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Further insights on structural modifications of muramyl dipeptides to study the human NOD2 stimulating activity

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Abstract: A series of muramyl dipeptide (MDP) analogues with structural modifications at the C4 position of MurNAc and on the D-*iso*-glutamine (*iso*Gln) residue of the peptide part were synthesized. The C4-diversification of MurNAc was conveniently achieved by using CuAAc click strategy to conjugate an azido muramyl dipeptide precursor with structurally diverse alkynes. D-Glutamic acid (Glu), replaced with *iso*Gln, was applied for the structural diversity through esterification or amidation of the carboxylic acid. In total, 26 MDP analogues were synthesized and bio-evaluated for the study of human NOD2 stimulation activity in the innate immune response. Interestingly, MDP derivatives with an ester moiety are found to be more potent than reference compound MDP itself or MDP analogues containing an amide moiety. Among the varied lengths of the alkyl chain in ester derivatives, the MDP analogue bearing the D-glutamate dodecyl (C12) ester moiety showed the best NOD2 stimulation potency.

Introduction

Peptidoglycans (PGNs) are the major components of bacterial cell walls. They comprise of a repeating disaccharide unit commonly known as *N*-acetyl glucosamine-(1→4)-*N*-acetyl-muramic acid, which is cross-linked by short peptide chains (in many Gram-positive bacteria the third amino acid is L-Lys; in most Gram-negative bacteria it is *meso*-DAP) to form the rigid structure of the cell wall.[1,2] The PGNs as well as these biomolecules are potential pathogen-associated molecular patterns (PAMPs), which can be recognized by pattern recognition receptors (PRRs) to induce immune response.[3,4] During bacterial infection, PGNs and their fragments are recognized by PRRs, located on either the membrane surface, like TLR2, NLRP3 and other peptidoglycan recognition proteins, or inside the cytoplasm, like Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and NOD1.[5–8] The *NOD2* gene is the first gene to be identified to associate with Crohn's disease (CD) susceptibility,[9] and recent studies revealed that functional loss of NOD2 due to mutations correlates with Crohn's disease, a chronic inflammatory gastrointestinal disease.[10,11]

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This intracellular NOD2 can be activated by PGN fragments to trigger innate immune responses through the NF-κB pathway, a ubiquitous transcription factor which induces expression of pro-inflammatory cytokines.[4,12–14] Structurally, *N*-acetyl muramyl dipeptide (MurNAc-L-Ala-D-*iso*-Gln, MDP, (**1**) [15] is the common motif shown in both Gram-positive and Gram-negative bacteria (Figure 1). The previous studies of the macrophage migration inhibition and adjuvant activity reported by Tanaka[15] showed that the structure, composition, and stereochemistry of two amino acids in DMP play an important role for activity. After that, MDP was found as one of NOD2 ligands and it was also claimed as the minimum structure required for the human NOD2 stimulation activity.[16–20] Several synthetic efforts to study NOD2 agonists focused on the non-sugar molecules[21–23] or diverse PGN-typed molecules with a longer glycan chain.[24–26]

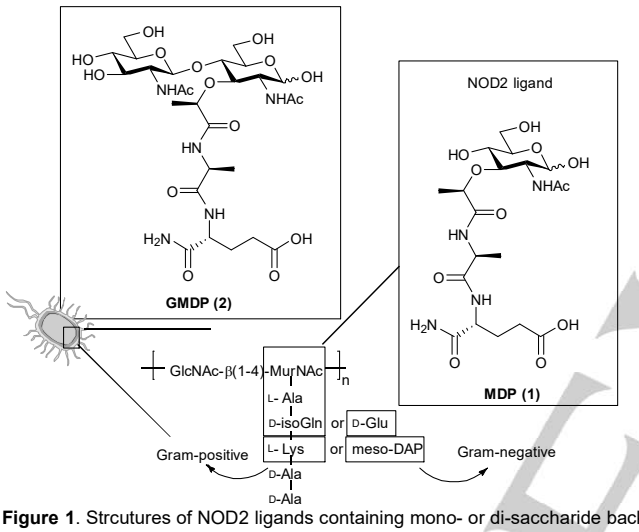


Figure 1. Strcutures of NOD2 ligands containing mono- or di-saccharide back-bone and their naturally occurring peptidoglycans of Gram-positive and Gram negative bacteria.

Earlier, our preliminary study revealed that the PGN disaccharide fragment, GlcNAc-MurNAc-L-Ala-D-*iso*-Gln (GMDP, **2**) is also a NOD2 ligand with a close activity profiling compared to MDP (see Figure S1). Similar observation was also reported by Fukase and Fujimoto.[26-28] Notably, they observed that the longer glycan chains of peptidoglycan analogues dramatically impair the NOD2 stimulation activity. The interesting activity of GMDP, which structurally contains the glycosyl moiety (GlcNAc) at the C4 position of MurNAc, prompted us to further investigate the effect of C4 substitution of MDP on NOD2 stimulation activity. Previous works demonstrated the modifications or the removal of the methyl group on the lactate of MurNAc and (or) on L-Ala of the peptide part in MDP could dramatically impair the activity by us and others (Figure 2).[27,28]

