# Synthesis and Immunostimulating Properties of Novel Adamant-1-yl Tripeptides

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The aim of this work was to prepare L- and D-(adamant-1-yl)-Gly-L-Ala-D-isoGln peptides in order to study their adjuvant (immunostimulating) activities. Adjuvant activity of adamant-1-yl tripeptides was tested in the mouse model using ovalbumin as an antigen and in comparison to the peptidoglycan monomer (PGM; $\beta$-D-GlcNAc-(1$\rightarrow$4)-D-MurNAc-L-Ala-D-isoGln-*meso*DAP($\varepsilon$NH$_2$)-D-Ala-D-Ala) and structurally related adamant-2-yl tripeptides.

**Introduction.** – Peptidoglycans are the ubiquitous constituents of bacterial cell walls, built of large polysaccharide chains and short peptide units. Natural and synthetic peptidoglycans, as well as their high- or low-molecular-mass fragments were widely tested due to their remarkable biological activites, particularly as potent immunomodulators [1][2]. Smaller-size peptidoglycans, as well as some peptidoglycan fragments of which the most widely known are muramyl peptides, had been extensively studied, since such compounds could be considered for use as adjuvants for human and animal vaccines [3–5]. Muramyl peptides represent structural analogs of the peptidoglycan monomer (PGM; **1**; *Fig. 1*) of bacterial cell walls, and MDP (muramyl dipeptide; *N*-acetyl-muramyl-L-alanyl-D-isoglutamine) has been known as the smallest synthetic adjuvant-active molecule capable of replacing whole *Mycobacteria* in *Freund*'s adjuvant [5].

On the other hand, compounds containing adamantyl (=tricyclo[3.3.1.1$^{3,7}$]decyl) residues are known to exhibit various biological activities, they are used as antiviral agents, drugs in the treatment of *Parkinson*'s disease, and depression [6–8]. Recently, the role of adamantane conjugates with mannose in the process of preventing microbial adhesion to host cells was discussed as well [9]. Furthermore, it is known that introducing an adamantyl moiety into substances with known biological activity improves their pharmacological properties and enhances their activity [10].

Several hundred chemically defined MDP analogs and derivatives were synthesized, in order to modulate, preferably improve, the properties of the parent molecule. Among those was a new class of compounds where L-Ala-D-isoGln was linked to adamantanamine [11–15] and a group of desmuramyl peptides comprising a phthalimido group instead of *N*-acetylmuramic acid [16–18]. Our research in the field of potential adjuvants aims at the synthesis of desmuramyl peptides that comprise an adamantyl moiety as a liphophylic part of the newly synthesized substances, and we

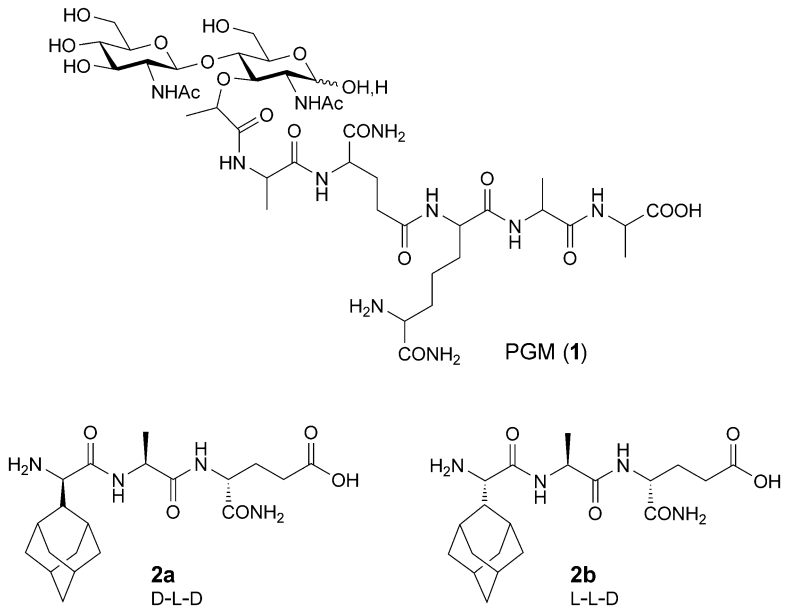



Fig. 1. *Structures of the PGM* (**1**) *and adamant-2-yl tripeptides* **2a** *and* **2b** [19]

previously reported the synthesis of derivatives in which the *N*-acetylmuramic portion of MDP was replaced with adamantylglycine. Previously synthesized admantyl tripeptides, **2a** and **2b** (*Fig. 1*), had the peptide portion linked at C(2) of the adamantyl moiety [19]. These diastereoisomers were tested separately and were shown to differ in biological activity in several model systems [19–22].

In this work, adjuvant (immunostimulating) activity of synthetic adamantyl tripeptides (AdTP), diastereoisomers of D,L-(adamant-2-yl)-Gly-L-Ala-D-isoGln ($Ad_2TP1$ (**2a**) and $Ad_2TP2$ (**2b**)) and the newly synthesized diastereoisomers of D,L-(adamant-1-yl)-Gly-L-Ala-D-isoGln ($Ad_1TP1$ (**3a**) and $Ad_1TP2$ (**3b**); *Fig. 2*), which comprise in their structure the adamantylglycyl moiety linked to the dipeptide L-Ala-D-isoGln, characteristic of the peptide portion of natural peptidoglycans and synthetic muramyl dipeptides, were studied. The adamantyl moiety was used in order to increase the lipophilic properties of this dipeptide, but it was also assumed that it might enhance its adjuvant activity. The adjuvant activities of adamantyl tripeptides and PGM (**1**) (peptidoglycan monomer, GlcNAc-MurNac-L-Ala-D-isoGln-*meso*DAP($\varepsilon NH_2$)-D-Ala-

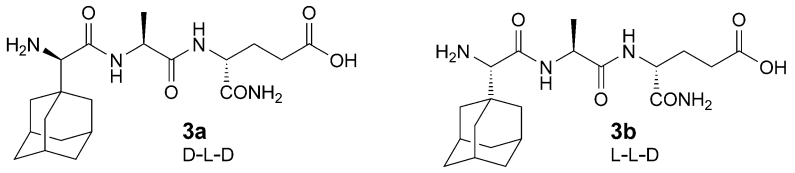



Fig. 2. *Structures of* D- *and* L-*(adamant-1-yl)-Gly*-L-*Ala*-D-*isoGln diastereoisomers* **3a** *and* **3b**

D-Ala) were compared. PGM (**1**) was established as a reference for adjuvant activity in a previously well-defined experimental model *in vivo*, established as suitable for comparison of the observed effects [23].

