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# Synthesis of Novel Adamantylacetyl Derivative of Peptidoglycan Monomer—Biological Evaluation of Immunomodulatory Peptidoglycan Monomer and Respective Derivatives with Lipophilic Substituents on Amino Group

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**Abstract**—Novel synthetic analogue of immunomodulatory peptidoglycan monomer **1** (PGM), (adamant-1-yl)−$CH_2$CO-PGM (**2**), was prepared by acylation of ε-amino group of diaminopimelic acid with symmetrical (adamant-1-yl)-acetic acid anhydride in the presence of triethylamine. The product was isolated by gel filtration on Sephadex G-25, followed by ion exchange chromatography on SP-Sephadex C-25. The susceptibility of (adamant-1-yl)−$CH_2$CO-PGM to hydrolysis with *N*-acetylmuramyl-L-alanine amidase was demonstrated, and the product of hydrolysis, (adamant-1-yl)−$CH_2$CO-pentapeptide **3**, was characterized. Both **2** and **3** are water soluble and non-pyrogenic compounds. Immunomodulatory activity of PGM (adamant-1-yl)−$CH_2$CO-PGM and structurally related derivative Boc-Tyr-PGM was compared in experiments in vivo, in mice, using ovalbumin (OVA) as an antigen. All three tested compounds exhibited comparable immunostimulating effects with respect to the induction of anti-ovalbumin immunoglobulin G. The results of evaluation of biological activity show that the substitution of free amino group in the parent peptidoglycan molecule with bulky lipophilic substituents did not affect the susceptibility to hydrolysis with *N*-acetylmuramyl-L-alanine amidase and did not alter markedly the immunostimulating activity. The results also indicate that the free amino group in the peptide chain is not a necessary requirement in the mechanism of immunostimulation of tested immunomodulators. 

## Introduction

Peptidoglycan is the structural element of the bacterial cell wall that determines cell shape and is responsible for physical integrity of bacteria. Peptidoglycans also possess diverse biological activities that depend upon the size and composition of the peptidoglycan fragments.[1–3] PGM,[4] the disaccharide pentapeptide used in this work, is the basic repeating unit of the *Brevibacterium divaricatum* cell wall peptidoglycan of the following structure: β-D-Glc*p*NAc-(1→4)-D-Mur*p*NAc-L-Ala-D-*iso*Gln-*meso*$A_2$pm(ε$NH_2$)-D-Ala-D-Ala (Fig. 1).[5,6] It exhibits strong immunomodulating, antitumour and antimetastatic activities.[7] Our interest has been focused on the preparation of novel peptidoglycan monomer derivatives that might possess different biological characteristics. It might be expected that even small structural changes can alter the biological activity of the compound. In our previous studies[8,9] we reported on the introduction of more lipophilic substituents on the free amino group of diaminopimelic acid in the parent PGM molecule. The synthetic analogue of peptidoglycan monomer, Boc-Tyr-PGM, was synthesized[8,9] by condensation of PGM and active esters of Boc-Tyr-OH. Boc-Tyr-PGM is a water-soluble, non-pyrogenic molecule. In several experimental models, Boc-Tyr-PGM exhibited immunostimulating and antimetastatic activities,[10] very similar to the activity of PGM, although the free amino group in a new derivative was blocked. Recently, we also reported on the syntheses of the novel conjugates[11] comprising Boc-Tyr-PGM coupled to glucuronoxylomannan from *Cryptococcus neoformans* type B and dextran, respectively.

In a continuation of our work, in the present paper we describe the preparation of the new derivative of

Abbreviations: PGM, peptidoglycan monomer; Ada, adamantyl; Glc*p*NAc, *N*-acetyl-D-glucosamine; Mur*p*NAc, acetyl-D-muramic acid; Ala, alanine; *iso*Gln, *iso*-glutamine; *meso*$A_2$pm, *meso*-diaminopimelic acid; Tyr, tyrosine; Glu, glutamic acid; Boc, *tert*-butyloxycarbonyl; OVA, ovalbumin; anti-OVA, anti-ovalbumin antibody; IgG, immunoglobuline G.

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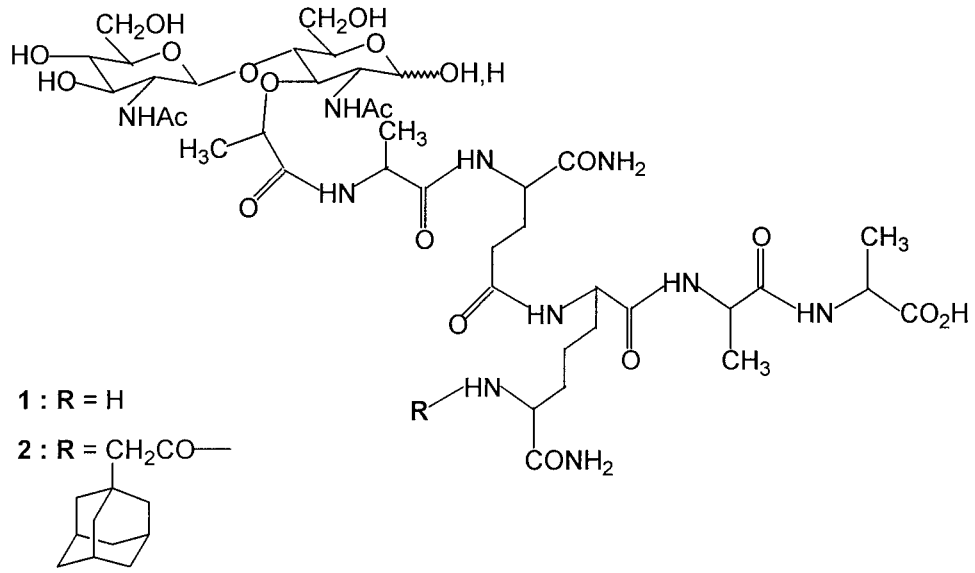


**Figure 1.** Structures of the peptidoglycan monomer (**1**, PGM) and (adamant-1-yl)-acetyl-peptidoglycan monomer [**2**, (Ada-1-yl)−$CH_2CO$-PGM].

peptidoglycan monomer **2**, which contains adamantylacetyl residue also coupled to PGM via amino group in diaminopimelic acid. It has been shown[12,13] that the conjugates of muramyl dipeptide analogues comprising adamantyl residue exhibit various biological activities, including immunomodulating and antiviral activity. However, the adamantyl residue has not been attached to the whole disaccharide pentapeptide molecule so far. Therefore, we examined the structure–activity relationship of the parent molecule, PGM, and of two derivatives having large lipophilic substituents, namely of Boc-Tyr-PGM prepared earlier and of a new derivative, described in this paper, (adamant-1-yl)−$CH_2CO$-PGM. Two aspects were studied:

(a) Immunomodulating activity was tested in vivo, in an experimental model in mice, using ovalbumin as an antigen. As described in our previous paper,[14] PGM exhibited strong immunostimulating effect in this model, and therefore, in the present work, comparison of activity of Boc-Tyr-PGM, (adamant-1-yl)−$CH_2CO$-PGM and the parent PGM as a reference was carried out in parallel.

