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Synthesis and Proinflammatory Properties of Muramyl Tripeptides Containing Lysine and Diaminopimelic Acid Moieties

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*The unusual amino acid diaminopimelic acid (DAP) was prepared by cross metathesis of appropriately protected vinyl glycine and allyl glycine derivatives. Catalytic hydrogenation of the cross-coupling product resulted in reduction of the double bond and the removal of protecting groups. The resulting compounds were appropriately protected for the polymer-supported and solution-phase synthesis of muramyl tripeptides **2** and **3**, which differ in the amidation of the α-carboxylic acids of the isoglutamine and DAP moieties. Muramyl dipeptide (**1**, MDP), the DAP-containing muramyl tripeptide **3**, and the lysine-containing muramyl tripeptides **4** and **5** induced TNF-α gene expression without TNF-α protein production in a human monocytic cell line. The observed block in translation could be removed by co-incubation with LPS, resulting in an apparent synergistic effect. Compound **2** did not induce TNF-α gene expression, neither did it exhibit a synergistic effect with LPS; this indicates that amidation of the α-carboxylic acids of the isoglutamine and DAP moieties results in a loss of biological activity. It is proposed that amidation of α-carboxylic acids is a strategy that may be used by pathogens to avoid detection by the innate immune system. Furthermore, the pattern recognition receptors Nod1 and Nod2 have been implicated in the possible induction of a synergistic effect of muropeptides with LPS.*

Introduction

The ability to recognize microbial components rapidly and to respond by initiating an acute inflammatory response is a crucial first line of defense against a microbial challenge. Overactivation of this inflammatory response, however, can result in the clinical symptoms of septic shock, which in the United States results in 100 000 deaths annually.[1] It has been estimated that 1% of hospitalized patients and 20–30% of patients in intensive care units develop sepsis. The advent of new antimicrobial resistance patterns, the increasing use of chemotherapeutic agents, and the emergence of diseases characterized by immunosuppression has caused the incidence of septic shock to increase dramatically.

Lipopolysaccharides (LPSs), peptidoglycans (PGNs), and lipoteichoic acid (LTA) comprise three principal bacterial cell wall components implicated in inducing the clinical manifestations of septic shock.[2] These components exert their biological effects by stimulating the host's monocytes and macrophages to produce proinflammatory mediators, such as TNF-α, IL-1, and IL-6. These mediators in turn elicit a variety of inflammatory responses in the host.

LPSs, vital components of the outer leaflet of the Gram-negative bacterial outer membrane, are comprised of three structural units: an outer polysaccharide component, a core oligosaccharide region, and the innermost portion, lipid A.[3,4] The lipid A region is largely responsible for the proinflammatory activity of LPSs and generally consists of a hexaacylated bis-1,4′-phosphorylated glucosamine disaccharide.

PGNs are particularly abundant in Gram-positive bacteria, in which they account for approximately half of the cell-wall mass. On the other hand, Gram-negative bacteria contain only a relatively thin PGN layer in the periplasmic space.[5–7] PGNs are large polymers composed of alternating β(1–4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, cross-linked by short peptide bridges (Scheme 1). These peptides consist of four or five alternating L- and D-amino acids that are attached to the carboxylic acid of MurNAc.[8,9] Lysine is commonly the third amino acid in the peptide moieties of PGNs of Gram-positive bacteria, while Gram-negative bacteria have a diaminopimelic acid (DAP) residue at this position.[10] Muramyl dipeptide (MDP) is the minimal structural subunit of PGN, accounting for some of its immunogenicity.[11,12]

The discovery of the Toll-like receptors (TLRs) less than a decade ago has advanced our understanding of early events in microbial recognition and response.[13–19] To date, eleven members of the mammalian TLR family have been identified, each potentially recognizing a discrete class of pathogen-associated

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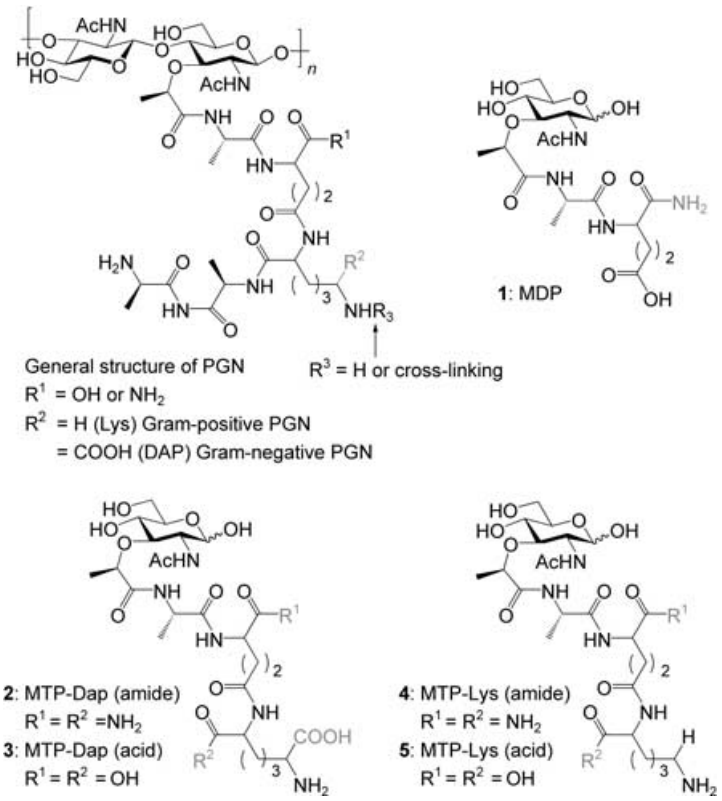
compounds.[20] Lipopolysaccharides (LPSs), for example, are recognized by TLR4, bacterial flagellin by TLR5, double-stranded RNA by TLR3,[20] and bacterial DNA by TLR9. The most recently discovered member of this family, TLR11, plays a critical role in the recognition and control of uropathogenic bacteria. Two recent studies have demonstrated that TLR3 is involved in recognition of single-stranded viral RNA. Contrary to initial beliefs that TLR2 in combination with TLR1 or TLR6 recognizes peptidoglycans (PGNs), recent studies with highly purified PGNs indicate that this might not be the case.[21] Instead it appears that Nod receptors (Nod1 and Nod2)[22] and peptidoglycan recognition proteins (PGRPs)[23–27] are the pattern recognition receptors that recognize PGNs (Figure 1).

We recently demonstrated that MDP induces TNF-α gene expression, but without significant TNF-α protein production.[28] This block in translation is removed, however, in the presence of LPSs and PGNs, which accounts for an apparent synergistic effect of MDP with LPSs or PGNs on TNF-α protein production. It is unclear whether any PGN breakdown products other than MDP can induce a synergistic effect with LPSs and PGNs. In this respect, it has been found that *Bordetella pertussis*, the causative agent of the respiratory disease pertussis, releases relatively large quantities of the muramyl peptide tracheal cytotoxin (TCT), a subunit of Gram-negative bacterial peptidoglycans.[29] When applied to hamster trachea epithelial cells, TCT and LPSs were found to be highly synergistic in the induction of IL-1α and nitric oxide synthase.[29] Others, however, have found that this compound and other PGN part-structures do not induce a synergistic effect.[30]

As part of a study to determine the immunological properties of PGN part-structures in detail, we report here the synthesis of muramyl tripeptides **2**, **3**, **4**, and **5**, each containing either a diaminopimelic acid (DAP) or a lysine residue. The ability of these compounds to induce TNF-α mRNA and protein production in a human monocytic cell line was determined and the results were compared with previously obtained data for MDP. Furthermore, the effect of preincubation of human monocytes with **2**, **3**, **4**, and **5** and MDP on TNF-α secretion induced by LPSs was compared to that of incubation with LPSs alone. These studies established that the DAP- and lysine-containing tripeptides **3**, **4**, and **5** induce a synergistic effect with LPSs. Furthermore, the observation that compound **2**—the α-carboxylic acids of which are modified as amides—has no activity indicates that these functionalities play crucial roles in the observed biological activity.



Scheme 1. Structures of peptidoglycans (PGNs) and synthetic compounds **1**–**5**.