Our current study should provide information on structure–activity relationship concerning the possible difference in the activity of the diastereoisomers at C(1) ($Ad_1$TP1 (**3a**) and $Ad_1$TP2 (**3b**)) and C(2) ($Ad_2$TP1 (**2a**) and $Ad_2$TP2 (**2b**)) of the adamantyl moiety. Comparative study of adjuvant effects of adamantyl tripeptides on humoral IgG (immunoglobulin G) immune response was estimated and carried out in our well-defined experimental mouse model *in vivo* [23][24]. Ovalbumin was used as a customary antigen for studying the adjuvant effect. The comparison of induced anti-OVA IgG levels was carried out quantitatively, and the subclasses of IgG, IgG1, and IgG2a, as indicator of Th1 or Th2 type of immune response, were also determined.

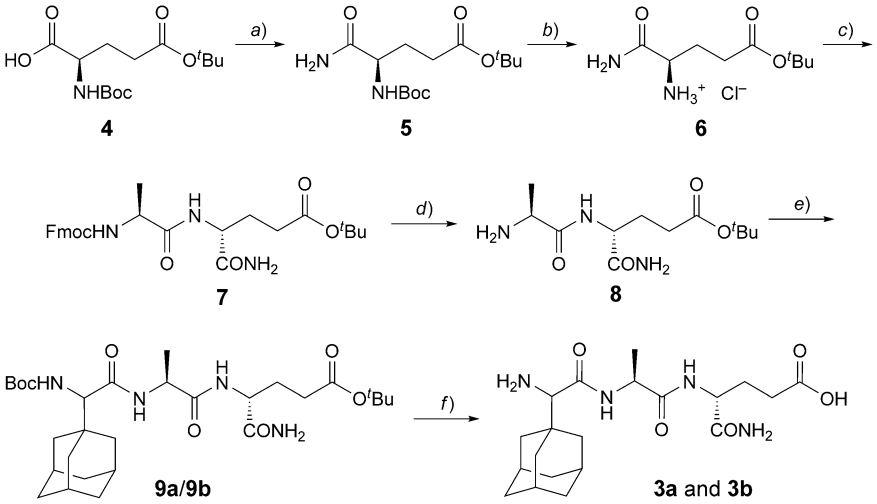
Only a few adjuvants have been approved for prophylactic vaccination of humans, in spite of decades of research and a few hundreds of pre-clinical candidates [25][26]. Obviously, there is a problem in designing a potent and well tolerated adjuvant that is needed today with the new generation of vaccines. These highly purified recombinant proteins (as antigens) are mostly poorly immunogenic and require adjuvants to become effective vaccines.

Immunomodulating characteristics of natural peptidoglycan molecules are well-known [2][27–29], and some of these compounds were considered for use as adjuvants for human and animal vaccines. Furthermore, several successful attempts to synthetically modify PGM (**1**) as a parent compound were undertaken in spite of its complex structure. In previous reports, we described the synthesis of **1** modified with Boc-tyrosine [30], adamant-1-yl [31], as well as with mannopyranosyl [32] residues. Smaller synthetic molecules such as muramyl dipeptides and tripeptides were investigated as well, and a number of structure–activity studies were reported. Thus, several synthetically modified MDPs, syntheses of which were patented, have already been included in clinical trials [33]. Furthermore, formulations in liposomes of these compounds were found to be of interest. Therefore, modifications with lipophilic substituents are of special relevance, since lipophilic parts in parent molecules render them better constituents for incorporation in liposomes [34][35]. In view of these reports including some of our previous work [30][32], it seemed justified to further explore the influence of the adamantyl moiety with its lipophilic character on biological properties of the dipeptide building block of MDP, with emphasis on its adjuvant activity.

**Results and Discussion.** – *Chemistry.* In this work, the adjuvant (immunostimulating) activities of synthetic adamantyltripeptides $Ad_2$TP1 (**2a**) and $Ad_2$TP2 (**2b**) [19], and newly synthesized $Ad_1$TP1 (**3a**) and $Ad_1$TP2 (**3b**) were studied.

D- and L-(adamant-1-yl)glycyl-L-alanyl-D-isoglutamine (**3a** and **3b**, resp.) were prepared by a sequence of steps as described in the *Scheme*. (*tert*-Butoxy)carbonyl-D-glutamic acid 5-*tert*-butyl ester (Boc-D-Glu-O$^t$Bu; **4**) was converted to an amide (*via* the carbamate intermediate) to give the isoglutamine derivative **5** in excellent yields [36]. D-Isoglutamine *tert*-butyl ester hydrochloride (**6**) was then successfully prepared by removing the *N*-Boc group in the presence of the *tert*-butyl ester, another acid-sensitive group, by treatment with HCl gas in AcOEt [37][38]. The isolation of

Scheme. *Synthesis of Adamant-1-yl tripeptides* **3a** *and* **3b**.




*a*) ClCOOEt, $Et_3N$, $NH_3$(g), $CHCl_3$. *b*) HCl(g) in AcOEt. *c*) 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC)·HCl, 1-hydroxybenzotriazole (HOBt)·$H_2O$, *N*-ethylmorpholine, Fmoc-L-Ala (Fmoc = (9*H*-fluoren-9-yl)methoxycarbonyl), dioxane/$CH_2Cl_2$ 1:1. *d*) 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), octane-1-thiol, THF. *e*) EDC·HCl, HOBt·$H_2O$, $Et_3N$, Boc-(1-Ad)Gly (Boc = (*tert*-butoxy)carbonyl), dioxane/$CH_2Cl_2$ 1:1. *f*) $CF_3COOH$ (TFA)/$CH_2Cl_2$ 1:1.