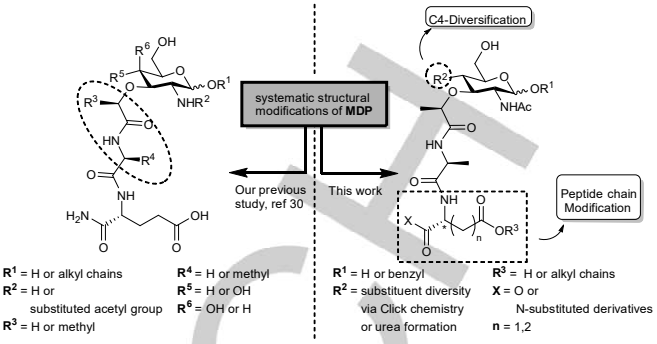
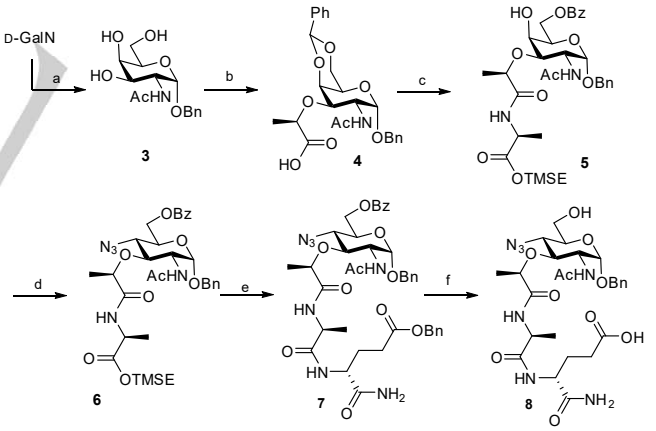


Figure 2. The scope of structural diversity of MDP analogues in this study and our previous work.[28]

To the best of our knowledge, the systematic investigation of the second amino acid, D-*iso*-Gln has not been extensively explored toward NOD2 activity. In order to have better understanding of the structure activity relationship of MDP-based analogues toward NOD2 stimulation activity, we decided to design and synthesize a series of muramyl dipeptide (MDP) analogues with structural modifications at the *C*4 position of MurNAc and also on *iso*-glutamine (*iso*Gln) in the peptide part. These MDP analogues were planned to be evaluated through the accessible NOD2 dependent assay.

Results and Discussion

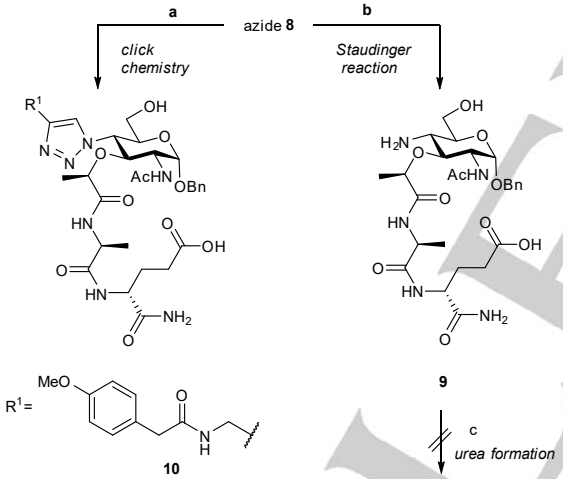


Scheme 1. Synthetic route for the preparation of azide **8**. *Reagents and conditions:* (a) i. NaOMe, Ac_2O, MeOH, 2 h, rt; ii. BnOH, HCl, 80 °C, 75% over 2 steps; (b) i. PhCHO, $ZnCl_2$, rt; ii. NaH, 2-(*S*)-chloropropionic acid, 1,4-dioxane, 70 °C, 73% over two steps; (c) i. H-Ala-OTMSE, HBTU, Et_3N, CH_2Cl_2/THF, rt; ii. *p*-TSA, MeOH, 60 °C, 0.5 h; ii. BzCl, pyridine, CH_2Cl_2, −78 °C to rt, 60% over 2 steps; (d) i. Tf_2O, pyridine, 0 °C, 1 h; ii. NaN_3, DMF, 60 °C, 24 h, 73% over two steps; (e) i. TBAF, THF, 30 min, rt; ii. D-*iso*Gln(OBn), HBTU, HOBT, DIPEA, CH_2Cl_2, rt, 4 h, 60% over two steps; (f) 2 N $LiOH_{(aq)}$, THF/MeOH=1:1, 50%.