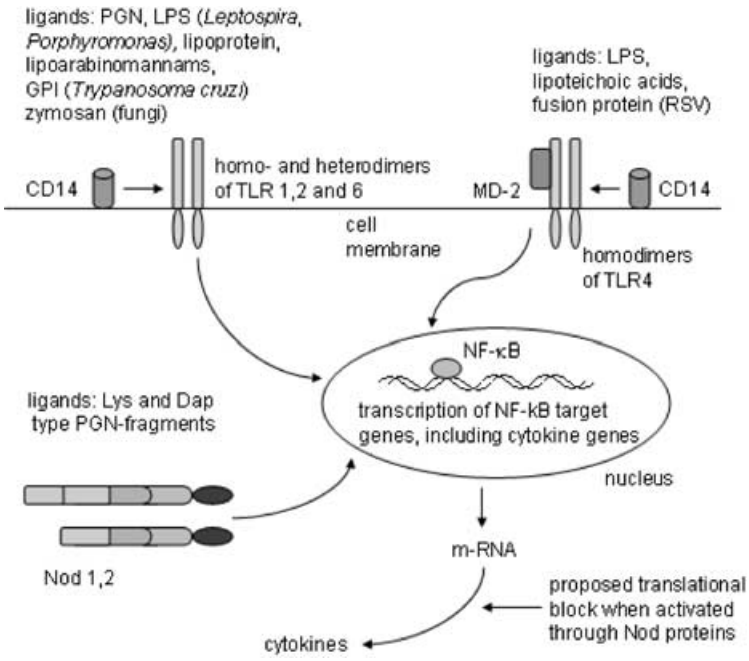
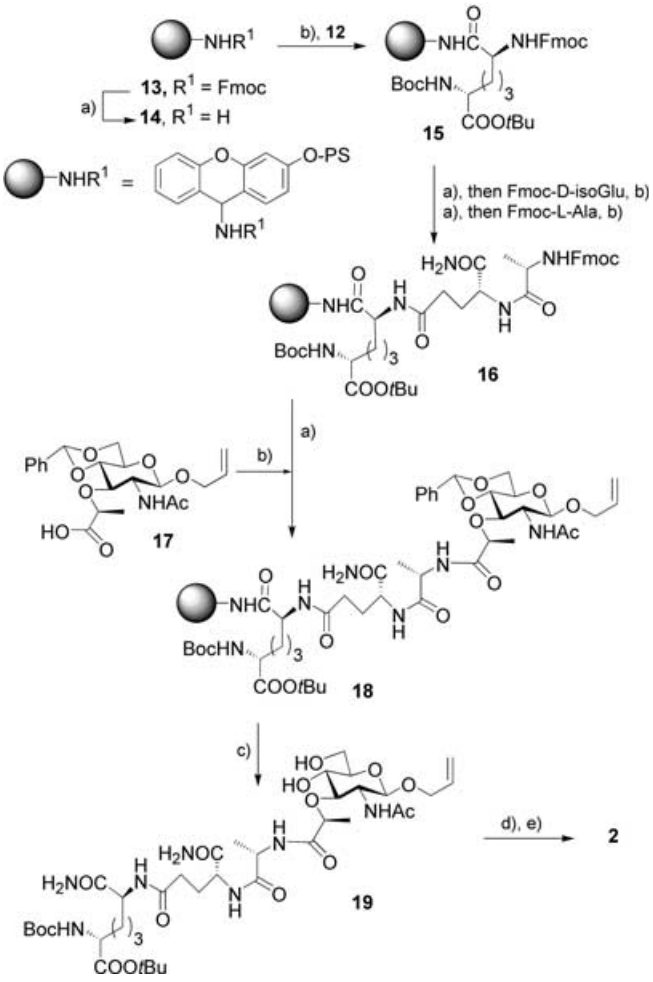


Figure 1. Cellular activation by TLRs and Nod proteins. Recognition of microbial components by the extracellular domains of TLRs leads to homo- or heterodimer formation. Subsequently, the intracellular domains of TLRs recruit adaptor proteins, which initiate the activation of a cascade of kinases, ultimately resulting in the activation of the transcription factor NF-κB. This transcription factor activates numerous genes for cytokines and chemokines. In mammals, Nod proteins mediate the intracellular recognition of peptidoglycan part-structures. Although the cellular activation of Nod proteins is independent of TLRs, it leads to activation of NF-κB. The results described in this paper indicate that cell signaling through Nods leads to the transcription of mRNA for cytokines such as TNF. However, no proteins are formed due to a translational block.

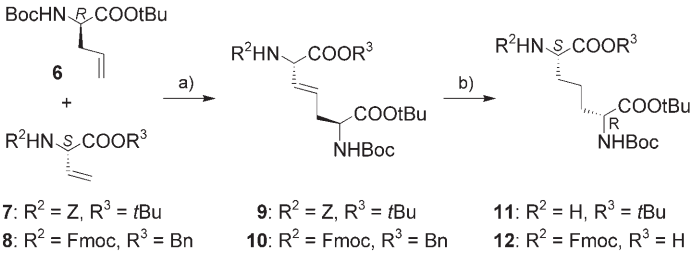
Results and Discussion

In general, lysine-containing muramyl peptides can be conveniently synthesized by standard solution- or polymer-supported approaches.[31,32] The chemical synthesis of DAP-containing fragments, however, is hampered by the difficulty involved in obtaining this unusual amino acid. The reported synthetic procedures for DAP either are lengthy or require the separation of enantiomers or epimers.[32–35]

We envisaged that DAP might be conveniently prepared by cross metathesis between readily available allyl glycine and vinyl glycine derivatives, followed by reduction of the double bond of the resulting compound.[36] High selectivity in olefin cross metathesis reactions has been achieved when two olefins have significantly different reactivities. Furthermore, the introduction of Grubbs' second-generation catalyst has made it possible to perform cross metathesis reactions with sterically demanding substrates,[37–40] so it was expected that a reaction between suitably protected vinyl glycine and allyl glycine derivatives in the presence of this catalyst should give a cross-coupling product in good yield. Indeed, reactions between *tert*-butyl carbamate-protected allyl glycine **6** and small excesses (1.8 equiv.) of suitably protected vinyl glycines **7** or **8** in the presence of Grubbs' second-generation catalyst gave, after a reaction time of 16 h, the cross-coupling products **9** and **10**, respectively, in acceptable yields (Scheme 2). Apart from these compounds, unreacted vinyl glycine and homodimerized allyl glycine were also present, but could easily be removed by silica gel column chromatography. The yield of this reaction was not improved by use of a larger excess of vinyl glycine or of a prolonged reaction time of 2 days. The protecting groups of **9** and **10** were selected because they allow conversion of these derivatives into amino acid building blocks suitable for solid-supported and solution-phase peptide synthesis. Thus, catalytic hydrogenation of **9** over Pd/C resulted in reduction of the double bond with concomitant removal of the benzylcarbonate (Z) group to give **11**, which is suitably protected for solution-phase peptide synthesis. Careful hydrogenation of **10** over Pt/C in a mixture of MeOH, H_2O, and HOAc resulted in the formation of compound **12** without the Fmoc protecting group being affected.[41] This derivative is suitably protected for solid-supported peptide synthesis.



Scheme 3. a) Piperidine in DMF (20%). b) PyBOP, HOBt, DIPEA in DMF. c) TFA in DCM (2%). d) TFA in DCM (20%). e) Pd/C, EtOH/H_2O/HCl (1 N; 4:2:0.01).



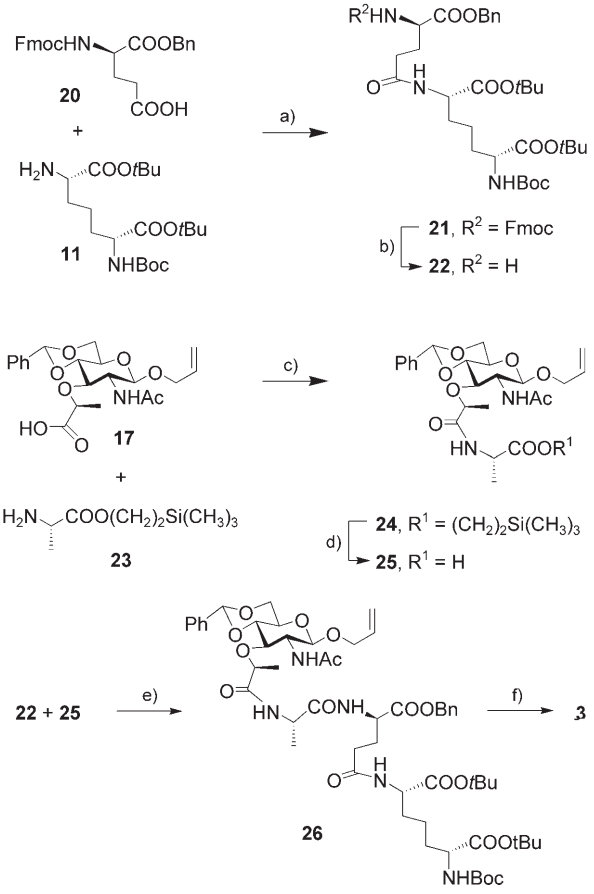
Scheme 2. a) Grubbs' II gen., DCM, (yields for compound 9 and 10, 55%, 64%, respectively). b) For **11**: 10% Pd/C, H_2 gas, EtOH/H_2O/DCM (9:1:1), 99%. For **12**: 3%Pt/C, H_2 gas, MeOH/H_2O/DCM (9:1:1), 97%.