compound **6** was facile, although we have shown that other procedures may be used, as well. Thus, concentrated $H_2SO_4$ in AcO$^t$Bu or methanesulfonic acid (MsOH) in AcO$^t$Bu/$CH_2Cl_2$ were shown to be efficient reagents for the selective Boc deprotection of compound **5**. The main disadvantage of these methods was the partial solubility of the starting amino acid **5** in AcO$^t$Bu, causing some difficulties in the separation of the unreacted **5** and the product **6** which precipitates during the reaction progress. Dipeptide **7** was prepared next by condensation of **6** with [(9*H*-fluoren-9-yl)methoxy]carbonyl-L-alanine (Fmoc-L-Ala), followed by removing the *N*-Fmoc protection [39] to give L-alanyl-D-isoglutamine *tert*-butyl ester (**8**). This method was much more efficient than the one using piperidine/THF due to the low volatility of the solvent. *rac*-(Adamant-1-yl)glycine hydrochloride was prepared by the Co-mediated $\beta$-keto ester alkylation, followed by *Schmidt* rearrangement and acid hydrolysis [40]. Subsequently, Boc-L,D-(adamant-1-yl)glycine (Boc-(1-Ad)Gly) was synthesized by *N*-Boc protection of *rac*-(adamant-1-yl)glycine hydrochloride. The racemic amino acid was used, even though the optically pure (adamant-1-yl)glycines and their derivatives are known [40][41]. The diastereoisomeric mixture Boc-D/L-(adamant-1-yl)glycyl-L-alanyl-D-isoglutamine *tert*-butyl ester (**9a**/**9b**, resp.) was prepared by the condensation of dipeptide **8** with *rac*-Boc-(1-Ad)Gly [9][32]. The tripeptide was deprotected in the next step without previous separation of the diastereoisomers. The protecting *N*-Boc and $^t$BuO groups were then removed in one step [42] to yield the mixture D-/L-(adamant-1-yl)glycyl-L-alanyl-D-isoglutamine (**3a**/**3b**, resp.) in good yields. Pure **3a** and **3b** were obtained after separation by column chromatography on silica gel, and were

unequivocally identified by NMR spectroscopy and enzymatic oxidations. Special batches of endotoxin-free **3a** and **3b** were prepared for testing their biological activities.

The absolute configurations of the diastereoisomers **3a** and **3b** were established by enzymatic oxidations of their hydrolysates using L-amino acid oxidase [19][43] at pH 7. Total acid hydrolysis of **3a** and **3b** with 6M HCl gave hydrolysates, which were incubated, after the removal of HCl, with L-amino acid oxidase. The resulting incubation mixtures were compared with the starting hydrolysates by TLC. Thus, no L-Ala was detected in the mixture resulting from the incubation of the hydrolysate of **3a**, since L-Ala was oxidized. On the other hand, only D-Glu (from D-isoGln) was detected in the oxidation mixture of the hydrolysate of **3b**, since L-(Ad-1-yl)Gly and L-Ala were oxidized.

The described amino acid oxidase experiments not only established the absolute configurations of **3a** and **3b**, but also documented the preservation of the L-Ala configuration during the peptide synthesis.

*Testing the Immunostimulating Activity.* Adjuvant activities of adamantyl tripeptides **2a**, **2b**, **3a**, and **3b** were evaluated by their immunostimulatory effects on secondary humoral response to ovalbumin (antigen) in mice of two genetically different strains, CBA (H-$2^k$) and NIH/OlaHsd (H-$2^q$), according to our previously described mouse model *in vivo* [23][24]. Anti-OVA IgG, anti-OVA IgG1, and anti-OVA IgG2a were determined in the mice sera after the second booster (*Figs. 3* and *4*).

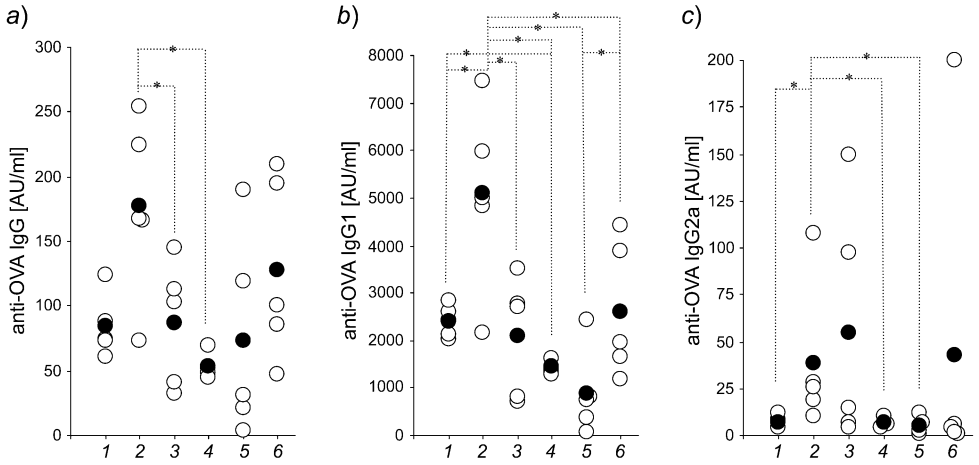



Fig. 3. *The effect of $Ad_1TP1$* (**3a**)*, $Ad_1TP2$* (**3b**)*, $Ad_2TP1$* (**2a**)*, and $Ad_2TP2$* (**2b**) *on the production of total anti-OVA IgG* (*a*)*, and its subtypes anti-OVA IgG1* (*b*) *and anti-OVA IgG2a* (*c*)*, respectively, in CBA (H-$2^k$) mice.* Mice were immunized with OVA as an antigene and sera were analyzed after second booster. Experimental groups: *1*, OVA alone; *2*, OVA + PGM (**1**); *3*, OVA + **3a**; *4*, OVA + **3b**; *5*, OVA + **2a**; *6*, OVA + **2b**. ●, roup mean value; ○, each serum separately. ∗: $p < 0.05$ in comparison to the group connected with dashed line.

In general, in both mice strains, no significant enhancement in total anti-OVA IgG antibody production was observed, when both adamant-1-yl and adamant-2-yl tripeptides were applied in comparison to no adjuvant (OVA alone) treated group. In comparison to the control, PGM-injected group, no better immunostimulating effect