As shown in scheme 1, benzyl *N*-acetyl-D-galactosamine **3**, was prepared from D-galactosamine (D-GalN) following the literature procedure.[29] After benzylidination of **3**, followed by *O*-alkylation at the *C*3-hydroxy position with 2-(*S*)-chloro-propionic acid under basic condition (NaH, 1,4-dioxane, 70 °C), acid **4** was obtained in 73% yield over two steps. Subsquently, **4** was

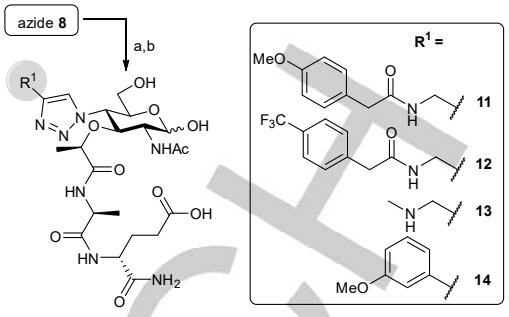
coupled with H-Ala-OTMSE in the presence of HBTU, followed by benzylidene acetal deprotection and selective *O*-benzoxylation at the *C*6-hydroxy group gave **5** in 60% yield over two steps. The Lattrell-Dax protocol[30] was utilized to convert alcohol **5** into azide **6** through the O-triflation in 73% yield over two steps. After TMSE deprotection of **6** with TBAF, the intermediate was coupled with γ-benzyl-D-isoglutamine in the presence of HBTU and HOBT to give **7** in 60% yield over two steps. Compound **7** was hydrolyzed under basic condition to afford azide **8** in moderate yield.

To achieve the desired C4-substituent diversity in MDP analogues initially conjugation with various alkyl and aryl isocyanates was planned. Towards this endeavor, the required amine precursor **9** was obtained from azide **8** *via* Staudinger reaction in 61% yield. Unfortunately, our attempt to conjugate amine **9** with an isocyanate such as 1-isocyanato-3-methoxybenzene, did not work well; presumably due to the C4 steric hindrance hampering the reactivity of amine **9**. To overcome this difficulty in C4 diversification, we adopted the copper (I)-catalyzed azide alkyne cycloaddition (CuAAC) strategy. Accordingly, when azide **8** was conjugated with a terminal alkyne, 2-(4-methoxyphenyl)-*N*-(prop-2-yn-1-yl)acetamide, smoothly generated required triazole **10** in 63% yield (Scheme 2).



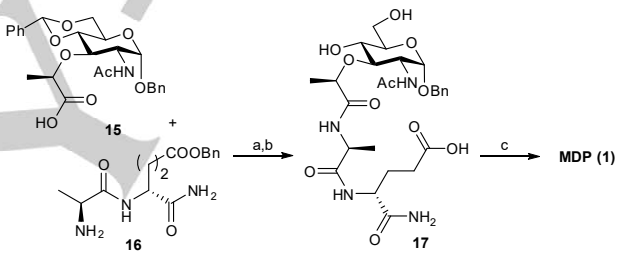
Scheme 2. Model studies of the C4 modification on MDP analogues. *Reagents and conditions:* (a) 2-(4-methoxyphenyl)-*N*-(prop-2-yn-1-yl) acetamide, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, $MeCN/H_2O$=1:1, 80 °C, 24 h; (b) PPh_3, THF/H_2O=3:1, 65°C, 24 h, 61%; (c) 1-isocyanato-3-methoxybenzene, DIPEA, CH_2Cl_2, 12 h, rt.

With this successful model reaction in hand, azide **8** was further subjected for parallel conjugation reactions with different readily prepared alkynes through the CuAAC protocol, followed by global deprotection to achieve the desired C4-substituted (alkyl, aryl, with amide group, and with secondary amine group) MDP analogues **11-14** in moderate to good yields as presented in scheme 3.



Scheme 3. Synthetic route of *C*4 diversified MDP **11** to **14** via click reaction. *Reagents and conditions:* (a) alkynes, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, $MeCN/H_2O$=1:1, 80 °C, 24 h; (b) H_2 (80 psi), $Pd(OH)_2$/C, MeOH, cat. AcOH, rt, 24 h.

The MDP analog **17** with the O-benzyl moiety at C-1 position was prepared *via* EDCI mediated amide coupling reaction in between acid **15**[31,32] and amine **16** and further debenzylation of **17** afforded MDP (**1**) in 55% yield over two steps as depicted in scheme 4.



Scheme 4. Preparation of MDP (**1**) *via* *C*1-OBn MDP (**17**). *Reagents and conditions:* (a) EDCI, DIPEA, CH_2Cl_2, rt; (b) i. TFA, CH_2Cl_2, rt, 30 min; ii. LiOH, H_2O, MeOH, rt, 2 h, 55% over three steps; (c) H_2 (80 psi), $Pd(OH)_2$/C, MeOH, cat. AcOH, rt, 16 h.

With these nine compounds (eight test compounds and one reference MDP (**1**) in hand), their bio-evaluation toward the human NOD2 stimulation activity was performed. As shown in figure 3, **17** with the *O*-benzyl moiety at the C1 alpha position dramatically lost the activity compared to MDP (**1**), suggesting that the orientation and substituted moiety at the C1 position might play an important role for the NOD2 stimulation activity. Indeed, the activity potency of **8**, **9**, and **10** were not attractive and azide **8** exhibited a weak response only at 1000 nM concentration. For the C4 structural modification, **10** and **11-14** indicated no NOD2 agonistic effect. These results implied that compounds with a substituted triazole moiety at the C4 position of MurNAc dramatically weaken the interactions or recognition with the NOD2 receptor. Although the triazole substitution at the C4 position of MDP impaired the NOD2 stimulation ability, our expeditious synthesis could be a useful approach for the structural diversification at the C4 position of MurNAc or other monosaccharide scaffolds via click chemistry.