Muramyl tripeptide **2** was assembled by classical 9-fluorenylmethoxycarbonyl (Fmoc) chemistry in combination with standard manual solid-phase peptide synthetic techniques (Scheme 3). The extremely acid-sensitive Sieber Amide resin (**13**) was selected as the polymeric support to allow the release of protected peptide amides by treatment with 2% trifluoroacetic acid (TFA). It was expected that these cleavage conditions would not affect other protecting groups of the glycopeptide, thus facilitating the purification of the released derivatives. Sieber Amide resin[42] (**13**) was swelled in dry dimethylformamide (DMF) and treated with piperidine in DMF (20%) to remove the Fmoc protecting group of the resin. The resulting amine **14** was coupled with Fmoc-protected derivative **12** by use of benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP),[43,44] 1-hydroxy-1*H*-benzotriazole (HOBt),[45] and *N*,*N*-diisopropylethylamine (DIPEA) as the activating reagent to give resin-bound **15**. Each reaction component was used in twofold excess with respect to the loading of the resin, and progress of the reaction was monitored by the Kaiser test.[46] After completion of the coupling, the resin was washed with DMF, and the

Fmoc protecting group of **15** was removed with piperidine in DMF (20%). The reaction cycle was repeated by subsequent use of Fmoc-D-isoglutamine, Fmoc-L-alanine, and 2-*N*-acetyl-1-β-*O*-allyl-4,6-benzylidene-3-muramic acid (**17**)[47] to give resin-bound glyco-tripeptide **18**. This glycopeptide was released from the solid support by treatment of the resin with TFA in dichloromethane (DCM; 2%) to give partially deprotected glycopeptide **19**. Glycopeptide **19** was treated with TFA in DCM (20%) to remove the Boc and the *tert*-butyl protecting groups of the DAP fragment. The anomeric allyl moiety was removed by isomerization and in situ cleavage of the intermediate vinyl ether with Pd/C in an EtOH/H_2O/HCl mixture to give the target compound **2** after purification by G-15 size exclusion column chromatography.

The target compound **3**, containing α-carboxyl groups, was prepared by a solution-phase approach because the alternative polymer-supported synthesis would require a large excess of a suitably protected DAP derivative, thus making the synthesis impractical (Scheme 4). The suitably protected DAP-containing derivative **11** was coupled with Fmoc-protected glutamic acid **20** in the presence of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate (HATU)[48] and DIPEA in DMF to give the protected dipeptide **21** in a yield of 88%. Attempts to perform this coupling by use of PyBOP, HOBt, and DIPEA resulted in lower yields and more difficult purifications. The Fmoc group of compound **21** was removed with piperidine in DMF (20%) to give the free amine **22** in a yield of 91%. In the first instance, the latter dipeptide was coupled with Fmoc-L-Ala to give a tripeptide, which was subjected to piperidine in DMF (20%) followed by coupling with **17** by use of HATU and DIPEA. The free amine of the L-Ala moiety of the intermediate tripeptide, however, entered into an intramolecular cyclization to give a diketopiperazine derivative as the major product. To avoid this side reaction, a suitably protected alanine derivative was first coupled with muramic acid derivative **16**, followed by attachment to dipeptide **22**. The carboxyl group of Boc-L-Ala was thus protected as a trimethylsilylethyl (TMSE) ester[49] by treatment with 2-(trimethylsilyl)ethanol, 4-dimethylaminopyridine (DMAP), and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide, followed by removal of the Boc protecting group with TFA in DCM (20%) to afford amine **23**. Next, compound **23** was coupled with suitably protected muramic acid derivative **17** in the presence of HOAt, DIC, and DIPEA in DMF to give MurNAc-L-Ala 24 in a good yield of 77%. MurNAc-L-Ala **24** was treated with TBAF in THF to unmask the carboxylic acid of the alanine moiety (**25**), which was coupled with dipeptide **22** with the aid of HOAt, DIC, and DIPEA in DMF to give the desired muramyltripeptide **26** in an acceptable yield of 64%. Finally, the target muramyl tripeptide **3** was obtained by removal of the benzylidene acetal, *tert*-butyl esters, and Boc groups from **26** by use of TFA in DCM (20%) for 5 h, followed by cleavage of the allyl moiety by treatment with Pd/C in a mixture of EtOH, H_2O, and HOAc.

The lysine-containing muramyl tripeptide **4** was prepared by a procedure similar to that employed for **2**, but in which Fmoc-L-Lys(Mtt)-OH was used instead of the DAP derivative **12** (Scheme 3). The advantage of using Mtt (4-methyltrityl) as a side-chain protecting group is that it can be removed with 1% TFA in DCM, so cleavage of the glycopeptide (MTP-Lys) from the Seiber amide resin also resulted in cleavage of the Mtt protecting group. The lysine-containing compound **5** was also assembled by a polymer-supported approach, but in this case the HMPB-AM resin was employed as the solid support. Release of the compound from this support results in the formation of a terminal acid. Fmoc-L-Lys(MTT)-OH was coupled with the HMPB-AM resin[50] by an anhydride formation protocol employing DIC and DMAP. After coupling of the first amino acid the synthesis was continued in a manner similar to that described for **4** to yield compound **5**.



Scheme 4. a) HATU, DIPEA, DMF, 88%. b) Piperidine in DMF (20%), 91%. c) HOAt, DIC, DIPEA, DMF, 77%. d) TBAF/THF, 84%. e) HOAt, DIC, DIPEA, 64%. f) i) TFA in DCM (20%), ii) Pd/C, EtOH/H_2O/HOAc (4:2:2), iii) Pd/C, EtOH/H_2O/HOAc (4:2:2), H_2.

Biological evaluation

The synthetic MTP derivatives **2**–**5** were tested for their ability to induce TNF-α protein and mRNA production in a human monocytic cell line (Mono Mac 6 cells), and the results were compared with similar data for MDP (**1**). Furthermore, the effect of preincubation of human monocytes with MDP (**1**) or **2**–**5** on TNF-α secretion induced by LPSs was compared with that observed on incubation with LPS alone.

It was found that the incubation of Mono Mac 6 cells with compounds **1**–**5** (100 μM) for 5 h resulted in each case in minimal production of TNF-α protein (Figure 2). On the other hand, MDP (**1**) and the muramyl tripeptides **3**, **4**, and **5** were each able to stimulate the production of significant levels of TNF-α mRNA after an incubation time of 1.5 h (Figure 3). Surprisingly, derivative **2** did not induce any production of TNF-α mRNA. Similar results were obtained for each compound when the cells were co-incubated with polymyxin B, indicating that the mRNA production was not a result of contamination of the samples (data not shown).

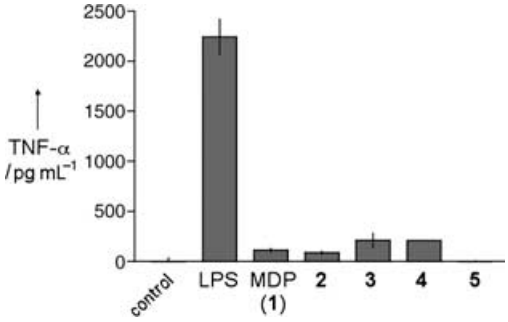


Figure 2. TNF-α protein production by Mono Mac 6 cells incubated with **1**–**5**. Mono Mac 6 cells were incubated with LPS (0.5 ng mL^{-1}), the synthetic compounds MDP (**1**), **2**, **3**, **4**, and **5** (100 μM each), or medium as control for 5 h, after which TNF-α was determined by ELISA. Treatment with LPS, MDP (**1**), **2**, **3**, **4**, and **5** did not affect cell viability, as judged by cellular exclusion of trypan blue.

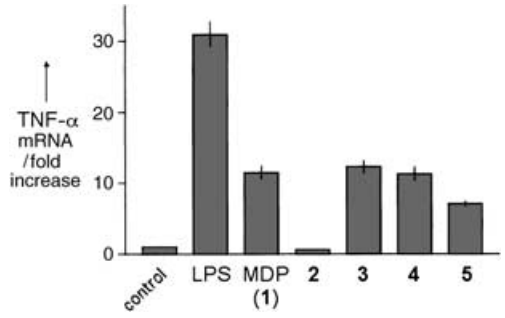


Figure 3. Induction of TNF-α mRNA by Mono Mac 6 cells incubated with MDP (**1**), **2**, **3**, **4**, and **5**. Mono Mac 6 cells were stimulated with medium alone, medium containing LPS (10 ng mL^{-1}), or the synthetic compounds MDP (**1**), **2**, **3**, **4**, or **5** (100 μM each) for 1.5 h, after which RNA was isolated for RT-PCR analysis of TNF-α message (40 PCR cycles). mRNA of the 18S ribosomal gene amplified under the same conditions was used as an internal control.

It was next investigated whether the observed block in translation of TNF-α mRNA could be removed by co-incubation with LPSs, as had been reported for MDP (**1**).[28] Treatment of the Mono Mac 6 cells with a wide range of LPS concentrations resulted in a clear dose/response curve with maximum supernatant concentrations of 3723 pg mL^{-1} TNF-α caused by 10 ng mL^{-1} of LPS. The results of these experiments yielded an EC_{50} (concentration producing 50% activity) value of 0.5 ng mL^{-1} and a Hill slope of 1.4 (Figure 4). Pretreatment of cells with MDP (**1**; 100 μM) followed by the addition of LPSs resulted in substantial increases in supernatant concentrations of TNF-α relative to results obtained with LPSs alone. This preincubation yielded a maximum level of 8880 pg mL^{-1} TNF-α, a Hill slope of 1.4, and an EC_{50} of 0.3 ng mL^{-1}. Next, the effect of co-incubation of compounds **2**–**5** with LPSs at the EC_{50} concentration was studied. Preincubation of cells with **3**, **4**, and **5** (100 μM) resulted in two- to threefold increases in TNF-α protein production, similar to the increase observed with 100 μM MDP (**1**; Figure 5). As expected, co-incubation of LPSs with compound **2** did not result in an increase in TNF-α protein production.