(b) Both PGM and Boc-Tyr-PGM are good substrates for *N*-acetylmuramyl-L-alanine amidase,[10,15] which cleaves the peptidoglycans giving the disaccharide GlcNAc-MurNAc and the respective peptides. The structure of the resulting disaccharide was confirmed[5] by mass spectrometry and acid hydrolysis. Also, it was synthesized[16] and fully characterized by $^1H$ and $^{13}C$ NMR. The corresponding peptides obtained by enzymatic hydrolyses were characterized[10,15,17] as well. In the present study, the susceptibility of (adamant-1-yl)−$CH_2CO$-PGM to the hydrolysis with this enzyme was investigated.

## Results

### Chemistry

**Synthesis of symmetrical anhydride of 1-adamantylacetic acid.** Activation of carboxyl group of (adamant-1-yl)-acetic acid was achieved by condensation of 2 mmol of the acid in the presence of dicyclohexylcarbodiimide. The urea derivative was removed by filtration and, upon evaporation of filtrate, a solid was obtained. Several attempts of purification by crystallization failed. The structure of (adamant-1-yl)-acetic acid anhydride was supported by IR spectrum, which revealed characteristic carbonyl band of anhydride at 1810 $cm^{-1}$. $^1H$ NMR spectrum in $CDCl_3$ revealed the expected signals. The crude anhydride was used in the next step without further purification.

**Preparation of (adamant-1-yl)-$CH_2CO$-PGM (2).** (A) Synthetic derivative of peptidoglycan monomer (adamant-1-yl)−$CH_2CO$-PGM (**2**) was prepared by condensation of unprotected peptidoglycan monomer **1** with an excess of symmetrical anhydride of (adamant-1-yl)-acetic acid in the presence of triethylamine in dimethylformamide. The reaction was monitored by TLC in solvent system B (7/3); no side products were detected. Unreacted anhydride was separated from the reaction product by extraction with organic solvent and the obtained product **2** was isolated by column chromatography on Sephadex G-25 in water (Fig. 2). TLC analysis revealed that the product still contained small amounts of unreacted PGM, which could be completely removed by ion exchange chromatography on SP-Sephadex C-25 in water (Fig. 3). The yield was 75.8%. The structure of (adamant-1-yl)−$CH_2CO$-PGM (**2**) was confirmed by $^1H$ and $^{13}C$ NMR spectroscopy, mass spectrometry, total acid hydrolysis and treatment of **2** with 2,4-dinitrofluorobenzene (DNP-F). Full assignment of $^1H$-signals was not possible, because of severe overlaps. Partial assignments have been deduced by comparison with $^1H$ NMR spectra of PGM.[6,18] $^{13}C$ NMR spectrum of **2** contained all expected carbon signals and corroborated the structure proposed. Mass spectrum of **2** revealed the molecular ion $[M + H]^+$ at $m/z$ 1186, indicating only one adamantylacetyl group in the molecule. Total acid hydrolysis of (adamant-1-yl)−$CH_2CO$-PGM with 6 M hydrochloric acid at 100 °C, for 16 h, followed by thin-layer

chromatography (TLC) in solvent system B (7/3), revealed the expected amino acid composition (Ala, Glu and $A_2pm$). Also, hydrolysis of **2** with 2 M hydrochloric acid at 100 °C for 2 h and TLC in solvent system B (7/4) revealed $GlcNH_2$ and $MurNH_2$. In order to undoubtedly prove the site of binding the adamantylacetyl residue, product **2** was treated with 2,4-dinitrofluorobenzene, followed by total acid hydrolysis. The hydrolysate contained the same amino acid composition as mentioned above. The absence of DNP-derivative of the diaminopimelic acid corroborated the assumption that in **2**, adamantylacetyl residue is bound on the peptide moiety of PGM. Also, this observation was supported by amidase hydrolysis of **2**, after which the (Ada-1-yl)–$CH_2CO$-pentapeptide **3** was obtained (see later).

Additional data on the structure of **2** and also of the related (adamant-1-yl)–$CH_2CO$-pentapeptide (**3**, see later) and their conformational properties in DMSO, using 2-D NMR spectroscopy and molecular modelling, were published separately.[18]

(Adamant-1-yl)–$CH_2$-CO-PGM is water-soluble despite increased lipophilicity. It is non-pyrogenic, as tested in rabbits.

(B) In order to obtain the better yield, the $^{14}C$-labelled **2** was prepared by condensation of [$^{14}C$]peptidoglycan monomer and a higher excess of the (adamant-1-yl)-acetic acid anhydride. TLC in solvent system B (7/3) revealed only one new spot ($R_f$ 0.33) assigned to **2**. Despite a higher excess of the acylating reagent the same reaction product was isolated by column chromatography on a Bio Gel P-2 superfine in water (Fig. 4) and on an SP Sephadex C-25, respectively. The product was indistinguishable from that obtained in (A). A slightly different procedure for the isolation of radioactive **2** was applied, avoiding extraction with organic solvent. This procedure also gave chromographically pure **2** as a hygroscopic amorphous solid, in a very good yield (78%), as in (A), and consisted of very simple column chromatographies.