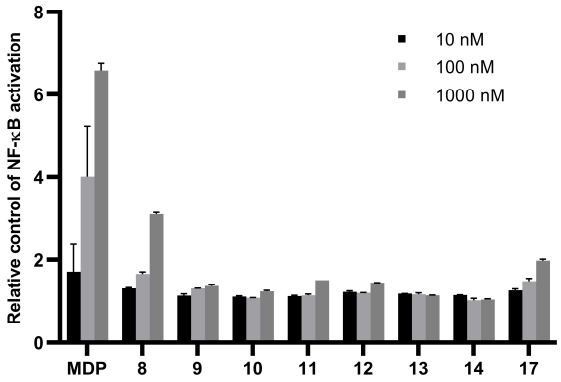


Figure 3. Stimulation of NOD2 by the C4 modified MDPs with a C4 substituted diveristy.

After analyzing these primary results, next we moved our attention to the structural modification of the dipeptide part on MDP as planned. Based on our structural design and the similar synthetic route in scheme **4**, six MDP analogues (**18-23,** Figure 4) were prepared. Notably, **15** was used as a common precursor, and protected dipeptides containing L-glutamic acid, D-glutamic acid or D-aspartic acid as building blocks were utilized.

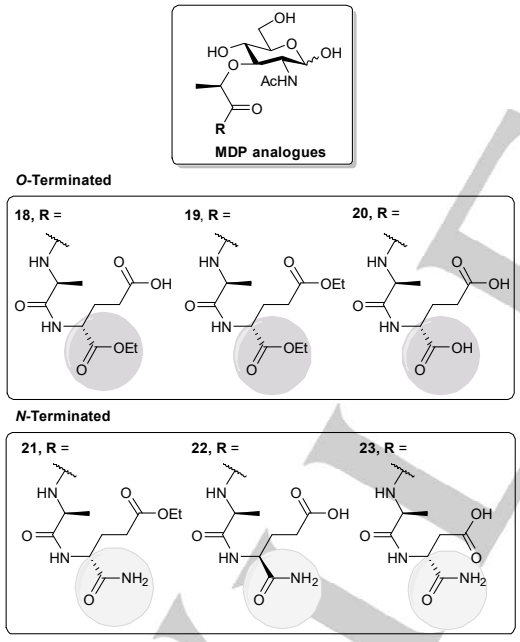


Figure 4. Structures of MDPs with modified dipeptides (**18-23**).

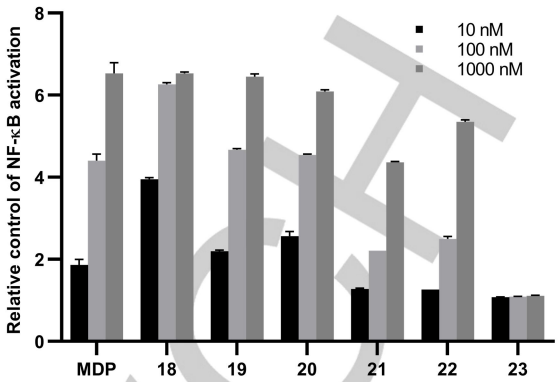


Figure 5. Stimulation of NOD2 by MDPs with modifications of isoglutamine **(18-23)**. HEK-Blue hNOD2 cells were incubated with different concentrations of MDP analogues for 16 h. SEAP were quantified as described in the Experimental Section. Data are presented as mean ± standard deviation (SD) (n = 3).

The biological results of MDP analogues **18-23** were shown in figure 5 and MDP (**1**) was applied as our reference compound. A brief summary was as follows: (1) When the D-chirality of *iso*-glutamine was changed to the L-form, the activity was found to be reduced (MDP (**1**) vs. **22**), and this result is consistent with early reported in the literature.[27] (2) The NOD2 stimulation activity dramatically lost for the MDP analogues possessing the shorter alkane spacer in the second amino acid from *iso*-glutamine to *iso*-asparagine (MDP (**1**) vs. **23**). (3) Analogue **21** bearing the *iso*-glutamine 5-ethyl ester moiety exhibited the less activity potency than MDP (**1**) with the free carboxylic acid of *iso*-glutamine. (4) The activity potency of MDP analogue **20** bearing glutamic acid manifested the similar results compared to MDP, which implied that the carboxylic acid might be a surrogate moiety of unsubstituted amide. (5) Gratifyingly, analogue **19** bearing the diethyl glutamate was found to be slightly more potent than MDP (**1**) at low concentration (10 nM and 100 nM), whereas analogue **18** (10 nM) with the only monoethyl ester moiety displayed a stronger activity than both MDP (**1**) and analogue **19** (Figure 5).