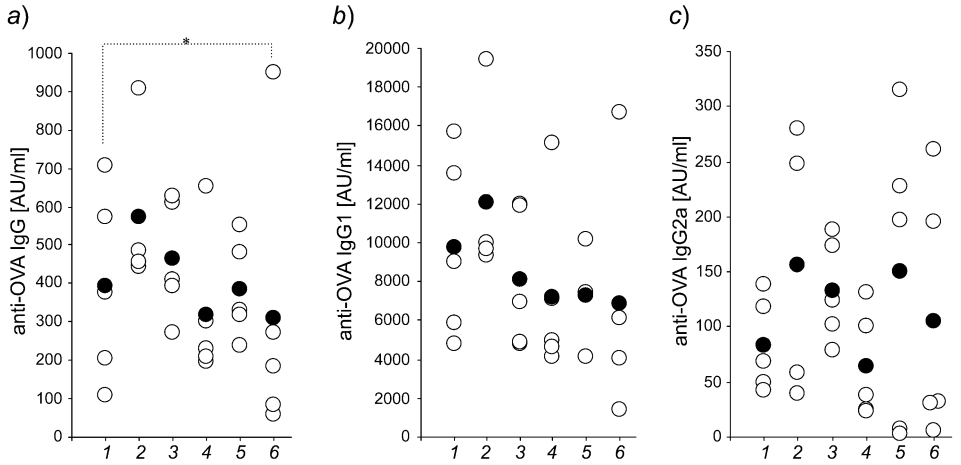



Fig. 4. *The effect of $Ad_1TP1$* (**3a**)*, $Ad_1TP2$* (**3b**)*, $Ad_2TP1$* (**2a**)*, and $Ad_2TP2$* (**2b**) *on the production of anti-OVA IgG* (*a*)*, and its subtypes anti-OVA IgG1* (*b*) *and anti-OVA IgG2a* (*c*)*, respectively, in NIH/OlaHsd (H-2$^q$) mice.* Mice were immunized with OVA as an antigene, and sera were analyzed after second booster. Experimental groups: *1*, OVA alone; *2*, OVA + PGM (**1**); *3*, OVA + **3a**; *4*, OVA + **3b**; *5*, OVA + **2a**; *6*, OVA + **2b**. ●, Group mean value; ○, each serum separately. ∗: $p < 0.05$ in comparison to the group connected with dashed line.

of tested adjuvants was observed either. However, comparing the production of anti-OVA IgG antibodies in mice of two different strains, a difference was observed. On the whole, the measured quantity of anti-OVA IgG antibodies was higher in NIH/OlaHsd (H-2$^q$) mice than in CBA (H-2$^k$) mice. The same trend was detected in both mice strains, when $Ad_1TP1$ (**3a**) and $Ad_1TP2$ (**3b**) were applied; a weak stimulation of antibody production was achieved with $Ad_1TP1$ (**3a**), whereas $Ad_1TP2$ (**3b**) was devoid of any stimulation. But, when adamant-2-yl tripeptides were applied, a different trend in antibody production was detected. In CBA (H-2$^k$) mice, the $Ad_2TP2$ (**2b**) isomer showed a higher stimulation of anti-OVA IgG antibodies production when compared to $Ad_2TP1$ (**2a**). When NIH/OlaHsd (H-2$^q$) mice were used in the experiment, the reversed situation was observed: $Ad_2TP1$ (**2a**) elicited the antibody production better than $Ad_2TP2$ (**2b**).

Since it is well-known that vaccine adjuvants can enhance or modulate the Th1/Th2 bias of induced immune response [44][45], we were also interested in the effect of tested adjuvants on the type of immune response. In this study, the type of generated immune response was indirectly estimated by quantification of OVA (antigen)-specific IgG1 (for activation of Th2 type) and IgG2a (for activation of Th1 type), and calculation of the respective IgG1/IgG2a ratio (*Table*).

When the amount of anti-OVA IgG1 antibodies was measured in two mice strains injected with adamantyl tripeptides, it was observed that the response in the NIH/OlaHsd (H-2$^q$) mice was higher than in CBA (H-2$^k$) mice. However, the obtained values could be considered as a slight suppression in the production of anti-OVA IgG1 antibodies in comparison with the results obtained when OVA alone or OVA with PGM (**1**) were administered. For $Ad_1TP2$ (**3b**), the response was a little lower than for

$Ad_1$TP1 (**3a**), and, for $Ad_2$TP2 (**2b**), it was significantly higher than for $Ad_2$TP1 (**2a**) in CBA (H-$2^k$) mice. Basically, it was the same pattern as for anti-OVA IgG antibodies. In NIH/OlaHsd (H-$2^q$) mice, there was essentially no difference in anti-OVA IgG1 antibody amount observed, neither when $Ad_1$-tripeptides nor $Ad_2$-tripeptides were applied.

When anti-OVA IgG2a antibodies were measured, the same trend as for anti-OVA IgG1 antibodies and anti-OVA IgG antibodies was observed in both mice strains. Hence, in comparison to the experimental group injected with OVA alone, without tested adjuvants, the amount of anti-OVA IgG2a in CBA(H-$2^k$) mice was higher, when $Ad_1$TP1 (**3a**) and $Ad_2$TP2 (**2b**) were applied, and, in NIH/OlaHsd (H-$2^q$) mice, when $Ad_1$TP1 (**3a**) and $Ad_2$TP1 (**2a**) were administered.

From IgG1/IgG2a ratio calculated for each serum (obtained after the second booster) and each experimental group of two different mice strains, it could be observed that neither of tested adamantyl tripeptides significantly shifted the IgG1/IgG2a ratio in comparison to no adjuvant-treated group of animals (*Table*). Accordingly, the tested adamantyl tripeptides did not stimulate immunomodulatory activity towards more pronounced Th1 or Th2 type of immune response in comparison to antigen alone.

Table. *The Ratio of anti-OVA IgG1 and anti-OVA IgG2a* (IgG1/IgG2a) *in NIH/OlaHsd (H-$2^q$) and CBA (H-$2^k$) Mice.* For each mouse serum, obtained after the second booster, $\log_{10}$ IgG1/IgG2a was calculated, and the result for each experimental group ($n=5$) is presented as average ± standard deviation (S.D.).

| Exsperimental groups | NIH/OlaHsd (H-$2^q$) Mice | CBA (H-$2^k$) Mice |
|---|---|---|
| No adjuvant | 2.07 ± 0.1 | 2.55 ± 0.2 |
| PGM (**1**) | 2.01 ± 0.5 | 2.23 ± 0.4 |
| $Ad_1$TP1 (**3a**) | 1.65 ± 0.1 | 1.87 ± 1.0 |
| $Ad_1$TP2 (**3b**) | 2.11 ± 0.3 | 2.33 ± 0.2 |
| $Ad_2$TP1 (**2a**) | 1.83 ± 0.6 | 2.12 ± 0.2 |
| $Ad_2$TP2 (**2b**) | 2.03 ± 0.5 | 2.59 ± 0.7 |

**Conclusions.** – L- and D-(adamant-1-yl)-Gly-L-Ala-D-isoGln peptides were prepared in six steps. All intermediate products and the final adamantyl peptides were fully characterized. The desired diastereoisomers, D-(adamant-1-yl)-Gly-L-Ala-D-isoGln ($Ad_1$TP1; **3a**) and L-(adamant-1-yl)-Gly-L-Ala-D-isoGln ($Ad_1$TP2, **3b**), were separated, and their absolute configurations were unequivocally determined.

