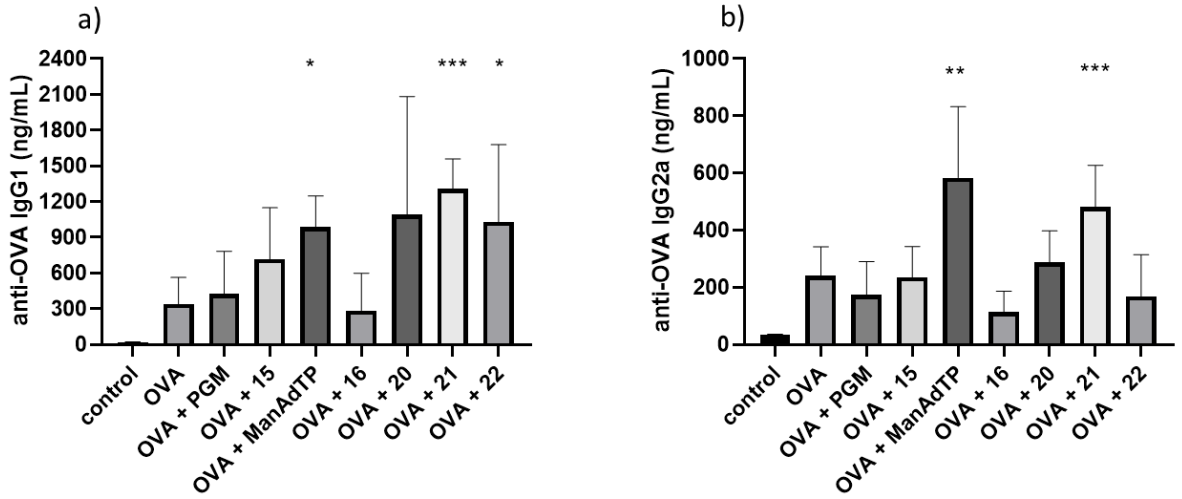


Figure 4: The effect of mannosylated desmuramyl peptides on production of anti-OVA IgG subtypes, anti-OVA IgG1 (a) and anti-OVA IgG2a (b) respectively, in

BALB/c mice immunized with OVA as an antigen. Bar graphs represent average values from individual mice from each group (*n*=5). *p<0.05 and ***p<0.001 denote statistical significance in comparison to the control group.

Statistically significant enhancement in anti-OVA IgG2a production (Figure 4b) was observed in groups immunized with mannosylated adamantyl-tripeptides, ManAdTP and **21**. The type of immune response was indirectly determined by quantification of IgG1 (for activation of Th2-type) and IgG2a (for activation of Th1-type) antibody for each serum (obtained after the second booster) and calculation of the IgG1/IgG2a ratio (Figure 5).

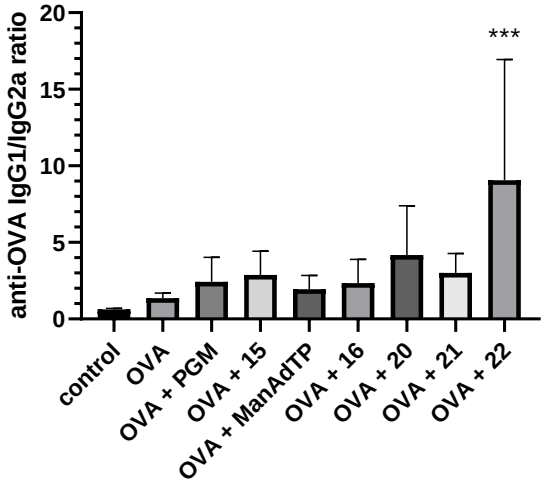


Figure 5: The ratio of anti-OVA IgG1 and anti-OVA IgG2a (IgG1/IgG2a) in BALB/c mice. For each mouse serum IgG1/IgG2a was calculated and the result for each experimental group (*n*=5) is presented as average ± standard deviation (SD). ***p<0.001 denote statistical significance in comparison to the control group.

From the IgG1/IgG2a ratio it is evident that all groups treated with tested adjuvants have higher values than the group treated with OVA alone, indicating the slight shift

toward more pronounced Th2 type of immune response. Compound **22** significantly switches immune response toward pronounced Th2 type, due to the predominant appearance of IgG1 antibodies. MDP and PGM dominantly induce IgG1 antibody production and stimulate Th2-polarized immune response as well [11,39]. It is well known that muropeptides act as NOD2 agonists and induce predominant Th2-biased response [40]. NOD2 agonists have the ability to act synergistically and augment the adjuvant activity of TLR ligands [10,41-43]. Furthermore, a combination of PRR ligands, such as NLR/TLR, induce Th1-polarized response. This NLR/TLR crosstalk is essential for modulation of innate and adaptive immune responses and leads to development of new approaches for the design of novel vaccines. Application of multi-PRR activation approaches can increase immunity significantly [44]. Another example of dual adjuvant system is represented by the activation of dendritic cells via combined macrophage-inducible CLR and TLR ligands [45]. Mannose receptors make one group in CLRs family which exist as soluble and trans-membrane receptors [4]. Like TLRs they initiate innate immune responses and activate acquired immunity. Mannose structures on the other hand, are one of the glycan structures that build up tumor antigens and regulate immune reaction by specific binding to CLRs. Therefore, compounds with expressed CRL agonist or antagonist properties could also be considered as potential agents for cancer immunotherapy [46,47]. Mannosylated liposomes with incorporated MDP have proved to be effective carriers for target inhibition of liver metastasis [48]. Therefore, presented mannosylated desmuramyl peptides with incorporated adamantane will be further explored in order to get a better insight into possible PRR crosstalk. Namely, inclusion of adamantane into carriers such as liposomes can additionally affect Th1/Th2 switch of immune response [49]. Presented results demonstrate the great immunostimulating potential of glycolyl-modified desmuramyl peptides. Peptidoglycan fragments with

mycobacterial structural features, such as synthesized compounds **20**-**22**, could efficiently link innate and adaptive immunity, similarly as *N*-glycolyl MDP enhances the innate immune response and T cell-mediated immunity [50]. NOD2-activation and interaction with CLR should be further explored, as well as a potential for synergistic multi-PRR activation.

Conclusion

A series of novel mannosylated desmuramyl peptides were prepared and characterized. In their structures, all glycopeptides comprised of mannose and key pharmacophore – desmuramyl peptide. These moieties are connected through glycolyl or hydroxyisobutyryl linker and additionally modified on N/C terminus with an adamantane subunit. The immunostimulating activities of tested compounds were compared to hit compounds ManAdTP and PGM. In experiments *in vivo*, all mannopeptides with glycolyl linker exhibited higher adjuvant activity than analogues with hydroxyisobutyryl linker indicating that the introduction of the glycolyl moiety plays a significant role in stimulation of the immune response. In particular, compound **21** was identified, so far, as the most potent adjuvant in this class of mannosylated desmuramyl peptides. It should be also noted that the compound **21** is stable, non-pyrogenic and water-soluble what makes it potentially applicable as an adjuvant for vaccines.

Supporting Information

Supporting Information File 1:

Experimental and characterization data

Acknowledgements

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