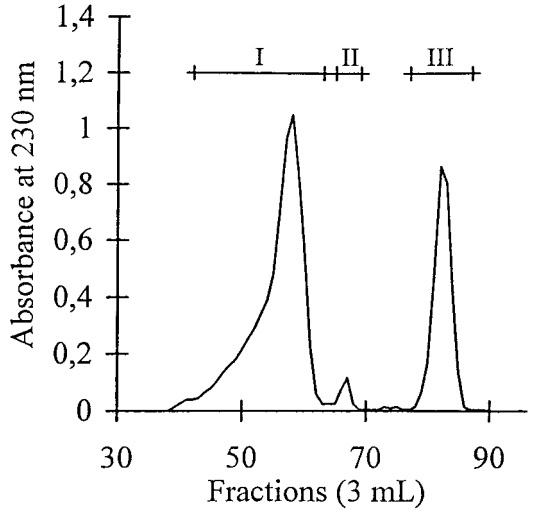


**Figure 2.** Separation of (Ada-1-yl)–$CH_2CO$-PGM (**2**) on a Sephadex G-25 (fine) column (90×2.5 cm) in water. Fractions containing **2** were collected as indicated by the solid bar (I).

## Biological assays

**Testing of immunostimulating activity.** Based on our previous experience with PGM as an adjuvant,[14] two derivatives, namely Boc-Tyr-PGM and (adamant-1-yl)-$CH_2CO$-PGM, were tested in the same manner, in parallel with PGM. Mice were immunized subcutaneously with suboptimal dose of ovalbumin (50 μg/mouse). Tested compounds (200 μg/mouse) were administered to experimental groups mixed with the antigen, in first immunization, as well as in the boosters. Two separate experiments were carried out. In the first experiment, mice received only one booster dose. (Adamant-1-yl)–$CH_2CO$-PGM exhibited adjuvant effect comparable to PGM (Fig. 5), inducing significantly higher anti-OVA

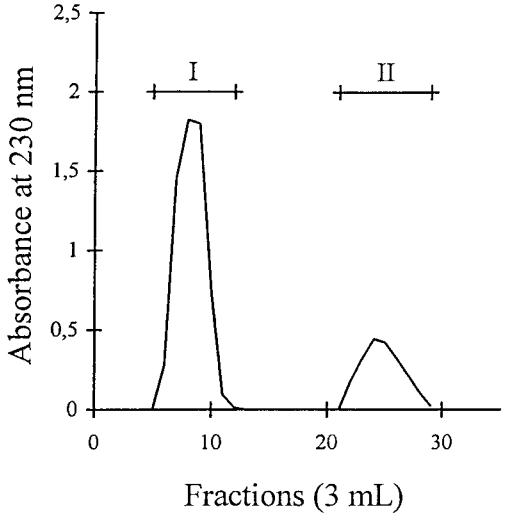


**Figure 3.** Purification of (Ada-1-yl)–$CH_2CO$-PGM (**2**) on an SP-Sephadex C-25 column (30×2.5 cm) in water. Peak I contains **2** and peak II unreacted PGM. Fractions containing **2** were collected as indicated by the solid bar.

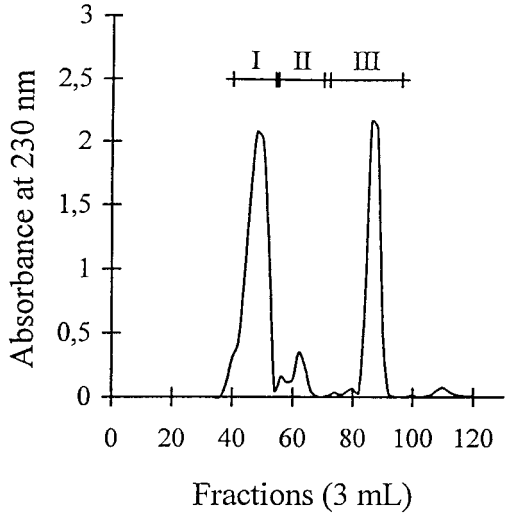


**Figure 4.** Isolation of $^{14}C$-labelled (Ada-1-yl)–$CH_2CO$-PGM (**2**) on a Bio Gel P-2 (superfine) column (90×2.5 cm) in water. Peak I contains the product **2** and peak II unreacted PGM. Fractions containing **2** were collected as indicated by the solid bar.

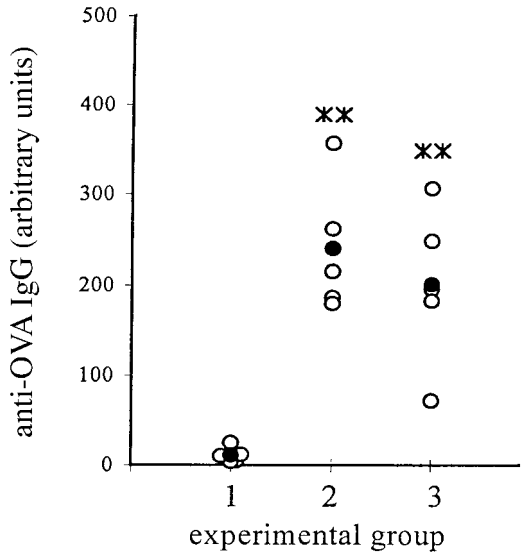


**Figure 5.** Effect of PGM and (Ada-1-yl)–$CH_2CO$-PGM, respectively, on anti-OVA IgG values in C57BL/6 mice after the first booster, expressed in arbitary units. Tested compounds were administered mixed with OVA. (1) Control group, receiving 50 μg of OVA; (2) group treated with 200 μg of PGM; (3) group treated with 200 μg of (Ada-1-yl)–$CH_2CO$-PGM. ● denotes group mean value; $^{**}P < 0.01$.

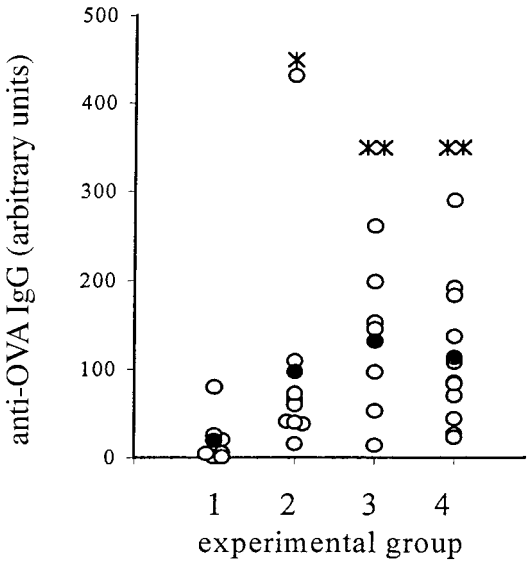


**Figure 6.** Effect of PGM, Boc-Tyr-PGM and (Ada-1-yl)–$CH_2CO$-PGM, respectively, on anti-OVA IgG values in C57BL/6 mice after the second booster, expressed in arbitary units. Tested compounds were administered mixed with OVA. (1) Control group, receiving 50 μg of OVA; (2) group treated with 200 μg of PGM; (3) group treated with 200 μg of Boc-Tyr-PGM; (4) group treated with 200 μg of (Ada-1-yl)–$CH_2$-CO-PGM. ● denotes group mean value; $^{*}P < 0.05$, $^{**}P < 0.01$.