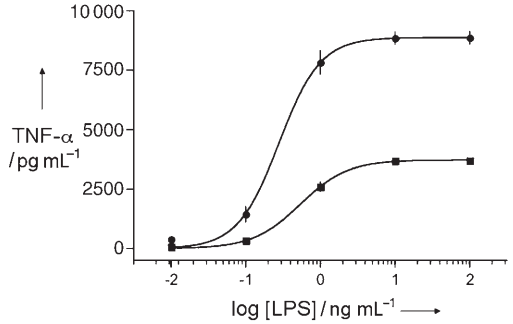


Figure 4. Effect of MDP (**1**) on LPS dose response. Mono Mac 6 cells were preincubated with MDP (**1**; 100 μM; ●) or medium (■) as control for 30 min at 37 °C. Increasing concentrations of LPS were added and, after an incubation period of 5 h, TNF-α protein was measured by ELISA. Stimulation with MDP (**1**) alone (100 μM) resulted in a TNF-α concentration of 112 ± 20 pg mL^{-1}.

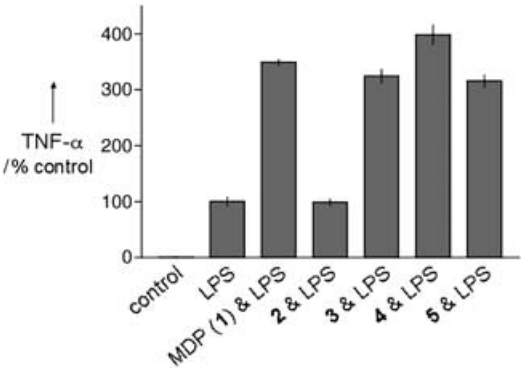


Figure 5. Induction of TNF-α production by Mono Mac 6 cells treated with MDP (**1**), **2**, **3**, **4**, or **5** and LPS. Mono Mac 6 cells were preincubated with the synthetic compounds MDP (**1**), **2**, **3**, **4**, or **5** (100 μM each) or medium as control for 30 min at 37 °C. After 5 h of stimulation with LPS (0.5 ng mL^{-1}), TNF-α was determined. TNF-α values were normalized for LPS alone (100%).

The results presented here demonstrate that lysine- and DAP-containing muropeptides **1** and **3**–**5** induce TNF-α gene expression without significant TNF-α translation. The block in translation is removed in the presence of LPSs, however, ac-

counting for an apparent synergistic effect of these compounds on TNF-α protein production. Importantly, compound **2** did not induce TNF-α gene expression, and nor did it exhibit a synergistic effect with LPSs. The structure of **2** is similar to that of the DAP-containing muramyl tripeptide **3** except for the fact that the α-carboxylic acids of the isoglutamine and DAP moieties are modified as amides. The cellular activation results thus suggest that at least one of these carboxylic acids of **3** is critical for induction of TNF-α gene expression. Interestingly, the α-carboxylic acids of the isoglutamine and the lysine moieties of compound **4** are also modified as amides, but this modification apparently does not affect the biological properties of this derivative. The observation that the DAP- and lysine-containing muramyl tripeptides display different structure/function relationships indicates that they initiate cellular activation through different receptors.

Initially, TLR2 was implicated in the initiation of cellular activation by peptidoglycans. However, recent studies have demonstrated that purification of PGNs results in a loss of activity on exposure to cells transfected with CD14 and TLR2.[21] It appears that the Nod receptors (Nod1 and Nod2)[22] and peptidoglycan recognition proteins (PGRPs) [23–27] are the pattern recognition receptors that sense PGNs. Although crystal structures of mammalian PGRPs are beginning to shed light on the ligand requirements of these proteins,[51] little is so far known about their modes of cellular activation. The Nod proteins have been studied in greater detail. There is evidence, for example, supporting recognition of DAP-containing muropeptides by Nod1, whilst Nod2 has been implicated in cellular activation by MDP and lysine-containing muropeptides. Furthermore, several studies have shown that mice pretreated with MDP are sensitized to endotoxic shock induced by LPSs whereas Nod2-deficient mice were resistant to such a challenge.[52] In addition, it has been observed that a synergistic effect of lipopeptide $Pam_3C(K)_4$ and MDP in wild-type mice is absent in mice macrophages deficient in Nod2. Although more research is required, it appears that the Nod proteins are involved in the apparent synergistic effect of muropeptides with LPSs.

The observation that small structural differences in muropeptides, such as in compounds **2** and **3**, have a major impact on the apparent synergistic effect with LPSs may explain conflicting reports.[29,30] Furthermore, amidation of the carboxylic acids of PGN fragments may be a strategy that could be used by pathogens to avoid host recognition and immune responses. To this end, PGN fragments from several bacilli such as *Bacillus licheniformis* and *Bacillus subtilus* are modified by amidation at the D-Glu and *meso*-DAP residues, respectively.[53] Notably, these pathogens possess reduced Nod1-stimulatory activity relative to their amidation free counterparts.[54–56]

Conclusion

In conclusion, it has been shown that MDP is not the only muropeptide able to induce a synergistic effect with LPSs. This effect arises from the removal of a block in translation by coincubation with LPSs. Small structural modifications such as the amidation of α-carboxylic acids may result in loss of biological activity.

Experimental Section

Synthesis

General methodology: Chemicals were purchased from Aldrich, Fluka, and Novabiochem and were used without further purification. Allyl glycines were purchased from Peptech. All solvents were dried in the appropriate manner and stored over 4 Å molecular sieves. All reactions were performed under anhydrous conditions under argon and monitored by TLC on Kieselgel 60 F254 (Merck). Detection was by examination under UV light (254 nm) and by charring with 10% sulfuric acid in methanol or ninhydrin in ethanol. Extracts were concentrated under reduced pressure at $<40\,^{\circ}C$ (bath). Silica gel column chromatography was performed on Merck 70–230 mesh. ^1H NMR (1D, 2D) and ^{13}C NMR spectra were recorded on Varian Merc300 and Varian 500 MHz spectrometers supported by Sun workstations. High-resolution mass spectra were obtained with a Voyager extraction STR instrument with 2,5-dihydroxybenzoic acid as matrix.

General method for cross-metathesis between allyl glycine and vinyl glycine derivatives: A solution of allyl glycine derivative **6** (0.46 mmol) and vinyl glycine derivative **7** or **8** (0.83 mmol) in DCM (1.5 mL) was placed under an atmosphere of Ar. Grubbs' 2nd generation catalyst (0.02 mmol) was added and the reaction mixture was stirred at room temperature for 18 h. The mixture was concentrated in vacuo and the residue was purified by flash silica gel column chromatography (eluent: hexane/ethyl acetate 8:1 *v/v*) to afford **9** or **10**, respectively.

1,7-Di-*tert*-butyl (2*S*,6*R*)-2-[(benzyloxycarbonyl)amino]-6-[(*tert*-butyloxycarbonyl)amino]hept-3-enedioate (9): Yield: 55%, $R_f = 0.71$ (eluent: hexane/ethyl acetate 2:1 *v/v*); $[\alpha]_D^{23} = +30.2$; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.28$–7.32 (m, 5H; arom., Z), 5.50–5.68 (m, 2H; γ,δ-C*HCH*), 5.42 (d, $J = 7.8$ Hz, 1H; N*H*), 5.08 (s, 2H; CH_2, Bn, Z), 4.99 (d, $J = 6.9$ Hz, 1H; N*H*), 4.70, 4.20–4.23 (m, 2H; α-C*H*, δ-C*H*), 2.47–2.48 (m, 2H; β-CH_2), 1.42 ppm (br s, 27H; C(CH_3)$_3$, 2×*t*Bu, Boc); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 182.45$, 182.44, 170.72, 169.53, 155.42, 155.13, 155.11, 136.24, 129.07, 128.47, 128.11, 127.88, 82.60, 82.02, 79.70, 66.94, 56.01, 53.17, 35.23, 29.65, 28.29, 27.98, 27.90 ppm; HRMS- MALDI-TOF calcd for $C_{28}H_{42}N_2O_8$ [*M*+Na]: 534.2941; found 534.2568.