Adjuvant activities of adamant-1-yl and adamant-2-yl tripeptides were tested in mice of two genetically different strains, CBA (H-$2^k$) and NIH/OlaHsd (H-$2^q$). All tested diastereoisomers elicited immune response but lower than that of PGM (**1**) in both mouse strains.

Comparing the diastereoisomers of adamant-1-yl tripeptides revealed that the induction of all three antibody types was better, when $Ad_1$TP1 (**3a**) was applied in CBA (H-$2^k$) mouse strain. On the contrary, when administered in CBA (H-$2^k$) mice, adamant-2-yl tripeptides revealed the opposite behavior: $Ad_2$TP2 (**2b**) elicited better immune response with respect to IgG, IgG1, and IgG2a induction. In NIH/OlaHsd (H-$2^q$) mouse strain, the pattern of IgG, IgG1, and IgG2a induction was basically the same

for both adamant-1-yl and adamant-2-yl tripeptides; $Ad_1TP1$ (**3a**) and $Ad_2TP1$ (**2a**) were always slightly better than $Ad_1TP2$ (**3b**) and $Ad_2TP2$ (**2b**), respectively, in inducing immune response.

In both CBA (H-$2^k$) and NIH/OlaHsd (H-$2^q$) mice strains, it was demonstrated that the immune response specific for OVA alone was Th2-biased due to the predominant appearance of IgG1 antibodies. PGM (**1**), as well, dominantly induced IgG1 antibody production and stimulated Th2-biased immune response.

In comparison to antigen alone, all tested adamantyl tripeptides did not show a tendency to modulate the OVA-specific response towards more pronounced Th1 or Th2 type of immune response. But a pronounced difference in IgG1 response was observed, when different mouse strains were used in the study. Regarding the IgG1 antibody production, the immune response in NIH/OlaHsd (H-$2^q$) mouse strain was approximately three times higher than in CBA (H-$2^k$) mouse strain.

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## Experimental Part

*General.* Bovine serum albumin (BSA), *Tween 20*, monoclonal anti-chicken egg albumin (clone OVA-14, mouse IgG1 isotype), avidin peroxidase, and *ortho*-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). L-Amino acid oxidase (EC 1.4.3.2.) from *Crotalus adamanteus*, type IV, was purchased from *Sigma* (USA). Chemicals for buffers and solns. were from *Kemika*, Croatia, unless stated otherwise. Peptidoglycan monomer was prepared in *PLIVA* (*Chemical and Pharmaceutical Works*, HR-Zagreb), according to the procedure described in [46]. (Adamant-2-yl)glycine [47], and adamant-2-yl tripeptides, $Ad_2TP1$ (**2a**) and $Ad_2TP2$ (**2b**) [19] were prepared at the Institute of Immunology, as described earlier. Racemic (adamant-1-yl)glycine hydrochloride was prepared as described in [40]. Chemical reagents used in syntheses were obtained from *Fluka* and *Aldrich Corp*. All org. solvents were purified using standard procedures.

Column chromatography (CC, solvents and proportions are given in the text) was performed on *Merck* silica gel 60 (70–230 mesh ASTM). For TLC monitoring, *Fluka* silica gel (*60 $F_{254}$*) plates (0.25 mm) were used. Visualization was effected with UV light, $I_2$, and ninhydrin. M.p.: in open capillaries with a *Büchi B-540* apparatus. Optical rotations $[\alpha]_D$: at r.t. with the *Schmidt+Haensch Polartronic NH8*. NMR Spectra: *Bruker Avance* (300 MHz) spectrometer; $\delta$ in ppm rel. to TMS, *J* in Hz. MS: *Waters MS-Quattro micro* instrument; in *m/z*. C, H, and N analyses were provided by the Analytical Services Laboratory of the Ruđer Bošković Institute, Zagreb.

HPLC Analyses (*Thermo Separation Products*, *Spectra SYSTEM 2000*) were performed on an *RP-18* column (*Vydac*, 5 μm, 218 × 4 mm) at r.t. at a flow rate of 1.0 ml/min, and the eluate was monitored at 214 nm. The gradient solvent system used consisted of MeCN containing 0.035% TFA, and $H_2O$ containing 0.05% TFA. The percentage of MeCN at 0, 20, and 21 min was 3, 17 and 3, resp., and the running time was 25 min.

*[(*tert-*Butoxy)carbonyl]*-D-*isoglutamine* tert-*Butyl Ester* (**5**). To a soln. of Boc-D-Glu-O$^t$Bu (**4**; 2.5 g, 8.24 mmol) in dry $CHCl_3$ (30 ml) at −15°, $Et_3N$ (1.26 ml, 9.06 mmol, 1.1 equiv.) and ClCOOEt (866 μl, 9.06 mmol, 1.1 equiv.) were added. The mixture was stirred for 0.5 h at −15°. Dry $NH_3$(g) was then passed through the stirred mixture for 2 h at 0°. The soln. was diluted with $CHCl_3$, extracted twice with 7% $K_2CO_3$, and washed twice with $H_2O$. The org. layer was dried ($MgSO_4$) and, after filtration, evaporated to yield **5** (2.38 g, 95%). White solid. $R_f$ ($^i$PrOH/petroleum ether (PE)/$H_2O$, 5 : 3 : 1) 0.82. M.p. 122.7–124.5°. $[\alpha]_D = -4.6$ ($c = 0.5$, MeOH). $^1$H-NMR ($CDCl_3$): 6.46 (*s*, NH); 5.81 (*s*, NH); 5.41 (*d*, *J* =

7.3, NH); 4.21–4.13 (*m*, H–C($\alpha$)); 2.49–2.27 (*m*, $CH_2(\gamma)$); 2.16–2.04 (*m*, 1 H, $CH_2(\beta)$); 1.96–1.84 (*m*, 1 H, $CH_2(\beta)$); 1.45 (*s*, Boc); 1.44 (*s*, $^t$Bu). $^{13}$C-NMR ($CDCl_3$): 174.10 ($CONH_2$); 172.74 (*C*OO$^t$Bu); 155.75 (CO of Boc); 80.93 ($Me_3C$); 53.54 (CH($\alpha$)); 31.79 ($CH_2(\gamma)$); 28.30 (*Me*$_3$C of Boc); 28.06 (*Me*$_3$C); 27.73 ($CH_2(\beta)$).