IgG values than the antigen alone. In the second experiment, additional group was treated with Boc-Tyr-PGM and all groups received two booster injections. All three compounds, PGM, Boc-Tyr-PGM and (adamant-1-yl)–$CH_2CO$-PGM, stimulated humoral immune response to a similar extent (Fig. 6). Since anti-OVA was determined after the second booster, the difference between control group and treated groups was not so marked as in the first experiment, and the individual values were more scattered, but mean values for treated groups were still significantly higher than for the control. These findings are in accordance with our previous results on the effects of PGM.[14]

**Hydrolysis with *N*-acetylmuramyl-L-alanine amidase.** The susceptibility of (adamant-1-yl)–$CH_2CO$-PGM to hydrolysis with *N*-acetylmuramyl-L-alanine amidase was investigated in a manner described previously.[15] The treatment with partially purified amidase, isolated from human serum according to known procedure[15] in Tris–HCl buffer, pH 7.9, resulted in the regioselective hydrolysis of the lactylamide bond between the disaccharide and peptide moiety (Fig. 7). The reaction was monitored by TLC in solvent system B (7/3). The products of the reaction, the disaccharide GlcNAc-MurNAc ($R_f$ 0.55) and the (adamant-1-yl)-$CH_2CO$-pentapeptide **3** ($R_f$ 0.49), were isolated by column chromatography on Sephadex LH-20 column in 50% ethanol (Fig. 8). Eluted first was the disaccharide, followed by (adamant-1-yl)–$CH_2CO$-pentapeptide (**3**). The obtained disaccharide co-migrated with the authentic sample[5] of GlcNAc-MurNAc formed by hydrolysis of PGM with the *N*-acetylmuramyl-L-alanine amidase. The characterization of the authentic disaccharide GlcNAc-MurNAc was performed earlier[5] by HPLC separation of the α- and β-anomers ($t_R$ 5.2 and 7.2 min, respectively), followed by mass spectrometry. The molecular ion $[M + H]^+$ at *m*/*z* 497 and acid hydrolysis confirmed the structure proposed. Also, it was synthesized[16] and fully characterized by $^1H$ NMR and $^{13}C$ NMR.

Acid hydrolysis of disaccharide obtained by enzymatic hydrolysis of (adamant-1-yl)–$CH_2CO$-PGM with 2 M hydrochloric acid at 105 °C, for 2 h, gave expected monosaccharides (glucosamine and muramic acid).

(Adamant-1-yl)-$CH_2CO$-pentapeptide (**3**) obtained by enzymatic hydrolysis was identified and its structure was confirmed by amino acid analysis and spectroscopic methods. Mass spectrum of **3** showed the presence of molecular ion $[M + H]^+$ at *m*/z 708.5. $^1H$ and $^{13}C$ NMR spectra revealed the expected signals. Total acid hydrolysis of **3** with 6 M hydrochloric acid at 105 °C, for 24 h, gave expected amino acid composition (Ala, Glu, and $A_2pm$).

To facilitate the study of the susceptibility of **2** to *N*-acetylmuramyl-L-alanine amidase, the hydrolysis was also carried out with $^{14}C$-labelled **2** as a substrate. The reaction was followed by TLC in the more suitable solvent system D. Essentially the same results were obtained in both cases. Radioactive products, the disaccharide ($R_f$ 0.19) and (adamant-1-yl)–$CH_2CO$-pentapeptide **3** ($R_f$ 0.36) were isolated (Fig. 9) and characterized.

## Discussion

Peptidoglycan molecules, depending on their composition and size, exhibit various biological activities, but most prominent and most often investigated seems to be