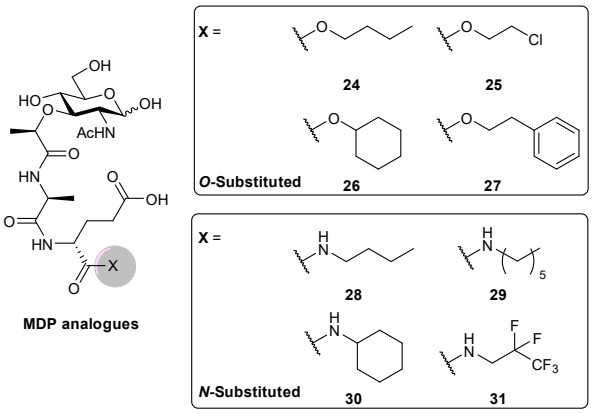


Figure 6. Structures of MDPs with a glutamate alkyl ester (**24-27**) or amide (**28-31**) moiety.

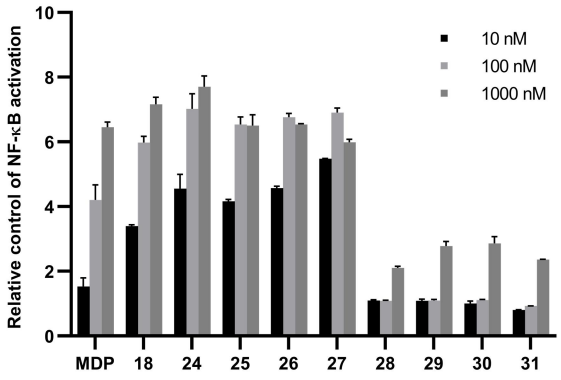


Figure 7. Stimulation of NOD2 by MDPs with a glutamate alkyl ester (**24-27**) or amide (**28-31**) moiety.

These exciting results (Figure 5), especially the structures of active hits (**18** and **19**), inspired us for further study the structural modifications of MDPs. Following the similar synthetic approaches we described above, eight MDP analogues including four *O*-substituted derivatives and four *N*-monosubstituted derivatives (analogues **24**-**31**, Figure 6) were prepared. The bio-evaluation results are shown in figure 7. Compared to **18**, MDP analogue **24** with a longer alkyl chain in the *O*-substituted glutamate ester demonstrated a better activity. In contrast, MDP analogue **25** with the chlorinated ethyl ester did not significantly increase the activity. MDP analogue **26** with a cyclohexane moiety or **27** with a phenethyl group presented the similar activity potency with analogue **19** even at higher concentrations (100 nM and 1000 nM). However, at the concentration of 10 nM, analogues **26** and **27** displayed slightly better activity potency than **18**. Notably, analogues **25**, **26**, and **27** exhibited stronger potency than reference compound MDP (**1**), even at a lower concentration (10 nM). Surprisingly, MDP analogues which structurally contain a *N*-mono-substituted amide moiety including a varied alkyl chain (**28** and **29**), a cyclohexyl moiety (**30**), and a fluorine-containing alkane (**31**) were found to be inactive and these compounds illustrated some weak activities only at a higher concentration (1000 nM). Our observations indicated this one atom difference (O vs. N) in structurally similar MDP analogue pairs such as **24** vs. **28** or **26** vs. **30** dramatically affected the NOD2 stimulation activity for unknown reasons. Further studies such as their stability or permeability test remained to be investigated in the future.

These interesting results encouraged us to pursue further study by preparing four more MDP analogues (**32**-**35**, Figure 8) containing a glutamate ester moiety with a varied carbon alkyl chain length (C8-**32**, C12-**33**, C16-**34**, and C20-**35**). Our biological results are displayed in figure 9. Impressively, at the lower concentration (10 nM), MDP (**1**) and its accessible analogue, Murabutide (MB, MurNAc-L-Ala-D-GlnOBu)[33] were found to be inactive but our newly synthesized MDP analogues with an alkyl ester moiety presented increased activity potency. All the MDP analogues (**18**, **24**, **32-35**) were more active than MDP (**1**) and MB. In addition, we observed that MDP analogues bearing a longer alkyl chain lead to the better activity and the compound possessing the C12 alkyl chain reached to the best possible potency. The similar trend of the NOD2 stimulation activity was also observed at 100 nM concentration. Notably, the activity potency of **34** and **35** did not demonstrated a clear trend; presumably due to the hydrophobic property of the longer alkyl chain (C16 and C20) of **34** and **35** (1000 nM) might be inducing some non-specific interactions or even leading to aggregation.

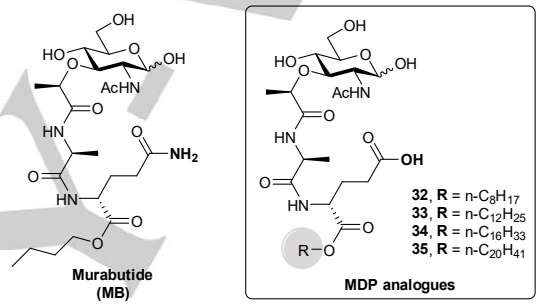


Figure 8. Chemical structures of MDPs with a glutamate alkyl ester moiety (**32-35**) and Murabutide (MB) as a reference compound.