1-Benzyl 7-*tert*-butyl (2*S*,6*R*)-2-[(fluoren-9-ylmethoxycarbonyl)-amino]-6-[(*tert*-butyloxycarbonyl)amino]hept-3-enedioate (10): Yield: 64%, $R_f = 0.70$ (eluent: hexane/ethyl acetate 2:1 *v/v*); $[\alpha]_D^{23} = +2.9$; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 7.77$ (d, $J = 7.5$ Hz, 2H; arom., Fmoc), 7.61 (d, $J = 6.9$ Hz, 2H; arom., Fmoc), 7.28–7.42 (m, 9H; arom., Fmoc, Bn), 5.66–5.69 (m, 2H; γ,δ-C*HCH*), 5.52 (d, $J = 8.1$ Hz, 1H; N*H*) 5.07–5.24 (m, 3H; CH_2, Bn, N*H*), 4.94 (br s, 1H; ε-C*H*), 4.36–4.41 (m, 2H; CH_2, Fmoc), 4.21–4.23 (m, 1H; α-C*H*, Fmoc), 2.42–2.53 (m, 2H; β-CH_2), 1.42 ppm (s, 18H; C(CH_3)$_3$, Boc, COO C(CH_3)$_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 170.68$, 170.40, 155.51, 155.17, 143.82, 143.71, 141.26, 135.04, 128.72, 128.62, 128.50, 128.22, 127.79, 127.68, 127.05, 125.07, 119.94, 82.10, 79.76, 67.48, 67.20, 55.44, 53.30, 47.09, 35.55, 31.55, 29.66, 28.29, 27.95, 22.61, 14.08 ppm; HRMS- MALDI-TOF calcd for $C_{38}H_{44}N_2O_8$ [*M*+Na]: 679.3098; found 679.2981.

Compound 2: The DAP-containing muramyl tripeptide (**2**) was synthesized by standard Fmoc solid-phase peptide synthesis. Sieber Amide resin (100 mg, 42 μmol; Novabiochem) was swelled in dry dimethylformamide (DMF; ~120 min, 3 mL), treated with 20% pi-

peridine in DMF (3×5 min, 3×2 mL), washed with freshly distilled DMF (3×3 mL), and then treated with DAP derivative **12** (38.7 mg, 42 μmol) in DMF in the presence of PyBOP (43.7 mg; Novabiochem), HOBt (11 mg; Aldrich), and DIPEA (29.2 μL; Alfa Aesar, Ward Hill, MA). Progress of the reaction was monitored by Kaiser test. After completion of the coupling, the resin was washed with DMF (3×3 mL), and the Fmoc protecting group was removed with piperidine in DMF (20%; 3×5 min, 3×2 mL). The reaction cycle was repeated with Fmoc-D-isoglutamine (30.94 mg, 84 μmol), Fmoc-L-alanine (26.12 mg, 84 μmol; Novabiochem), and subsequently 2-*N*-acetyl-1-β-*O*-allyl-4,6-benzylidene-3-*O*-muramic acid (**17**; 35.4 mg, 84 μmol). The resulting resin-bound glycopeptide (**18**) was washed with DMF (3×3 mL), dichloromethane (7×3 mL), and methanol (3×3 mL). The resin was dried in vacuo for 4 h, reswelled in DCM (~5 mL), and filtered. The glycopeptide (**19**) was released by treatment of the resin with trifluoroacetic acid in DCM (2%, 10×2 mL). The combined washings were concentrated under reduced pressure and coevaporated with toluene (3×10 mL) to remove traces of TFA. After cleavage from the resin, the MTP-DAP (**19**) was treated with TFA (20%) to remove the Boc and *t*Bu protecting groups. The deprotected derivative was precipitated from cold diethyl ether to afford an off-white product. A suspension of this compound and Pd/C (10%, 5 mg) in a mixture of EtOH/H_2O/HCl (1 N; 4:2:0.01, 0.6 mL) was stirred at r.t. for 16 h. The solution was filtered and the filtrate was purified by Sephadex G10 size exclusion column chromatography (eluent H_2O) to afford the target compound **2** as a mixture of α/β anomers (9.3 mg, 30% overall yield). $[\alpha]_D^{26} = +0.2$; ^{1}H NMR (500 MHz, D_2O): $\delta = 5.08$ (d, $J = 3.2$ Hz, 0.16 H; *H*-1(α)), 4.32 (d, $J = 8.4$ Hz, 0.84 H; *H*-1(β)), 4.07–4.23 (m, 5 H; α-C*H*×2, DAP, α-C*H*, Ala, α-C*H*, Glu, α-C*H*, Lac), 3.67–3.89 (m, 3 H; *H*2, *H*6ab), 3.38–3.50 (m, 3 H; *H*3, *H*4, *H*5), 2.27–2.36 (m, 2 H; γ-CH_2, Glu), 2.10 (m, 1 H; β-C*H*H, Glu), 1.65–1.93 (m, 8 H; β,δ-CH_2, Dap, β-CH*H*, Glu, NH(COCH_3)), 1.42–1.45 (m, 2 H; γ-CH_2, Dap), 1.29–1.35 ppm (m, 6 H; CH_3, Lac, CH_3, Ala); C-13 (HSQC): $\delta = 102.18$ (*C*-1-β), 91.74 (*C*-1-α), 83.31, 78.93, 76.24, 69.16, 60.66 (*C*6), 60.65, 60.06, 58.04, 55.68, 55.01, 54.33, 53.66, 50.29, 32.09 (γ-*C*, Glu), 30.75 (*C*-Dap), 27.71 (β-*C*, Glu), 23.00 (NHCH_3), 21.98, 19.29, 17.27 ppm; HRMS- MALDI-TOF calcd for $C_{25}H_{45}N_7O_{11}$ [*M*+HCl]: 700.1355; found 700.1058.

Compound 24: HOAt (107 mg, 0.79 mmol) and DIC (39 μL, 0.26 mmol) were added to a solution of 2-*N*-acetyl-1-β-*O*-allyl-4,6-benzylidene-3-*O*-muramic acid (**17**; 111 mg, 0.26 mmol) in dry DMF (1 mL). After the reaction mixture had been stirred for 30 min at RT, a mixture of H-L-Ala-OTMSE (**23**, 50 mg, 0.26 mmol) and DIPEA (93 μL, 0.52 mmol) in dry DMF (1 mL) was added and stirring was continued for 2 days. On completion of the reaction, the solution was filtered and the filtrate was concentrated in vacuo. The solid residue was dissolved in EtOAc (20 mL) and was washed with H_2O (5×2 mL), $NaHCO_3$ (5×2 mL), and brine (5×2 mL). The organic layer was dried ($MgSO_4$) and filtered, and the filtrate was concentrated in vacuo. The residue was purified by silica gel column chromatography (MeOH in DCM, 2%) to afford **24** as a white solid (121 mg, 77%). $R_f = 0.80$ (MeOH/DCM 5:95); $[\alpha]_D^{26} = -3.73$; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.20$–7.43 (m, 5 H; arom, Ph), 6.64 (d, $J = 7.8$ Hz, 1 H; N*H*), 5.76–5.89 (m, 1 H; OCH$_2$C*H*=CH$_2$), 5.48 (s, 1 H; C*H*, Ph), 5.21 (d, $J = 18.9$ Hz, 1 H; OCH$_2$CH=C*H*H), 5.12 (d, $J = 10.5$ Hz, 1 H; OCH$_2$CH=CH*H*), 4.80 (d, $J = 8.4$ Hz, 1 H; *H*1), 4.38 (t, $J = 6.9$ Hz, 1 H; α-C*H*, Lac), 4.26–4.32 (m, 3 H; *H*6a, *H*4, OC*H*HCH=CH$_2$), 4.12–4.21 (m, 3 H; COOCH_2, α-C*H*, Ala), 4.00–4.09 (m, 2 H; *H*3, OCH*H*CHCH$_2$), 3.75 (t, $J = 10.5$ Hz, 1 H; *H*6b), 3.57 (dd, $J = 9.3$ Hz, 1 H; *H*2), 1.93 (s, 3 H; NHCH_3), 1.34–1.40 (m, 6 H; 2×CH_3, Ala, Lac), 0.96 (t, $J = 16.8$ Hz, 2 H; CH_2Si), 0.00 ppm (s, 9 H; Si(CH_3)$_3$); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 173.01$, 171.06, 137.06, 133.64, 128.97, 128.22, 125.87, 117.43, 101.22, 99.96, 81.62, 78.66, 78.01, 70.09, 68.64, 65.84, 63.86, 57.09, 48.12, 41.91, 23.55, 19.38, 17.96, 17.27, −1.58 ppm; HRMS- MALDI-TOF calcd for $C_{29}H_{44}N_2O_9Si$ [*M*+Na]: 615.2706; found 615.2394.