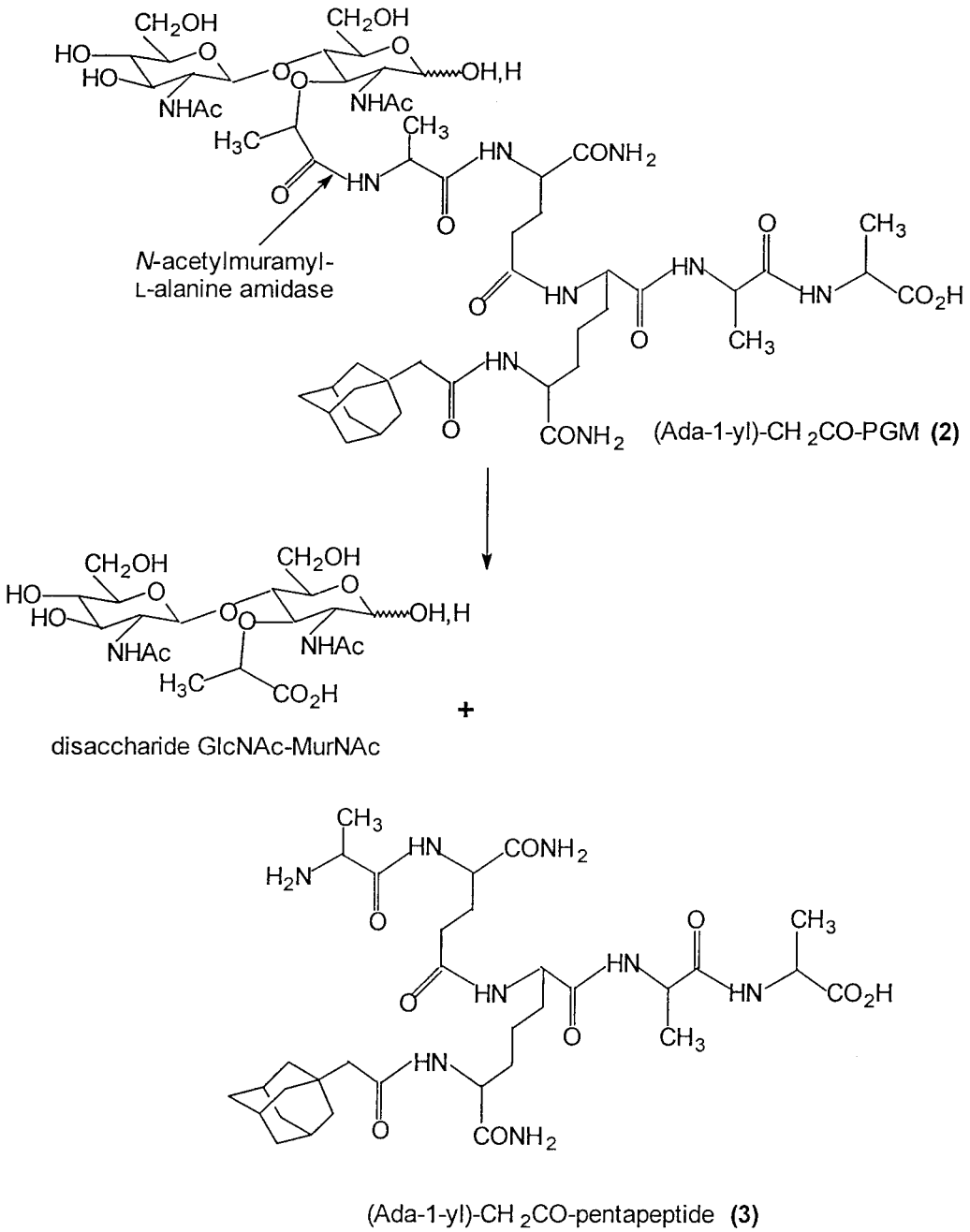


**Figure 7.** Products of the enzymatic hydrolysis of (Ada-1-yl)–$CH_2CO$-PGM (**2**) with *N*-acetylmuramyl-L-alanine amidase.

immunomodulating activity.[1–3,19] Structure–activity relationship studies on this topic have been numerous and well documented. Such studies address in the first step the synthetic modifications either in the saccharide portion of the molecule, or in the peptide chain coupled to the muramic acid. Synthetic modifications described so far were mostly carried out on muramyl dipeptides or tripeptides, and less often on more complex disaccharide peptides with longer peptide chains. It could be expected that the complex molecules comprising both the carbohydrate and peptide parts require protection of functional groups prior to coupling of new entities, and after the completed coupling, the appropriate deprotection is necessary.[20–23] Several muramyl peptide derivatives have already been included in clinical trials but their syntheses have been patented. Some of the prominent derivatives currently investigated[24] are muroctasin ($N^2$-muramyldipeptide-$N^6$-stearoyl-L-lysine) [MDP-Lys(L18)], muramyltripeptide-phosphatidylethanolamine (MTP-PE), 6-*O*-(2-tetradecylhexadecanoyl)-muramyldipeptide (B-30-MDP) and threonyl-muramyldipeptide (Thr-MDP) and their respective formulations in liposomes. Lipophilic substituents on muramyl peptides made such compounds much better constituents for incorporation into liposomes, in comparison to unsubstituted parent molecules. One of the aims of our work was also to prepare derivatives that could be more effectively incorporated into liposomes, since in our previous study[25] we showed that only up to 15% of PGM could be encapsulated in liposomes.

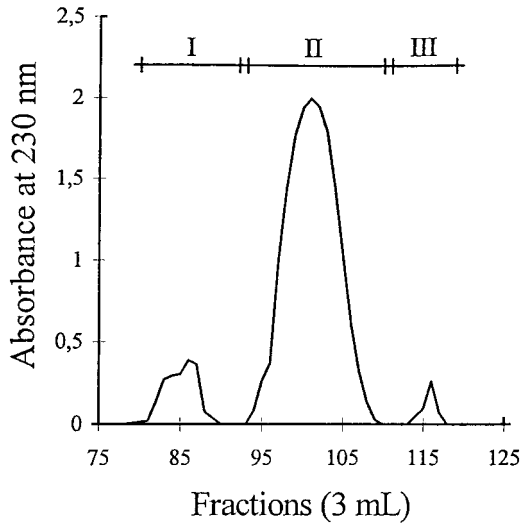


**Figure 8.** Separation of the products of the enzymatic hydrolysis of (Ada-1-yl)−$CH_2CO$-PGM on a Sephadex LH-20 (90×1.5 cm) column in 50% ethanol. Peak I contains the disaccharide and peak II (Ada-1-yl)−$CH_2CO$-pentapeptide (**3**). Corresponding fractions (denoted by solid bars) were collected and characterized.

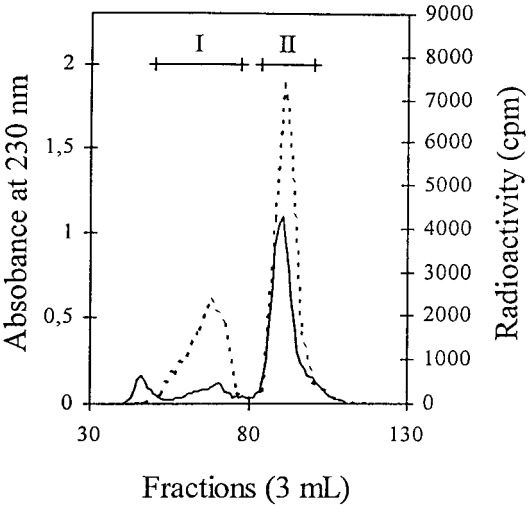


**Figure 9.** Separation of the products of the enzymatic hydrolysis of (Ada-1-yl)−$CH_2CO$-[$^{14}$C]PGM (**2**) on a Sephadex LH-20 column (90×1.5 cm) in 50% ethanol; (—) absorbance at 230 nm, (- - - -) radioactivity expressed in cpm. Peak I contains the disaccharide and peak II (Ada-1-yl)−$CH_2CO$-[$^{14}$C]pentapeptide (**3**). Corresponding fractions (denoted by solid bars) were collected and characterized.

It should be pointed out that synthesis of peptidoglycan derivative **2** presented in this paper was conducted with unprotected PGM molecule. PGM has several reactive groups, hydroxyl groups in the disaccharide, and the free carboxyl and amino groups in the pentapeptide moiety. Each of these functional groups could participate in chemical reactions. However, it has been shown[6] that in the preferential conformation of PGM in aqueous and dimethylsulfoxide solution, the ε-amino group of *meso*-diaminopimelic acid ($A_2$pm) is exposed and it is readily available for some reactions without the interference of other groups. In addition, *N*-hydroxysuccinimide esters and anhydrides probably react much faster with free amino group than with hydroxyl groups in carbohydrate moiety. This is in accordance with the greater nucleophilicity of amino group.[26] A report[27] revealed that the reaction of unprotected disaccharide-tripeptide and disaccharide-tetrapeptide (bacterial peptidoglycan analogues) with *N*-hydroxysuccinimide ester of appropriate carboxylic acid resulted in regioselective structural modification of the peptide moiety as well.