D-*Isoglutamine* tert-*Butyl Ester Hydrochloride* (**6**). A freshly prepared sat. soln. of dry HCl in dry AcOEt (40 ml), prepared by bubbling HCl into AcOEt for 1 h, was added to **5** (2 g, 6.6 mmol). The mixture was stirred for 3 h at r.t. During the reaction, a white solid precipitated. The mixture was evaporated, and $Et_2O$ was added to the residue. Product **6** (0.97 g, 62%) was filtered off as a white solid. $R_f$ ($^i$PrOH/PE/$H_2O$, 5 : 3 : 1) 0.51. M.p. 212.2–214.4°. $[\alpha]_D = -10$ ($c = 0.5$, MeOH). $^1$H-NMR ($D_2O$): 3.96 (*t*, $J = 6.6$, H–C($\alpha$)); 2.39 (*t*, $J = 7.5$, $CH_2(\gamma)$); 2.10–2.03 (*m*, $CH_2(\beta)$); 1.36 (*s*, $^t$Bu). $^{13}$C-NMR ($D_2O$, dioxane): 173.39 ($CONH_2$); 171.33 (*C*OO$^t$Bu); 83.05 ($Me_3C$); 52.18 (CH($\alpha$)); 30.43 ($CH_2(\gamma)$); 27.11 (*Me*$_3$C); 25.70 ($CH_2(\beta)$). ESI-MS: 203.20 ($M^+$, $C_9H_{19}N_2O_3^+$; calc. 203.26).

*{[(9*H*-Fluoren-9-yl)methoxy]carbonyl}*-L-*alanyl*-D-*isoglutamine* tert-*Butyl Ester* (**7**). To a soln. of Fmoc-Ala (1 g, 3.2 mmol) in dry $CH_2Cl_2$ (15 ml) at 0°, 3-[3-(dimethylamino)propyl]-1-ethylcarbodiimide (EDC·HCl; 738 mg, 3.85 mmol, 1.2 equiv.) and *N*-hydroxybenzotriazole (HOBt; 521 mg, 3.85 mmol, 1.2 equiv.) were added. The mixture was then stirred for 0.5 h at the same temp., and a soln. of **6** (789 mg, 3.85 mmol, 1.2 equiv.) in dioxane (30 ml) and *N*-ethylmorpholine (813 µl, 6.42 mmol, 2 equiv.) were then added. The mixture was stirred for 1 h at 0°, and for 48 h at r.t. The soln. was diluted with $CH_2Cl_2$, extracted with 0.5M HCl, and washed with sat. aq. $NaHCO_3$. The org. layer was dried ($Na_2SO_4$) and, after filtration, evaporated. The residue was purified by CC (silica gel; MeCN/$H_2O$ 5 : 1) to afford **7** (1.21 g, 76%). White solid. $R_f$ (MeCN/$H_2O$ 5 : 1) 0.78. M.p. 190.3–192°. $[\alpha]_D = +16.4$ ($c = 0.5$, DMF). $^1$H-NMR ($CDCl_3$): 7.74 (*d*, $J = 7.4$, H–C(4) and H–C(5) of Fmoc); 7.56 (*d*, $J = 6.8$, H–C(1) and H–C(8) of Fmoc); 7.41 (*d*, $J = 7.1$, NH); 7.39–7.28 (*m*, H–C(2), H–C(7), H–C(3), and H–C(6) of Fmoc); 4.46–4.43 (*m*, H–C($\alpha$) of Ala); 4.37 (*d*, $J = 6.9$, $CH_2$ of Fmoc); 4.23 (*t*, $J = 6.5$, H–C(9) of Fmoc); 4.18 (*t*, $J = 6.1$, H–C($\alpha$) of isoGln); 2.45–2.42 (*m*, 1 H, $CH_2(\gamma)$); 2.34–2.31 (*m*, 1 H, $CH_2(\gamma)$); 2.13–2.10 (*m*, 1 H, $CH_2(\beta)$); 2.00–1.94 (*m*, 1 H, $CH_2(\beta)$); 1.36 (*s*, $^t$Bu); 1.40 (*d*, $J = 5.9$, Me of Ala). $^{13}$C-NMR ($CDCl_3$): 173.72, 172.93 (CONH and *C*OO$^t$Bu); 158.25 (CO of Fmoc); 143.72, 143.65, 141.25 (arom. C of Fmoc); 127.73, 127.09, 127.06, 119.97 (arom. CH of Fmoc); 81.23 ($Me_3C$); 67.15 ($CH_2$ of Fmoc); 52.59 (CH($\alpha$) of isoGln); 50.90 (CH($\alpha$) of Ala); 47.05 (CH of Fmoc); 31.82 ($CH_2(\gamma)$); 28.01 (*Me*$_3$C); 27.10 ($CH_2(\beta)$); 18.32 (Me of Ala). ESI-MS: 496.30 ($[M+H]^+$, $C_{27}H_{34}N_3O_6^+$; calc. 496.24). Anal. calc. for $C_{27}H_{33}N_3O_6$: C 65.44, H 6.71, N 8.48; found: C 65.76, H 7.11, N 8.12.

L-*Alanyl*-D-*isoglutamine* tert-*Butyl Ester* (**8**). To a soln. of **7** (1000 mg, 2.02 mmol) in dry THF (20 ml) under $N_2$, octane-1-thiol (4.2 ml, 20.2 mmol, 10 equiv.) and DBU (99.5 µl, 0.67 mmol, 0.33 equiv.) were added. The mixture was stirred at r.t. for 16 h and monitored by TLC (MeCN/$H_2O$ 5 : 1). The mixture was then triturated with $Et_2O$, and the white solid residue was purified by CC (silica gel, $CHCl_3$/MeOH 1 : 1) to afford **8** (304 mg, 55%). Colorless solid foam. $R_f$ (MeCN/$H_2O$ 5 : 1) 0.28. $[\alpha]_D = 8.0$ ($c = 0.5$, MeOH). $^1$H-NMR ($CD_3OD$): 4.36 (*dd*, $J = 5.0, 6.7$, H–C($\alpha$) of isoGln); 3.56 (*q*, $J = 6.9$, H–C($\alpha$) of Ala); 2.34–2.31 (*m*, $CH_2(\gamma)$); 2.15–2.09 (*m*, 1 H, $CH_2(\beta)$); 1.91–1.84 (*m*, 1 H, $CH_2(\beta)$); 1.45 (*s*, $^t$Bu); 1.32 (*d*, $J = 6.9$, Me of Ala). $^{13}$C-NMR ($CD_3OD$): 176.99, 176.30, 173.82 (CONH and *C*OO$^t$Bu); 81.93 ($Me_3C$); 53.53 (CH($\alpha$) of isoGln); 51.25 (CH($\alpha$) of Ala); 32.66 ($CH_2(\gamma)$); 28.59 ($CH_2(\beta)$); 28.38 (*Me*$_3$C); 20.71 (Me of Ala).