Compound 25: TBAF (1 M in THF, 95 μL) was added to a cooled (0 °C) solution of **24** (47 mg, 0.08 mmol) in THF (1 mL), and the reaction mixture was stirred for 5 h at RT. On completion of the reaction (as indicated by TLC analysis), the reaction mixture was concentrated in vacuo and the residue was subjected to silica gel column chromatography (2% MeOH/DCM) to yield compound **25** as a colorless oil. (33 mg, 84%). $R_f = 0.30$ (MeOH in DCM: 8:92); $[\alpha]_D^{26} = -9.6$; ^{1}H NMR (500 MHz, CD_3OD): $\delta = 7.28$–7.39 (m, 5 H; arom, Ph), 5.79–5.83 (m, 1 H; OCH$_2$C*H*=CH$_2$), 5.56 (s, 1 H; C*H*, Ph), 5.18 (d, $J = 17.5$ Hz, 1 H; OCH$_2$CH=CH*H*), 5.07 (d, $J = 11.0$ Hz, 1 H; OCH$_2$C*H*=C*H*H), 4.51 (d, $J = 8.5$ Hz, 1 H; *H*1), 4.21–4.23 (m, 2 H; *H*6a, OC*H*HCH=CH$_2$), 3.99–4.10 (3 H; OCH*H*CH=CH$_2$, α-*H*, Ala, Lac), 3.88 (t, $J = 9.5$ Hz, 1 H; *H*2), 3.75 (t, $J = 10.5$ Hz, 1 H; *H*6b), 3.68 (t, $J = 9.5$ Hz, 1 H; *H*3), 3.58 (t, $J = 9.0$ Hz, 1 H; *H*4), 3.38–3.43 (m, 1 H; *H*5), 1.32 and 1.25 ppm (d, 6 H; 2×CH_3, Lac, Ala); ^{13}C NMR (75 MHz, CD_3OD): $\delta =$ 174.55, 174.23, 138.96, 135.23, 130.00, 129.16, 127.25, 117.27, 102.68, 102.51, 82.77, 81.08, 79.48, 71.25, 69.59, 67.34, 59.51, 59.47, 56.81, 24.79, 23.43, 20.71, 19.85, 19.21, 13.92 ppm; HRMS- MALDI-TOF calcd for $C_{24}H_{32}N_2O_9$ [*M*+Na]: 515.1198; found 515.1931.

1,7-Di-*tert*-butyl (2*S*,6*R*)-N^{α}-(*N*-fluoren-9-ylmethoxycarbonyl-D-isoglutamyl α-benzyl ester)-N^{ε}-(*tert*-butoxycarbonyl)-2,6-diaminopimelate (21): Pd/C (5%, 50 mg) was added to a solution of **9** (70 mg, 0.12 mmol) in a mixture of EtOH/H_2O/DCM (10:1:1 *v/v/v*, 2 mL). The mixture was placed under an atmosphere of hydrogen and stirred for 16 h. On completion of the reaction (TLC analysis), the suspension was filtered through a pad of celite, washed with EtOH, and the filtrate was concentrated in vacuo. The free amine (**11**) was used immediately in the next reaction step. HATU (49 mg, 0.12 mmol) and DIPEA (46 μL, 0.25 mmol) were added to a solution of FmocGluOBn (59 mg, 0.12 mmol) in dry DMF, and a solution of the free amine **11** in dry DMF and DIPEA (23 μL, 0.12 mmol) was then added. The resulting reaction mixture was stirred at RT for 20 h, after which it was concentrated in vacuo and the residue was subjected to size exclusion column chromatography over LH-20 (DCM/MeOH 1:1) to afford **21** (96 mg, 88%) as a colorless syrup. $R_f = 0.71$ (MeOH/DCM 5:95); $[\alpha]_D^{23} = +44.0$; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 7.78$ (d, $J = 7.5$ Hz, 2 H; arom., Fmoc), 7.59 (d, $J = 7.2$ Hz, 2 H; arom., Fmoc), 7.26–7.37 (m, 9 H; arom., Fmoc, Bn), 6.44 (d, $J =$ 7.5 Hz, 1 H; N*H*), 5.80 (d, $J = 7.5$ Hz, 1 H; N*H*), 5.15 (s, 2 H; CH_2Bn), 5.03–5.08 (m, 1 H; N*H*), 4.33–4.43 (m, 4 H; CH_2, Fmoc, α, ε-C*H*, Dap), 4.18 (t, 1 H; C*H*, Fmoc), 4.10 (m, 1 H; α-C*H*, Glu), 2.23 (m, 3 H; γ-CH_2, β-C*H*H, Glu), 1.54–1.98 (7 H; β-CH*H*, Glu, β,γ,δ-CH_2, Dap), 1.39 ppm (s, 27 H; C(CH_3)$_3$, Boc, 2×*t*Bu); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 171.85$, 171.68, 171.57, 171.32, 156.28, 155.46, 143.90, 143.68, 141.26, 135.17, 128.59, 128.46, 128.30, 127.66, 127.05, 125.15, 119.92, 82.05, 81.84, 79.63, 67.28, 67.10, 53.57, 53.49, 53.45, 52.57, 47.12, 32.68, 32.13, 31.82, 28.43, 28.29, 27.94, 20.87 ppm; HRMS- MALDI-TOF calcd for $C_{47}H_{61}N_3O_{11}$ [*M*+Na]: 866.4306; found 866.4518.

Compound 22: A solution of **21** (35 mg, 0.04 mmol) in piperidine in dry DMF (20%, 1 mL) was stirred at RT for 3 h. On completion of the reaction (as indicated by TLC analysis), the solvent was evaporated in vacuo and the residue was subjected to silica gel column chromatography (MeOH/DCM 2:98) to afford compound **22** as a colorless gel (24 mg, 93%). $R_f = 0.29$ (MeOH/DCM 4:96); $[\alpha]_D^{26} =$ −0.3; ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 7.29$–7.35 (m, 5 H; arom H, Bn), 6.49 (d, $J = 7$ Hz, 1 H; N*H*), 5.14 (s, 2 H; CH_2Bn), 5.04 (d, $J = 8$ Hz, 1 H; N*H*), 4.39–4.43, 4.10 (m, 2 H; α-C*H*, Dap, ε-C*H*, Dap), 3.52 (br s, 1 H; α-C*H*, Glu), 2.33–2.39 (m, 1 H; γ-CH*H*, Glu), 2.24–2.29 (m, 1 H; γ-C*H*H, Glu), 2.10–2.12 (m, 2 H; β-C*H*, Glu), 1.56–1.82 (m, 4 H; β,δ-CH_2,

Dap), 1.42 (br s, 27H; C(CH_3)$_3$, Boc, 2×*t*Bu), 1.25–1.36 ppm (m, 2H; γ-CH_2-Dap); ^{13}C NMR (HSQC): 128.76 (arom H, Bn), 68.04 (*C*HBn), 53.70, 55.61, 55.62 (α,ε-*C*H-Dap, α-*C*H-Glu) 33.14, 32.66, 31.70, 28.83(*C* (CH_3)$_3$, Boc, 2×*t*Bu) 21.66 ppm; HRMS- MALDI-TOF calcd for $C_{32}H_{51}N_3O_9$ [*M*+Na]: 644.3515; found 644.3002.

Compound 26: HOAt (16 mg, 90 μmol) and DIC (5.9 μL, 30 μmol) were added to a solution of **25** (19 mg, 38 μmol) in DMF (0.5 mL) and the mixture was stirred at RT for 1 h. A solution of **22** (24 mg, 38 μmol) and DIPEA (13.4 μL, 77 μmol) in dry DMF (0.05 mL) was added to the reaction mixture and stirring was continued at RT for 16 h. The solvents was evaporated and the residue was subjected to silica gel column chromatography (MeOH/DCM 2:98) to afford compound **26** as a colorless gel (27 mg, 64%). R_f = 0.56 (MeOH/DCM 4:96); $[\alpha]_D^{26} = -15.71$; ^{1}H NMR (500 MHz, CD_3OD): δ = 7.40 (m, 2H; arom H), 7.23–7.28 (m, 8H; arom H), 5.78–5.83 (m, 1H; $OCH_2CH{=}CH_2$), 5.56 (s, 1H; *CH*, Ph), 5.19 (d, *J* = 17.0 Hz, 1H; $OCH_2CH{=}CHH$), 5.07 (d, *J* = 11.0 Hz, 3H; CH_2Bn, $OCH_2CH{=}CHH$), 4.51 (d, *J* = 8.0 Hz, 1H; *H*1), 4.39–4.41 (m, 1H; α-*CH*, Glu), 4.22–4.30 (m, 5H; α-*CH*, Dap, α-*CH*, Ala, α-*CH*, Lac, *H*6a, $OCHHCH{=}CH_2$), 4.13 (t, *J* = 6.5 Hz, 1H; ε-*CH*, Dap), 3.97–4.01 (m, 1H; $OCHHCH{=}CH_2$), 3.86 (t, *J* = 9.0 Hz, 1H; *H*2), 3.75 (t, *J* = 10 Hz, 1H; *H*6b), 3.61–3.70 (m, 2H; *H*3, *H*4), 3.39–3.41 (m, 1H; *H*5), 2.21–2.24 (m, 2H; γ-CH_2, Glu), 2.09 (m, 4H; β-CH*H*, Glu, $NHCOCH_3$), 1.92–1.88 (m, 1H; β-*CH*H, Glu), 1.68–1.51 (6H; β,γ,δ-CH_2, Dap), 1.23–1.38 ppm (15H; C(CH_3)$_3$, Boc, 2×CH_3, Ala, Lac); ^{13}C NMR (75 MHz, CD_3OD): δ = 175.31, 174.85, 174.65, 174.63, 173.81, 172.88, 172.68, 172.67(*C*O), 139.01, 137.14, 135.39, 135.34 (*C*H-allyl), 130.44, 130.12, 129.97, 129.65, 129.60, 129.40, 129.34, 129.27, 129.17, 127.30, 127.27 (arom, Ph, Bn) 117.15 (*C*H-allyl), 102.65, 102.48 (Ph*C*H, *C*1), 82.80, 82.57, 82.49, 80.51, 78.98, (*C*(CH_3)$_3$×3, *C*3, *C*4) 71.07, 69.61, 68.07, 67.44, 56.81, 55.60, 54.48, 53.49, 50.24 (α-C, Ala, Glu, Dap, Lac) 32.82, 32.27, 32.04, 28.77, 28.46, 28.30, 28.17, 23.26, 19.78, 18.51 ppm (C(CH_3)$_3$×3, *C*H$_3$, Ala, *C*H$_3$, Lac); HRMS- MALDI-TOF calcd for $C_{56}H_{81}N_5O_{17}$ [*M*+H+Na]: 1120.2548; found 1120.2733.