In the present work, as well as in previous reports,[8,9] we have shown that the substitutions on this free amino group could be performed in a very simple, one step reaction. The bulky, lipophilic substituent could be either in the form of *N*-hydroxysuccinimide, as shown in the synthesis of Boc-Tyr-PGM,[8] or in the form of acid anhydride, as in the synthesis of (adamant-1-yl)−$CH_2CO$-PGM described in this paper.

The isolation of substituted peptidoglycan monomers and satisfactory purification could be achieved by simple column chromatography, preferably the combination of gel filtration and ion exchange chromatography.

Peptidoglycan monomer exhibits immunostimulating activity (adjuvant effect) in animal models in vivo.[7,10,14] In a representative model in mice, using ovalbumin (OVA) as an antigen, humoral immune response was markedly stimulated[14] by PGM, so we used this model for assessment of immunostimulating properties of two lipophilic derivatives, Boc-Tyr-PGM and (adamant-1-yl)−$CH_2CO$-PGM, and for comparison with the parent PGM. All three tested compounds exhibited comparable immunostimulating effect, i.e. induced significant increase in specific anti-OVA IgG. These results indicate that the bulky substituents (Boc-Tyr and adamantyl) do not alter the adjuvant effect of PGM.

Low molecular weight peptidoglycans are good substrates for the enzyme *N*-acetylmuramyl-L-alanine amidase from human sera.[28,29] Peptidoglycan monomer and its synthetic analogue Boc-Tyr-PGM were hydrolysed by the amidase yielding the disaccharide GlcNAc-MurNAc and the respective peptapeptide L-Ala-D-*iso*Gln-*meso*-$A_2$pm(ε$NH_2$)-D-Ala-D-Ala and hexapeptide L-Ala-D-*iso*Gln-*meso*-diaminopimelyl-$N^{\varepsilon}$-(Boc-Tyr)-D-Ala-D-Ala, respectively.[10,15] In several experimental models, the pentapeptide exhibited biological activity comparable with the original PGM molecule.[7] In this work we have shown that the introduction of bulky, lipophilic adamantylacetyl moiety into the peptide portion of PGM did not affect the susceptibility of lactylamide bond to hydrolysis with *N*-acetylmuramyl-L-alanine amidase.

## Conclusion

In summary, a novel derivative of peptidoglycan monomer was prepared in a simple reaction of unprotected starting compound and adamantylacetyl anhydride. The bulky, lipophilic substituent on amino group in the pentapeptide chain did not affect immunostimulating activity, which remained similar to the parent PGM and another derivative, Boc-Tyr-PGM. Such results indicate that the free amino group at the diami-

nopimelic acid residue in the peptide chain of PGM is not an essential requirement for immunostimulating effect. Also, the novel derivative remained a good substrate for *N*-acetylmuramyl-L-alanine amidase, i.e. the substituents on amino group did not interfere with susceptibility to the enzyme.

## Experimental

### Materials and methods

Peptidoglycan monomer was prepared in PLIVA, Chemical and Pharmaceutical Works (Zagreb, Croatia), according to the previously described procedure.[4]

$^{14}$C-labelled peptidoglycan monomer, with the label incorporated into the acetyl group of the sugar moiety (50%), and the glutaminyl residue of the peptide chain (50%) were obtained as described earlier[4] using [$^{14}$C]acetate as precursor. For current experiments peptidoglycan monomer (PGM) of 50,000 Bq/mg was used. Boc-Tyr-PGM was prepared as described earlier.[8,9]

Sephadex G-25, Sephadex LH-20 and SP-Sephadex C-25 were obtained from Pharmacia (Uppsala, Sweden). Bio-Gel P-2 was supplied from Bio-Rad Laboratories (Richmond, USA). *tris*-(Hydroxymethyl)-aminomethane, *o*-phenylendiamine (OPD), 2,4-dinitrofluorobenzene (DNP-F), and Dowex-50W×4 ($H^+$-form), Tween 20, anti-chicken egg albumin (clone OVA-14) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), (adamant-1-yl)-acetic acid, and *N,N*′-dicyclohexylcarbodiimide from Fluka Chemie AG (Switzerland), triethylamine from Merck (Darmstadt, Germany), and Aquasol as a scintillation cocktail from NEN (England).

TLC was performed on Kieselgel 60 $HF_{254}$ (Merck, Darmstadt, Germany) with solvent system: (A) benzene/ethyl acetate (3/1); (B) *n*-propanol/concd ammonia (proportions are given in the text); (C) *n*-butanol/ethanol/concd ammonia/water (5/3/2/2); (D) *n*-butanol/glacial acetic acid/water (60/15/25). The detection was effected by charring with ninhydrin, thymol, iodine or chlorine–starch–iodine reagent for peptides.

Radioactivity was measured by using a Beckman LS-100 liquid scintillation counter. The course of the reaction with $^{14}$C-labelled PGM was monitored by TLC. Portions of the plate were marked according to the position of reference PGM and (adamant-1-yl)-PGM, the adsorbent was scraped off, suspended in a mixture of 2 mL of 50% ethanol and 2 mL of scintillation cocktail, and the radioactivity was measured and expressed as a percentage of the total radioactivity on the plate. The radioactivity in fractions following column chromatography was determined in 0.5 mL fraction aliquots, mixed with 5 mL Aquasol.

Absorbance was measured on a Perkin–Elmer Lambda 3 UV/vis spectrometer. $^1$H and $^{13}$C NMR spectra were recorded with a Varian Gemini 300 spectrometer. IR spectra were recorded with a Perkin–Elmer Model 137 spectrometer. Mass spectra were recorded with a Sciex Perkin–Elmer API1.