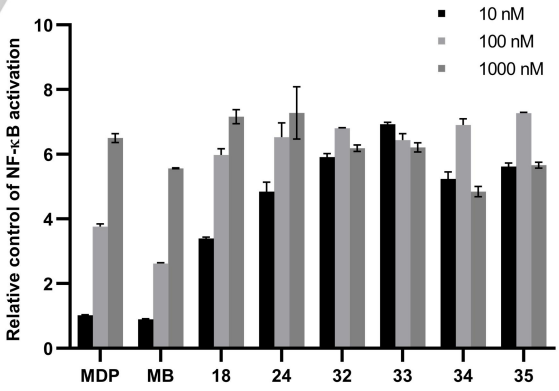


Figure 9. Stimulation of NOD2 by MDPs with a glutamate alkyl ester (**32-35** moiety. (MDP (**1**), MB, **18,** and **24** are reference compounds as control.)

Next, we took advantage of recent reports of the crystal structure of NOD2 to study the putative binding mode of **33** through our computational modeling.[34] The concave surface of NOD2^{LRR} (comprising of R823, F851, R877, G879, G905, W907, W931, S933, V935, E959, K989, S991 and C961 residues) is required for MDP recognition.[23,35–37] As displayed in Figure 10, we observed both **1** and **33** are interacting in a similar fashion. In a similar line with previous predictions,[23,36] we observed the MurNAc moiety of **1**/**33** found in a hydrophobic pocket (F851, W907 and W931 residues). The L-Ala-D-iGln part was found interacting with a positively charged pocket that involves R823, R877 and K989 residues. Further, in case of **33**, the C12 tail part was found to be placed in a hydrophobic pocket (F903, Y821, K847 A875, and W911 residues).

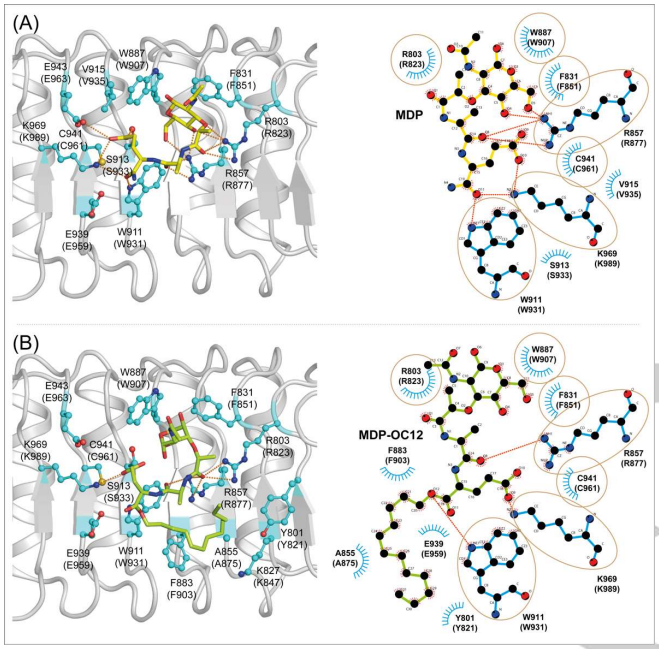


Figure 10. Predicted binding mode of (A) MDP (**1**) and (B) **33** on the concave surface of NOD2^{LRR}. Left panel indicates the 3D model interaction model, where the protein is visualized in gray cartoon, key interacting residues in ball-stick model (cyan) and the ligands are displayed in colored sticks models (**1**; yellow and **33**; lime). Right panel indicates the interaction of **1**/**33** with NOD2^{LRR} in 2D, where the key H-bond forming residues, **1** and **33** are displayed in line-art model, and the residues with hydrophobic interactions are showed in half-circles. The polar contacts or H-bonds are indicated in orange dashed lines and residues in parenthesis are of human NOD2.

Conclusions

We have successfully performed extensive structural modifications of MDP at the C4 position of MurNAc and on *iso*-glutamine (*iso*Gln) residue of the peptide part. A convenient method for the diversification at the C4 position of MurNAc was developed through a CuAAC click strategy to conjugate an azido muramyl dipeptide precursor with a wide range of structurally diverse alkynes. Although the substituted triazole moiety at the C4 position of MDP analogues did not exhibit promising results for NOD2 stimulation activity, our strategy could be useful for structural diversity of MurNAc or other monosaccharide analogues towards other research purposes or applications. Besides these studies, we also performed the systematic modifications of the *iso*-glutamine (*iso*Gln) moiety in MDP analogues, applied for the human NOD2 stimulation activity in the innate immune response. Delightfully, MDP derivatives with a substituted ester moiety are observed to be more potent than the reference compounds (MDP and MB) and the other MDP analogues containing an amide moiety. Interestingly, the general trend manifested MDP analogues bearing a longer alkyl chain in the D-glutamate ester moiety led to the better NOD2 stimulation activity. Among our synthetic MDP analogues, the most potent NOD2 activator was the MDP analogue bearing the D-glutamate dodecyl (C12) ester moiety. This new finding and new NOD2 activators would allow us to use them as chemical probes or ligands for further studies in the innate immune system, cytokine expression pattern, or autophagy research in the future. These results will be reported in due course.