Muramyl tripeptide 3: The fully protected muramyl tripeptide derivative **26** (11 mg, 0.001 mmol) was treated with TFA in DCM (20%, 0.5 mL) for 5 h. The solution was coevaporated from toluene to remove traces of TFA. The resulting white compound was dried in vacuo for 5 h. Pd/C (10%, 10 mg) was added to a solution of the crude compound in an EtOH/H_2O/HOAc (4:2:2, 0.8 mL) mixture and the reaction mixture was stirred for stirred for 5 days, after which it was filtered through a thin pad of celite and the resulting filtrate was concentrated under reduced pressure. A crude NMR confirmed the complete removal of the allyl group. The benzyl ester was removed by stirring the compound under H_2 in the presence of Pd/C in an EtOH/H_2O/HOAc (4:2:2, 0.8 mL) mixture for 16 h. On completion of the reaction, Pd/C was filtered off and the solvents were evaporated in vacuo. The crude product subjected to G-15 column chromatography (H_2O) to afford compound **3** as white solid (3.9 mg, 58%) upon lyophilization. $[\alpha]_D^{26} = -8.1$; ^{1}H NMR (500 MHz, D_2O): δ = 5.08 (d, 1H; *H*1), 3.59–4.59 (m, 10H; α,ε-*CH*, Dap, α-*CH*, Ala, α-*CH*, Glu, α-*CH*, Lac, *H*2, *H*3, *H*4, *H*5, *H*6), 2.46 (br s, 2H; γ-CH_2, Glu), 2.00–2.28 (m, 7H; β-CH_2, Glu, CH_2, Dap, $NHCOCH_3$), 1.87 (m, 2H; CH_2, Dap), 1.59–1.66 ppm (m, 6H; 2×CH_3, Ala, Lac); C-13 (HSQC): δ = 91.71 (*C*-1), 82.79 (*C*2), 82.33, 82.10, 78.68, 76.63 (α-*C*, Ala, Glu, Dap, Lac), 75.95, 69.79 (*C*2, *C*3), 60.09, 61.36, 59.99, 57.71, 56.11, 55.89, 55.43, 55.20, 50.41, 49.79, 32.78 (γ-C, Glu), 31.64, 31.41, 28.91, 28.68, 27.54, 22.52 (β-C, Glu), 21.84, 21.61, 17.96, 17.74 ppm; HRMS- MALDI-TOF calcd for $C_{26}H_{43}N_5O_{15}$ [*M*/2+Na]: 355.6268; found 355.5276.

MTP-Lys (**4**): Sieber Amide resin (100 mg, 42 μmol; Novabiochem) was swelled in dry dimethylformamide (DMF; 5 mL, ~120 min), treated with piperidine in DMF (20%, 3×5 mL, 3×2 mL), washed with freshly distilled DMF (3×3 mL), and then treated with Fmoc-L-Lys(Mtt)-OH (52.4 mg, 84 μmol; Novabiochem) in DMF in the presence of PyBOP (43.7 mg; Novabiochem), HOBt (11 mg; Aldrich), and DIPEA (29.2 μL; Alfa Aesar, Ward Hill, MA). Progress of the reaction was monitored by the Kaiser test. After completion of the coupling, the resin was washed with DMF (3×3 mL), and the Fmoc protecting group was removed with piperidine in DMF (20%, 3×5 min, 3×2 mL). The reaction cycle was repeated with Fmoc-D-isoglutamine (30.9 mg, 84 μmol), Fmoc-L-alanine (26.1 mg, 84 μmol; Novabiochem), and, subsequently, 2-*N*-acetyl-1-β-*O*-allyl-4,6-benzylidene-3-*O*-muramic acid (**17**, 35.4 mg, 84 μmol). The resulting resin-bound glycopeptide was washed with DMF (3×3 mL), dichloromethane (7×3 mL), and methanol (3×3 mL). The resin was dried in vacuo for 4 h, reswelled in DCM (~5 mL), and filtered. The glycopeptide was released by treatment of the resin with trifluoroacetic acid in DCM (2%, 10×2 mL). The resin washings were combined, concentrated under reduced pressure, and coevaporated with toluene to remove traces of TFA, and the residue was dried in vacuo. The crude product was subjected to TFA in DCM (20%, ~2 mL) to ensure complete removal of the benzylidene protecting groups. The resulting product was purified by Sephadex G15 size exclusion column (Amersham Biosciences) chromatography (eluent H_2O) to give [2-*N*-acetyl-1-β-*O*-allyl-3-*O*-muramyl]-L-alanyl-D-isoglutamyl-L-lysine (8.7 mg, 75%) as a white, amorphous solid. $[\alpha]_D^{26} = -5.9$; ^{1}H NMR (500 MHz, D_2O): δ = 5.75–5.80 (m, 1H; $OCH_2CH{=}CH_2$), 5.13 (dd, 2H; $OCH_2CH{=}CH_2$), 4.12 (d, *J* = 8.43 Hz, 1H; *H*1), 4.00–4.23 (m, 6H; α-*CH*, Ala, α-*CH*, Lys, α-*CH*, Glu, α-*CH*, Lac, $OCH_2CH{=}CH_2$), 3.83 (d, *J* = 10.7 Hz, 1H; *H6a*), 3.73 (t, 1H; *H2*), 3.63–3.67 (dd, 1H; *H6b*), 3.33–3.34 (m, 3H; *H3, H4, H5*), 3.05 (t, 2H; ε-CH_2, Lys), 2.27–2.31 (m, 2H; γ-CH_2, Glu), 2.05–2.09 (m, 1H; β-CH_2, Glu), 1.85–1.87 (m, 4H; β-CH_2, Glu, NH*Ac*), 1.67–1.69 (m, 1H; β-CH_2, Lys), 1.59–1.62 (m, 1H; β-CH_2, Lys), 1.37–1.42 (m, 2H; δ-CH_2, Lys), 1.32 (m, 5H; CH_3, Lac, γ-CH_2, Lys), 1.26 ppm (d, *J* = 7.13 Hz, 3H; CH_3, Ala); ^{13}C NMR (75 MHz, D_2O): δ = 177.06, 176.02, 175.99, 175.30, 175.25, 174.27, 133.52, 118.24, 100.27, 82.97, 78.41, 75.78, 70.67, 68.85, 60.86, 55.21, 53.63, 52.87, 49.97, 39.33, 31.57, 30.54, 26.93, 26.42, 22.34, 22.28, 18.89, 16.69 ppm; HRMS- MALDI-TOF calcd for $C_{28}H_{49}N_7O_{11}$ [*M*+Na]: 682.3380; found 682.3062.

The [2-*N*-acetyl-1-β-*O*-allyl-3-*O*-muramyl]-L-alanyl-D-isoglutamyl-L-lysine (8.7 mg, 10.3 μmol) was dissolved in an ethanol/acetic acid/water (2:1:1, 0.8 mL) mixture, and Pd on charcoal (10%, 9 mg) was added. After stirring at room temperature for 48 h, the reaction mixture was filtered, the filtrate was concentrated under reduced pressure, and the residue was coevaporated from toluene (3×10 mL). The residue was subjected to Sephadex G15 size exclusion column chromatography (eluent H_2O) to give the target compound (**4**) as a mixture of α/β anomers (5.3 mg, 61%). $[\alpha]_D^{26} = +20.6$; ^{1}H NMR (500 MHz, D_2O): δ = 5.16 (d, *J* = 3.3 Hz, 0.69H; *H1*-α anomer), 4.67 (d, *J* = 8.1 Hz, 0.31H; *H-1*-β anomer), 4.20–4.34 (m, 4H; α-*CH*, Lys, α-*CH*, Glu, α-*CH*, Ala, α-*CH*, Lac), 3.50–4.00 (m, 6H; *H2,H3,H4,H5,H6*), 3.01 (t, 2H; ε CH_2, Lys), 2.39–2.45 (m, 2H; γ CH_2, Glu), 2.15–2.23 (m, 1H; β CH_2, Glu), 1.65–2.00 (m, 8H; β CH_2, Glu, β,δ-CH_2, Lys, NH($COCH_3$), 1.37–1.47 ppm (m, 8H; γ CH_2, Lys, CH_3, Lac, CH_3, Ala); ^{13}C NMR (75 MHz, D_2O): δ = 177.06, 176.02, 175.25, 174.14, 101.03, 95.07, 91.13, 82.77, 79.85, 78.46, 78.24, 77.89, 75.87, 73.51, 71.66, 69.05, 60.88, 60.67, 56.34, 53.86, 53.63, 52.87, 49.98, 49.03, 39.33, 31.57, 30.53, 26.93, 26.42, 22.65, 22.37, 22.28, 22.15, 18.79, 16.70 ppm; HRMS- MALDI-TOF calcd for $C_{25}H_{45}N_7O_{11}$ [*M*+Na]: 642.3067; found 642.3777.