**Synthesis of symmetrical anhydride of (adamant-1-yl)-acetic acid.** To a cold solution of (adamant-1-yl)-acetic acid (970 mg, 5 mmol) in dry dichloromethane (25 mL), dicyclohexylcarbodiimide (530 mg, 2.6 mmol) was added. The reaction mixture was kept at ambient temperature for 16 h (monitoring by TLC in solvent system A). Dicyclohexylurea was filtered off, the filtrate was evaporated and the residue was dissolved in a small quantity of dichloromethane. The second crop of dicyclohexylurea was filtered off and the filtrate was evaporated under reduced pressure. The crude anhydride of adamantylacetic acid (0.93 g, 100% yield) was used in the next step. IR (KBr) ν 2900, 2850, 1810, 1795, 1710, 1450, 1058, 1033 cm$^{-1}$. $^1$H NMR ($CDCl_3$, TMS) δ 2.17 (s, 2H), 1.99 (s, 3H), 1.67 (s, 12H).

**Preparation of (adamant-1-yl)–$CH_2$CO-PGM (2).** To a solution of peptidoglycan monomer (**1**, PGM, 100 mg, 0.1 mmol) in dimethylformamide (DMF, 3 mL), triethylamine (30 μL) was added, followed by an excess of 1-adamantylacetic acid anhydride (40 mg, 0.108 mmol). The reaction was carried out at ambient temperature. The anhydride (20 mg) was added at 3, 5 and 16 h intervals and reaction was monitored by TLC in solvent system B (7/3). Other products except **2** were not detected. After 24 h, the solvent was evaporated in high vacuum, the residue dissolved in water (10 mL) and acidified with hydrochloric acid to pH 3. After extraction with dichloromethane (3×10 mL) the aqueous layer was concentrated and applied to a Sephadex G-25 column (2.5×90 cm) and eluted with water. Fraction (3 mL) absorbances were measured at 230 nm and those corresponding to (adamant-1-yl)−$CH_2$CO-PGM (**2**) were pooled and evaporated. Final purification of the product was achieved by chromatography on an SP-Sephadex C-25 column eluted with water and lyophilized, giving the chromatographically pure (adamant-1-yl)−$CH_2$CO-PGM as a fluffy mass (89.8 mg, 75.8%). IR (KBr) ν 3420–3250, 2900, 1800, 1685, 1530, 1320, 1200 cm$^{-1}$. $^1$H NMR ($D_2O$, TMS) δ 5.12 (d, $J_{1,2}$ = 3.1 Hz, < 1H), 5.20–3.31 (m, 21H), 2.33–2.26 (m, 2H), 2.06–1.40 (m, 33H), 1.31 (d, $J$ = 7.06 Hz, 3H), 1.33 (d, $J$ = 7.26 Hz, 3H), 1.25 (d, $J$ = 7.26 Hz, 3H) and 1.21 (d, $J$ = 7.26 Hz, 3H). $^{13}$C NMR ($D_2O$, dioxane) δ 179.33 ($CO_2$H), 177.81, 176.68, 176.51, 176.45, 175.81, 175.71, 175.46, 174.92, 174.67, 174.59 (lactyl *C*O, 5 *C*ONH, 2 $CH_3$*C*O, 2 *C*O$NH_2$), 101.31 (C′-1β), 91.08 (C-1α), 78.33, 77.05, 76.96, 76.31, 74.40, 71.84, 71.06 (*C*-3,3′,4,4′,5,5′; lactyl *C*H), 61.93, 60.54 (*C*-6,6′), 56.82, 54.85, 54.47, 54.26, 53.72, 53.61, 51.00, 50.81, 50.23 (*C*-2,2′; *C*H-α, 3 Ala, *iso*Gln, *meso*$A_2$pm; *C*H-ε, *meso*$A_2$pm, $CH_2$CO), 43.07 (3C, Ada), 37.01 (3C, Ada), 33.46, 32.39, 31.34 ($CH_2$-γ, *iso*Gln; $CH_2$-β,δ, *meso*$A_2$pm; *C* Ada), 29.23 (3C, Ada), 28.02 ($CH_2$-β, *iso*Gln) 22.93 (2 $CH_3$CO; $CH_2$-γ, *meso*$A_2$pm), 18.98 (lactyl $CH_3$), 17.76, 17.62, 17.47 ($CH_3$, 3 Ala). MS: $[M + H]^+$ at *m*/*z* 1186. Total acid hydrolysis revealed the expected composition (Ala, Glu, $A_2$pm and Ada-$CH_2$COOH).

To a solution of **2** (1 mg) in 0.1 M sodium hydrogen carbonate (1 mL), 1% 2,4-dinitrofluorobenzene (0.2 mL) was added. The mixture was left at ambient temperature

overnight in the dark. The mixture was acidified with 6 M hydrochloric acid (0.2 mL) and extracted three times with ether (3×3 mL). TLC in solvent system B (7/3) did not show a new product. Aqueous layer was evaporated, dissolved in 6 M hydrochloric acid (1 mL) and hydrolysed in vacuo at 105 °C for 16 h. The hydrolysate was evaporated and subjected to a Dowex 50W×4 ($H^+$-form) column (2 mL), rinsed with water and eluted with 2 M ammonia (25 mL). The composition of eluate (Ala, Glu and $A_2$pm) was determined by TLC in solvent system B (7/3).

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

**Preparation of (adamant-1-yl)–$CH_2CO$-[$^{14}$C]PGM.** $^{14}$C-Labelled **2** was prepared by a procedure slightly different from that described above. To a solution of [$^{14}$C]PGM (1.6 mg, 89900 Bq) and unlabelled PGM (101 mg) in dimethylformamide (3 mL), triethylamine (30 μL) was added. Five min later anhydride of adamantylacetic acid (227 mg) in DMF (2 mL) was added and reaction was carried out at room temperature for 22 h (TLC in solvent system B, 7/3). The reaction mixture was evaporated in high vacuum, the residue dissolved in water (2 mL) and filtered through a piece of cotton. Filtrate was concentrated under reduced pressure, acidified with hydrochloric acid to pH 3, and subjected to column chromatography on a Bio-Gel P-2 (90×2.5 cm) in water at 4 °C. Fraction (3 mL) absorbances were checked at 230 nm, radioactivity determined in fraction aliquots and those fractions corresponding to (adamant-1-yl)–$CH_2CO$-[$^{14}$C]PGM (92 mg, 78%, spec. activity 661 Bq/mg) were pooled and evaporated. Radioactive purity of $^{14}$C-labelled **2** was 93%. Final purification of **2** was carried out by chromatography on an SP-Sephadex C-25 column (2.5×50 cm) in water. Chromatographically pure (adamant-1-yl)–$CH_2CO$-[$^{14}$C]PGM was obtained.