Experimental Section

General Information

All solvents and reagents were obtained commercially and used without further purification. All reactions were monitored by analytical thin-layer chromatography (TLC) plates with silica gel 60 F_{254}. TLC plates were visualized by the exposure of ultraviolet light at 254 nm or by the immersion of staining solution (*p*-anisaldehyde, acidic ninhydrin, phosphomolybdic acid, or potassium permanganate) followed by heating. After the completion of all reactions, solvents were removed by rotary evaporation. The crude products were purified by column chromatography (cc) with 40–63 μm silica gel. NMR spectra were recorded by the Bruker AVANCE 600 spectrometer at ambient temperature. ^{1}H NMR spectra were referenced with deuterated solvents such as chloroform-*d* (δ=7.26), methanol-d_4 (δ=3.31), and deuterium oxide (δ=4.79). ^{13}C NMR were referenced with chloroform-*d* (δ=77.23 ppm of central line) and methanol-d_4 (δ=49.15 ppm of central line). High-resolution mass spectra were obtained by the Bruker Daltonics BioTOF III spectrometer (ESI-MS). HEK-Blue hNOD2 cells were incubated with different concentrations of MDP analogues for 16 h. SEAP (secreted alkaline phosphatase) were quantified as described in supporting information. Data are presented as mean ± standard deviation (SD) (n = 3). Synthetic procedures (**3**→**7** and **15**→**17**) and preparation of dipeptides (**S1**-**S17**) are shown in the supporting information.

Compound 8. $LiOH_{(aq)}$ (2.0 N, 0.47 mL, 0.94 mmol) was added to the solution of **7** (0.15 g, 0.19 mmol) in MeOH/THF (10 mL, v/v = 1:1). After 1 h, the mixture was neutralized by Dowex 50WX8 resin, and then the filtrate was concentrated. The crude product was purified by cc (*n*-PrOH/H_2O = 40:1 to 20:1, silica gel) to give **8** (72 mg, 0.12 mmol, 63%) as a white solid; ^{1}H NMR (600 MHz, D_2O) δ 7.35-7.28 (m, 5H), 4.78 (d, *J* = 3.5 Hz, 1H), 4.61 (d, *J* = 12.0 Hz, 1H), 4.43 (d, *J* = 12.0 Hz, 1H), 4.22-4.10 (m, 3H), 4.02-3.98 (m, 1H), 3.79-3.75 (m, 1H), 3.72-3.71 (m, 2H), 3.63-3.55 (m, 2H), 2.22-2.15 (m, 2H), 2.09-2.02 (m, 1H), 1.86-1.81 (m, 4H), 1.39-1.32 (m, 6H); ^{13}C NMR (150 MHz, D_2O) δ178.0, 175.8, 175.3, 174.4, 173.7, 136.8, 128.7, 128.6, 96.0, 95.8, 78.7, 78.0, 70.5, 69.7, 61.1, 60.6, 53.4, 52.7, 49.5, 31.2, 21.83, 21.80, 18.3, 18.2, 16.7, 16.6; HRMS (ESI-TOF) *m/z* calcd for $C_{26}H_{37}N_7O_{10}$ + H^+: 608.2675 $[M+H]^+$; found: 608.2676.

Compound 9. Triphenylphosphine (17 mg, 0.066 mmol) was added to the solution of **8** (20 mg, 0.033 mmol) in THF/H_2O (1 mL, v/v = 3:1), and the reaction was refluxed at 65 °C. After 24 h, the reaction mixture was concentrated. The residue was purified by cc (*n*-PrOH/H_2O = 20:1 to 7:1, silica gel) to afford **9** (15 mg, 0.026 mmol, 78%) as a white solid; ^{1}H NMR (600 MHz, D_2O) δ 7.37-7.32 (m, 5H), 4.80 (s, 1H), 4.65 (d, *J* = 11.9 Hz, 1H), 4.49 (d, *J* = 11.9 Hz, 1H), 4.14-4.08 (m, 3H), 4.06-4.04 (m, 2H), 3.91-3.89 (m, 2H), 3.73 (d, *J* = 3.7 Hz, 2H), 3.35-2.98 (m, 1H), 2.21-2.13 (m, 2H), 2.03-1.99 (m, 1H), 1.86-1.80 (m, 1H), 1.77 (s, 3H), 1.38 (s, 3H), 1.36 (s, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.5, 178.3, 177.7, 175.3, 173.8, 136.7, 128.7, 128.6, 128.4, 96.0, 77.6, 77.4, 69.8, 60.5, 54.5, 54.3, 53.3, 53.2, 51.5, 49.6, 49.5, 31.4, 31.3, 27.8, 27.7, 21.8, 18.8, 18.7, 16.6, 16.3; HRMS (ESI-TOF) *m/z* calcd for $C_{26}H_{39}N_5O_{10}$ + H^+: 582.2770 [*M*+H]$^+$; found: 582.2772.