*[(*tert*-Butoxy)carbonyl]*-L,D-*(adamant-1-yl)glycine* (Boc-(1-Ad)Gly). L,D-(Adamant-1-yl)glycine hydrochloride [40] (350 mg, 1.42 mmol) was suspended in dioxane/$H_2O$ 2 : 1 (12 ml). Di(*tert*-butyl) dicarbonate (467 mg, 2.84 mmol, 2 equiv.) and 1M NaOH (6.25 ml, 4.4 equiv.) were added. The mixture was stirred at r.t. for 24 h and than acidified (pH 3) with sat. aq. $KHSO_4$. The soln. was extracted twice with AcOEt, the org. layer washed with $H_2O$ and dried ($Na_2SO_4$). The product Boc-(1-Ad)Gly (392 mg, 89%) was isolated as a white solid. $R_f$ (AcOEt/AcOH/$H_2O$ 3 : 1 : 1) 0.80. M.p. 187.6–189.9°. $^1$H-NMR ($CDCl_3$): 7.27 (*s*, NH); 4.01 (*s*, H–C($\alpha$)); 2.01 (br. *s*, 3 H of Ad); 1.73–1.55 (*m*, 12 H of Ad); 1.45 (*s*, Boc). $^{13}$C-NMR ($CDCl_3$): 175.45 (COOH); 152.20 (CO of Boc); 62.40 (CH of Gly); 42.20, 40.24, 38.55, 36.64, 36.42, 36.35 (C and $CH_2$ of Ad); 28.36, 28.32, 28.26, 27.94 (CH of Ad, *Me*$_3$C).

*[(*tert*-Butoxy)carbonyl]*-D/L-*(adamant-1-yl)glycyl*-L-*alanyl*-D-*isoglutamine* tert-*Butyl Ester* (**9a**/**9b**). To a soln. of Boc-(1-Ad)Gly (257 mg, 0.83 mmol) in dry $CH_2Cl_2$ (5 ml) at 0°, EDC·HCl (191 mg, 1 mmol, 1.2 equiv.) and HOBt (135 mg, 1 mmol, 1.2 equiv.) were added. The mixture was stirred for 0.5 h at the

same temp., and a soln. of **8** (272 mg, 1 mmol, 1.2 equiv.) in dioxane (5 ml) and $Et_3N$ (813 µl, 6.42 mmol, 2 equiv.) were then added. The mixture was stirred for 1 h at 0° and than for 48 h at r.t. The soln. was diluted with $CH_2Cl_2$, extracted with 0.5M HCl, and washed with sat. aq. $NaHCO_3$. The org. layer was dried ($Na_2SO_4$) and, after filtration, evaporated. The residue was purified by CC (silica gel, AcOEt) to afford **9a**/**9b** (374 mg, 80%). Colorless solid foam. $R_f$ (AcOEt) 0.31. $^1$H-NMR (mixture of diastereoisomers; $CDCl_3$): 7.43 (*d*, *J* = 7.6, NH); 7.35 (*pt*, *J* = 9.4, 8.6, 2 NH); 6.98 (*s*, NH); 6.93 (*s*, NH); 6.07 (*s*, NH); 5.99 (*s*, NH); 5.40 (*d*, *J* = 8.0, NH); 5.36 (*d*, *J* = 9.0, NH); 4.59–4.56 (*m*, 2 H–C($\alpha$) of Ala); 4.53–4.48 (*m*, 2 H–C($\alpha$) of isoGln); 3.85 (*d*, *J* = 9.1, 1 H–C($\alpha$) of 1-AdGly); 3.81 (*d*, *J* = 7.9, 1 H–C($\alpha$) of 1-AdGly); 2.41–2.28 (*m*, 2 $CH_2(\gamma)$); 2.17–1.92 (*m*, 2 $CH_2(\beta)$); 1.99 (br. *s*, 3 CH of Ad); 1.97 (br. *s*, 3 CH of Ad); 1.73–1.52 (*m*, 12 $CH_2$ of Ad); 1.44 (*s*, 2 Boc); 1.43 (*s*, $^t$Bu); 1.42 (br. *s*, $^t$Bu, Me of Ala); 1.37 (*d*, *J* = 6.9, Me of Ala). $^{13}$C-NMR ($CDCl_3$): 173.73, 173.59, 172.59, 172.53, 172.41, 172.34, 170.53, 170.49 (CONH and *C*OO$^t$Bu); 156.11, 156.09 (CO of Boc); 81.86 ($Me_3C$); 63.28, 63.09 (CH of Gly); 52.55, 52.14 (CH($\alpha$) of isoGln); 49.17, 48.98 (CH($\alpha$) of Ala); 38.77, 38.63, 36.71, 36.69, 31.84, 31.79, 27.76, 27.35 ($CH_2$ of Ad, $CH_2(\gamma)$, C of Ad, $CH_2(\beta)$); 28.38, 28.37, 28.24, 28.20, 28.04 (CH of Ad, $Me_3$C of Boc); 18.30, 18.23 (Me of Ala). ESI-MS: 565.40 ($[M+H]^+$, $C_{29}H_{49}N_4O_7^+$; calc. 565.36).

D*A*-*(Adamant-1-yl)glycyl*-L-*alanyl*-D-*isoglutamine* (**3a**/**3b**). The mixture **9a**/**9b** (324 mg; 0.57 mmol) was suspended in $CH_2Cl_2$ (4.3 ml), and TFA (4.3 ml, 5.7 mmol, 10 equiv.) was added. The mixture was stirred at r.t. for 6 h and then concentrated *in vacuo*. The residue was purified by flash chromatography (FC; silica gel, $CHCl_3$/MeOH 1:1) to give **3a**/**3b** (206 mg, 88%). Colorless solid.

Pure diastereoisomers **3a** (88 mg) and **3b** (113 mg) were separated from the mixture by CC (silica gel, $CHCl_3$/MeOH 1:1). The retention times obtained by RP-HPLC separation were 11.96 min for **3a** and 7.72 min for **3b**.