**Hydrolysis of (adamant-1-yl)-$CH_2CO$-PGM with *N*-acetylmuramyl-L-alanine amidase.** A solution of (adamant-1-yl)−$CH_2CO$-PGM (**2**, 85 mg) 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[15] (900 μL, 0.816 i.u./mL) at 37 °C, for 48 h. The pH was maintained at 7.9 by the addition of 0.1 M NaOH and the reaction was monitored by TLC in solvent system B (7/3). Incubation mixture was evaporated under reduced pressure, the residue was dissolved in 50% ethanol, and solution chromatographed on a Sephadex LH-20 column (90×2.5 cm) in the same solvent. Absorbance of fractions (3 mL) was checked at 230 nm and those containing (adamant-1-yl)−$CH_2CO$-pentapeptide (**3**) were pooled, and concentrated (~2 mL) under reduced pressure. Final purification was carried out by chromatography on a Sephadex LH-20 column in sterile water. Fractions containing **3** were combined, concentrated, passed through 0.45 μm membrane and lyophilized, to give the pure **3** (38 mg, 75%). Total acid hydrolysis of product **3** in 6 M hydrochloric acid at 100 °C, for 16 h, followed by TLC in solvent system B (7/3), revealed the expected amino acid composition (Ala, Glu and diaminopimelic acid). $^1$H NMR ($D_2O$, TMS) δ 4.26–3.94 (m, 6H), 2.31–2.27 (near t, 2H), 2.07–1.48 (m, 25H), 1.41 (d, $J$ = 7.06 Hz, 3 H), 1.25 (d, $J$ = 7.26 Hz, 3H) and 1.21 (d, $J$ = 7.26 Hz, 3 H)). $^{13}$C NMR ($D_2O$, dioxane) δ 179.69 ($CO_2H$), 177.80, 176.43, 175.80, 175.66, 174.61, 174.44, 171.70 (5 *C*ONH, 2 *C*O$NH_2$), 54.63, 54.23, 53.70, 51.27, 51.05, 50.14, 49.90 (*C*H-α, 3 Ala, *iso*Gln, *meso*$A_2$pm; *C*H-ε, $A_2$pm; $CH_2$CO), 43.07 (3 $CH_2$, Ada), 37.00 (3 *C*H, Ada), 33.45, 31.95, 31.29 ($CH_2$-γ, *iso*Gln; $CH_2$-β,δ, *meso*$A_2$pm; *C*, Ada), 29.22 (3 $CH_2$, Ada), 27.74 ($CH_2$-β, *iso*Gln), 22.76 ($CH_2$-γ, *meso*$A_2$pm), 17.80, 17.56, 17.44 ($CH_3$, 3 Ala). MS: $[M + H]^+$ at *m*/*z* 708.5.

**Hydrolysis of (adamant-1-yl)−$CH_2CO$-[$^{14}$C]PGM with *N*-acetylmuramyl-L-alanine amidase.** A solution of (adamant-1-yl)-acetyl-[$^{14}$C]peptidoglycan monomer (49 mg, 26839 Bq), in the above-mentioned buffer system, was incubated with the purified[15] *N*-acetylmuramyl-L-alanine amidase (900 μL, 0.816 i.u./mL) at 37 °C. The reaction was monitored by TLC in solvent system D and stopped by addition of ethanol. Precipitated proteins were removed by centrifugation, and the concentrated supernatant was chromatographed on a Sephadex LH-20 column (90×2.5 cm) in water, to give [$^{14}$C]-labelled **3** (21 mg, 48.6% of the total radioactivity used).

## Testing of immunostimulating activity

**Mice.** Male inbred mice, at least 6 weeks old, 5–10 in a group, were used. C57BL/6 mice were obtained from the medical faculty at the University of Rijeka, Croatia. During the experimental period they were housed in the animal facility of the Institute of Immunology, Inc., Zagreb. Commercial food and water were provided ad libitum.

**Immunizations.** Control groups of mice were immunized s.c. into the tail base with 0.2 mL of solution containing 50 μg of ovalbumin (OVA) in saline. Groups receiving immunomodulators were immunized with 0.2 mL of solution containing 50 μg of antigen and 200 μg of respective tested immunomodulator in saline. Animals were immunized two or three times at two-week intervals. All mice were bled one week after the booster immunization, as specified under respective figures. All animals were anaesthetized prior to blood collection with ether. Individual sera were decomplemented, i.e. heated for 30 min at 56 °C and stored at −20 °C until tested. This treatment inactivates some peptidases and proteases and ensures more consistent and reproducible results.

All three tested compounds were dissolved in saline prior to use and were non-pyrogenic preparations. Endotoxin content was determined with QCL-1000 LAL test (Biowhittaker, USA) and pyrogenicity in vivo was tested in rabbits according to Ph. Eur. 1997: 2.6.8.

**Enzyme-linked immunosorbent assay.** Anti-OVA antibodies were measured by ELISA as described previously.[14] Briefly, microtitre plates were coated with OVA (150 μg/mL), blocked with 0.5% gelatin in Tween–PBS and washed. Mouse sera to be tested were added in serial 2-fold dilutions, incubated for 2 h at 37 °C and the

plates washed. For IgG assay, horseradish peroxidase-conjugated sheep anti-mouse IgG (Cappel, Organon Teknika Corp., Durham, NC, USA, 1 in 10,000 in PBS) was used and incubated for 1 h at 37 °C. After washing, OPD solution was added and incubated for 30 min at room temperature. The reaction was stopped by adding 1 M $H_2SO_4$. Absorbance was read at 492 nm. Arbitrary units of anti-OVA were calculated using parallel line assay. Several dilutions of each serum sample were tested and compared with the standard positive serum, which was run on each plate. Since no reference preparation of a declared value for anti-OVA IgG has been available, the commercial anti-OVA was used as a standard preparation and the arbitrary standard value was ascribed to it. The set of data obtained after measuring the absorbances was used in a computer program for parallel line assay, so arbitrary units for each sample were calculated with respect to the value of standard sera. These arbitrary units relate to the amount of anti-OVA IgG in each sample and were used instead of expressing these amounts in titres.

All samples were assayed in duplicate. Sera from naive mice were used as blank on each plate.

Stimulation index for any treated group was calculated by dividing the mean value (expressed in arbitrary units or as absorbance) of a treated group with the mean value of control group in the respective experiment.

### Statistical analysis

Differences between experimental groups were determined by a Mann–Whitney U test and $P < 0.05$ was considered as significant. Analysis was performed using Statistica 5.0 for Windows, StatSoft Inc.

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