MTP-Lys-acid (**5**): HMPB-AM resin (100 mg, 91 μmol; Novabiochem) was swelled in dry dimethylformamide (DMF; ~30 min). 1,3-Di-

isopropylcarbodiimide (DIC, 70 μL, 450 μmol) was added at 0 °C to a solution of Fmoc-Lys(Mtt)-OH (567 mg, 900 μmol) in dry DCM (~5 mL) and the resulting reaction mixture was stirred for 20 min. This solution was then concentrated to dryness (~1 h over vacuum pump) and dissolved in dry DMF (2 mL), which was added to the swelled resin. DMAP (11.1 mg, 90 μmol) was added to this solution, and the reaction was mixed by bubbling of N_2 gas for 6 h. The reaction mixture was then treated with piperidine in DMF (20%, 3× 5 min, 3×3 mL) to remove the Fmoc group on the Fmoc-Lys(Mtt)-OH moiety. Completion of reaction was monitored by Kaiser test. The reaction cycle was repeated with Fmoc-D-Glu(O*t*Bu) (77.1 mg, 182 μmol), Fmoc-L-alanine (56 mg, 182 μmol; Novabiochem), and, subsequently, 2-*N*-acetyl-1-β-*O*-allyl-4,6-benzylidene-3-*O*-muramic acid (**17**, 76 mg, 182 μmol). The resulting resin-bound glycopeptide was washed with DMF (3×2 mL), dichloromethane (5×2 mL), and methanol (3×2 mL). The resin was dried in vacuo for 4 h, reswelled in DCM (~5 mL), and filtered. The glycopeptide was released by treatment of the resin with trifluoroacetic acid in DCM (1%, 10× 2 mL). The resin washings were combined and concentrated under reduced pressure and the residue was dried in vacuo. The crude product was treated with TFA in DCM (20%, 2 mL) to ensure complete removal of the benzylidene protecting groups. The resulting product was purified by Sephadex G15 size exclusion column chromatography (Amersham Biosciences) to give **5** (8.7 mg, 75%) as a white, amorphous solid. ^{1}H NMR (500 MHz, D_2O): $\delta = 5.17$ (d, $J =$ 3.0 Hz, 0.41 H; *H*1(α)), 4.67 (d, $J = 8.5$ Hz, 0.59 H; *H*1(β)), 4.23–4.42 (m, 4H; α-C*H*, Lys, α-C*H*, Ala, α-C*H*, Glu, α-C*H*, Lac), 3.46–3.99 (m, 6H; *H*2, *H*6ab, *H*3, *H*4, *H*5), 3.00 (t, 2H; εCH_2, Lys), 2.40 (t, 2H; γCH_2, Glu), 2.21 (m, 1H; βCH*H*, Glu), 1.88–2.05 (m, 5H; NHCH_3, βC*H*H, Glu, C*H*, Lys), 1.70–1.88 (m, 3H; 3×C*H*, Lys), 1.36–1.45 ppm (m, 8H; γCH_2, Lys, CH_3, Lac, CH_3, Ala); C-13 (HSQC): $\delta = 95.13$ (αC-1), 91.51 (βC-1), 82.98, 81.69, 78.53, 78.25, 73.65 (α-*C*, Ala, Glu, Lys, Lac), 80.11, 76.09, 71.93, 66.34, 61.16, 61.02, 60.87, 59.27, 57.57, 55.85, 54.12, 53.26, 52.69, 50.25, 39.57 (εC Lys), 32.00 (γC-Glu), 30.11, 26.92 (βC-Glu), 26.80, 25.97, 22.66, 22.43, 19.12, 17.70, 17.22 ppm; HRMS- MALDI-TOF calcd for $C_{25}H_{43}N_5O_{13}$ [*M*]: 621.2857; found 621.2571.

Biological Studies

Reagents: MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) was obtained from Calbiochem, polymyxin B from Bedford Laboratories, and *E. coli* 055:B5 LPS from List Biological Laboratories. All studies were performed with a same batch of *E. coli* 055:B5 LPS, which is further referred to in the text as LPS.

Cell maintenance: Mono Mac 6 cells, provided by Dr. H.W.L. Ziegler-Heitbrock (University of Munich, Germany), were cultured in RPMI 1640 medium with L-glutamine (Mediatech) supplemented with penicillin (100 u mL^{-1})/streptomycin (100 μg mL^{-1}; Mediatech), OPI supplement (1%; Sigma; containing oxaloacetate, pyruvate, and bovine insulin), and fetal bovine serum (10%; HyClone). The cells were maintained in a humid 5% CO_2 atmosphere at 37 °C. New batches of frozen cell stock were grown up every 2–3 months and growth morphology was evaluated. Before each experiment, Mono Mac 6 cells were allowed to differentiate for 2 days in the presence of calcitriol (10 ng mL^{-1}; Sigma).

ELISA TNF-α: Cells were harvested by centrifugation and were gently resuspended (10^6 cells mL^{-1}) in prewarmed (37 °C) medium. Cells were then incubated for 5 h with different combinations of stimuli in the presence or absence of polymyxin B as described below. At the end of the incubation period, cell supernatants were collected and stored frozen (−80 °C) until assayed for TNF-α protein. Concentrations of TNF-α in culture supernatants were determined in duplicate by a solid-phase sandwich ELISA. Briefly, 96-well plates (Nalge Nunc International) were coated with purified mouse *anti*-human TNF-α monoclonal antibody (mAb; Pharmingen). TNF-α in standards and samples was allowed to bind to the immobilized mAb for 2 h at room temperature. Biotinylated mouse *anti*-human TNF-α mAb (Pharmingen) was then added, producing an antibody-antigen-antibody "sandwich". After addition of avidin-horseradish peroxidase conjugate (Pharmingen) and ABTS peroxidase substrate (Kirkegaard & Perry Laboratories), a green color was produced in direct proportion to the amount of TNF-α present in the sample. The reaction was stopped by addition of peroxidase stop solution (Kirkegaard & Perry Laboratories), and the absorbance was measured at 405 nm with a microplate reader (Bio-Tek Instruments). All data for TNF-α are presented as the means ± SD of duplicate cultures. Each experiment was repeated at least twice.

Evaluation of materials for contamination by LPS: To ensure that any increase in TNF-α production was not caused by LPS contamination of the solutions containing the various stimuli, the experiments were performed in the absence and in the presence of polymyxin B, an antibiotic that binds avidly to the lipid A region of LPS, thereby preventing LPS-induced monokine production.[57] TNF-α concentrations in supernatants of cells preincubated with polymyxin B (25 μg mL^{-1}) for 30 min before incubation with 0.5 ng mL^{-1} LPS for 5 h were reduced from 1738 ± 96 pg mL^{-1} to 3 ± 2 pg mL^{-1}, whereas preincubation with polymyxin B had no effect on TNF-α synthesis by cells incubated with 100 μM MDP (**1**; ~112 pg mL^{-1}) or the synthetic compounds **2**, **3**, **4**, and **5** (~87, ~209, ~206 and ~0 pg mL^{-1}, respectively). Therefore, LPS contamination of these preparations was inconsequential.

Preparation of RNA and quantification of TNF-α mRNA by real-time polymerase chain reaction (PCR) analysis: Cells were harvested by centrifugation and were gently suspended (2.25×10^6 cells mL^{-1}) in prewarmed (37 °C) medium. Cells were incubated with the indicated concentrations of the stimuli in the presence or absence of polymyxin B for 1.5 h, after which cells were harvested, and total RNA was isolated by use of the StrataPrep Total RNA Miniprep Kit (Stratagene) according to the manufacturer's protocol. TNF-α gene expression was quantified in a two-step reverse transcription-PCR (RT-PCR). In the RT step, cDNA was reverse transcribed from total RNA samples (0.625 μg/50 μL) by use of random hexamers from the TaqMan RT reagents (Applied Biosystems). In the PCR step, PCR products were synthesized from cDNA (11.25 ng/10 μL) by use of the Taqman universal PCR master mix and TaqMan PDARs for human TNF-α (Applied Biosystems). Measurements were carried out by use of the ABI Prism 7900 HT sequence detection system (Applied Biosystems), according to the manufacturer's protocols. As an endogenous control for these PCR quantification studies, 18S ribosomal RNA gene expression was measured by use of the TaqMan ribosomal RNA control reagents (Applied Biosystems). Results represent means ± SDs of quadruplicate measurements. Each experiment was repeated at least twice.

Data analysis: LPS concentration/response data for stimulation of TNF-α production in Mono Mac 6 cells were analyzed by nonlinear least-squares curve fitting in Prism (GraphPad Software, Inc.). These data were fitted with the following logistic equation:

$$Y = E_{max}/(1 + (EC_{50}/X)^{\text{Hill slope}}) \quad (1)$$

where Y is the TNF-α response, X is the LPS concentration, E_{max} is the maximum response, and EC_{50} is the concentration of LPS producing 50% stimulation.

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