D-*(Adamant-1-yl)glycyl*-L-*alanyl*-D-*isoglutamine* (**3a**). $R_f$ ($CHCl_3$/MeOH 1:1) 0.48. $[\alpha]_D = -76.3$ ($c = 0.5$, $H_2O$). $^1$H-NMR ($CD_3OD$): 4.39 (*q*, *J* = 7.3, H–C($\alpha$) of Ala); 4.08 (*dd*, *J* = 3.4, 7.7, H–C($\alpha$) of isoGln); 3.48 (*s*, H–C($\alpha$) of 1-AdGly); 2.49–2.41 (*m*, 1 H, $CH_2(\gamma)$); 2.28–1.91 (*m*, 1 H of $CH_2(\gamma)$, $CH_2(\beta)$); 2.05 (br. *s*, 3 CH of Ad); 1.80–1.61 (*m*, 6 $CH_2$ of Ad); 1.39 (*d*, *J* = 7.4, Me of Ala). $^{13}$C-NMR ($CD_3OD$): 182.35 ($CONH_2$); 177.20, 174.74 (CONH); 169.05 (COOH); 64.13 (CH of Gly); 56.73 (CH($\alpha$) of isoGln); 50.25 (CH($\alpha$) of Ala); 39.54, 37.53 ($CH_2$ of Ad); 35.78 (C of Ad); 35.16 ($CH_2(\gamma)$); 29.62 (CH of Ad); 27.86 ($CH_2(\beta)$); 17.77 (Me of Ala). ESI-MS: 409.30 ($[M+H]^+$, $C_{20}H_{33}N_4O_5^+$; calc. 409.25). Anal. calc. for $C_{20}H_{32}N_4O_5$: C 58.81, H 7.90, N 13.72; found: C 58.47, H 8.20, N 13.69.

L-*(Adamant-1-yl)glycyl*-L-*alanyl*-D-*isoglutamine* (**3b**). $R_f$ ($CHCl_3$/MeOH, 1:1) 0.30. $[\alpha]_D = +13$ ($c = 0.5$, $H_2O$). $^1$H-NMR ($CD_3OD$): 4.24 (*dd*, *J* = 3.7, 6.3, H–C($\alpha$) of isoGln); 4.16 (*q*, *J* = 7.1, H–C($\alpha$) of Ala); 3.36 (*s*, H–C($\alpha$) of 1-AdGly); 2.32 (*t*-like, *J* = 6.3, 6.9, $CH_2(\gamma)$); 2.16–1.92 (*m*, $CH_2(\beta)$); 2.03 (br. *s*, 3 CH of Ad); 1.79–1.64 (*m*, 6 $CH_2$ of Ad); 1.45 (*d*, *J* = 7.1, Me of Ala). $^{13}$C-NMR ($CD_3OD$): 181.34 ($CONH_2$); 176.93, 173.98 (CONH); 170.22 (COOH); 63.95 (CH of Gly); 55.38 (CH($\alpha$) of isoGln); 51.76 (CH($\alpha$) of Ala); 39.41, 37.56 ($CH_2$ of Ad); 36.26 ($CH_2(\gamma)$); 34.49 (C of Ad); 29.66 (CH of Ad); 28.17 ($CH_2(\beta)$); 16.88 (Me of Ala). ESI-MS: 409.30 ($[M+H]^+$, $C_{20}H_{33}N_4O_5^+$; calc. 409.25). Anal. calc. for $C_{20}H_{32}N_4O_5$: C 58.81, H 7.90, N 13.72; found: C 59.17, H 8.15, N 13.78.

For testing the biological activities, special batches of endotoxin-free **3a** and **3b** were prepared by passing the aq. soln. of **3a** and **3b** through a *Detoxy* gel column (*Pierce*, The Netherlands), followed by lyophilization.

*Enzymatic Oxidations with* L-*Amino Acid Oxidase. Hydrolysis and Enzymatic Oxidation of* **3a**. After total acid hydrolysis of **3a** (1 mg) in 6M HCl at 100° for 16 h, HCl was evaporated, and the residue was dissolved in phosphate buffer (pH 6.5). The pH of the soln. was adjusted to 7 by the addition of 1M NaOH. L-Amino acid oxidase from *Crotalus adamanteus*, type IV (80 µl, 1.0 mg protein/ml, 5.0 units/mg protein) was added to the hydrolysate, and the mixture was incubated for 24 h at 37°. TLC with BuOH/AcOH/$H_2O$ 6:1.5:2.5 and $^i$PrOH/$NH_3$ 7:3 revealed the expected amino acid composition: D-glutaminic acid, D-(adamant-1-yl)glycine, and no L-alanine.

*Hydrolysis and Enzymatic Oxidation of* **3b**. The same procedure (hydrolysis and enzymatic oxidation with L-amino acid oxidase) was repeated for **3b** (1 mg). TLC in the same solvent systems revealed only D-Glu. No L-(Ad-1-yl)Gly and L-Ala were detected.

*Experiments* in vivo. Experiments *in vivo* were performed on NIH/OlaHsd (H-2$^q$) and CBA (H-2$^k$) inbread mice strains. All mice used were females 2 to 2.5 months old. Commercial food and water were

provided *ad libitum*. During the experiments, animals were kept at the animal facility at the Institute of Immunology and all experiments were performed according to the Croatian Law on Animal Welfare (The Official Gazette 'NN' 135/06).

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

The dose of OVA (antigen) was 10 μg per mouse. The dose of PGM (**1**) and adamantyl tripeptides was 200 μg per mouse. OVA and tested substances were dissolved in saline, and the injection volume in all experimental groups was 0.1 ml per mouse.

*Enzyme Immunoassays for Qual. and Quant. Determination of OVA-Specific IgG* (anti-OVA IgG) *in Mice Sera.* Enzyme immunoassays (ELISA) were performed on flat-bottomed high-binding microtiter plates (*Costar*, USA) according to the procedures described in [32][48]. 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 ml (AU/ml) were voluntarily assigned [49].

*Enzyme Immunoassays for Qual. and Quant. Determination of OVA-Specific IgG Subtype Anti-OVA IgG1 and Anti-OVA IgG2a in Mice Sera.* ELISA for determination of anti-OVA IgG1 and anti-OVA IgG2a were performed as described in [24]. The relative quantities of antibody subtypes were determined by parallel line assay using appropriate standard preparation. The monoclonal anti-OVA IgG1 was a standard for relative quantification of anti-OVA IgG1 to which 400,000 AU/ml was assigned, while polyclonal mouse serum containing high levels of anti-OVA IgG2a was used as a standard for relative quantification of IgG2a-specific antibodies with 20,000 AU/ml. The ratio IgG1/IgG2a was used as indication of the Th1/Th2 bias of induced immune response.

*Statistics.* Statistical analyses were performed with Statistica 6.0 for *Windows*, *StatSoft Inc*. The significant difference between exper. groups was evaluated by *Kruskal–Wallis* ANOVA, followed by multiple *Mann–Whitney* U-nonparametric tests. A probability values less than 0.05 ($p < 0.05$) were considered significant.

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