Compound 10. *N*,*N*-Diisopropylethylamine (0.42 mL, 2.4 mmol) was added to the mixture of propargylamine (0.13 mL, 2.0 mmol), 4-methoxyphenylacetic acid (2.0 mmol), and EDCI (0.46 g, 2.4 mmol) in dry CH_2Cl_2 (5.0 mL). The mixture was stirred at rt. After 12 h, the mixture was quenched and washed with 1.0 N $HCl_{(aq)}$, and the organic layer was collected, dried over magnesium sulfate, and concentrated. The residue was directly applied for next step without further purification. The mixture of the residue (20 mg, 0.099 mmol), $CuSO_{4(aq)}$ (1.0 M, 3.3 μL, 0.0033 mmol) and sodium ascorbate$_{(aq)}$ (1.0 M, 9.9 μL, 0.0099 mmol) was added to the solution of **8** (20 mg, 0.033 mmol) in MeCN/H_2O (0.33 mL, v/v = 1:1). The reaction mixture was stirred at 80 °C. After 24 h, the reaction mixture was concentrated and purified by cc (*n*-PrOH/H_2O = 20:1 to 7:1, silica gel) to afford **10** (17 mg, 0.021 mmol, 63%) as a white solid; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.86 (d, 1H, *J* = 6.6 Hz), 7.47-7.41 (m, 5H), 7.19 (d, 2H, *J* = 7.8 Hz), 6.92 (d, *J* = 8.4 Hz, 2H), 5.0 (s, 1H), 4.81 (s, 1H), 4.70 (t, *J* = 9.6 Hz, 1H), 4.63 (d, *J* = 12.0 Hz, 1H), 4.41 (s, 1H), 4.44-4.40 (m, 1H), 4.26-4.20 (m, 4H), 3.78 (s, 3H), 3.71-3.66 (m, 1H), 3.51 (s, 2H), 3.45 (d, *J* = 12.6 Hz, 1H), 3.16 (dd, *J* = 12.6, 3.6 Hz, 1H), 2.27-2.10 (m, 3H), 1.88 (d, *J* = 10.2 Hz, 3H), 1.38-1.36 (m, 3H), 0.44-0.41 (m, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 178.1, 178.0, 176.3, 174.7, 174.6, 174.0, 173.9, 173.8, 157.9, 145.1, 136.7, 130.3, 130.2, 128.7, 128.7, 128.4, 127.3, 124.8, 114.3, 96.2, 96.2, 78.2, 78.1, 77.8, 70, 70, 60.9, 59.4, 55.3, 53.6, 53.3, 49.4, 49.2, 41.4, 34.2, 31.2, 37.3, 27.2, 21.8, 17.4, 16.8, 16.6; HRMS (ESI-TOF) *m/z* calcd for $C_{38}H_{50}N_8O_{12}$ + H^+: 811.3621 [*M*+H]$^+$; found: 811.3640.

Compound 11. A mixture of **10** (7.0 mg, 0.009 mmol), $Pd(OH)_2$/C (3.5 mg) and AcOH (5.2 μL, 0.09 mmol) in MeOH (0.90 mL) was stirred under hydrogen (80 psi). After 24 h, the reaction was filtered through a pad of celite, and then concentrated. The residue was purified by cc (*n*-PrOH/H_2O = 20:1 to 5:1, silica gel) to give **11** (3.5 mg, 0.005 mmol, 56% yields) as a white solid; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.25β) δ 7.86-7.84 (m, 1H), 7.16-7.14 (m, 2H), 6.89 (d, *J* = 8.4 Hz, 2H), 5.20 (d, *J* = 3.2 Hz, 1H), 4.43-4.37 (m, 2H), 4.23-4.03 (m, 4H), 3.77-3.68 (m, 4H), 3.49-3.42 (m, 3H), 3.18-3.14 (m, 1H), 2.21-2.12 (m, 2H), 2.07-1.99 (m, 1H), 1.91-1.90 (m, 3H), 1.85-1.81 (m, 1H), 1.36-1.33 (m, 3H), 0.41-0.36 (m, 3H). ^{13}C NMR (150 MHz, D_2O) δ 178.6, 177.5, 175.2, 174.5, 173.4, 160.1, 131.2, 128.8, 126.3, 126.1, 115.0, 92.9, 78.7, 78.6, 78.2, 71.1, 62.9, 61.5, 56.2, 55.7, 50.5, 42.8, 35.8, 33.0, 32.7, 30.7, 30.3, 29.9, 23.7, 22.8, 18.5, 18.4, 17.8, 14.5; HRMS (ESI-TOF) *m/z* calcd for $C_{28}H_{39}N_7O_{11}$ + H^+: 721.3151 [*M*+H]$^+$; found: 721.3162.

Compound 12. The reaction was carried out as described above in **10**, followed the reaction which was carried out as **11**. The final product **12** (8.0 mg, 0.011mmol, 33% over three steps) was obtained from 4-(Trifluoromethyl)phenylacetic acid; ^{1}H NMR (600 MHz, D_2O) δ 8.15-8.01 (m, 1H), 7.75-7.60 (m, 2H), 7.55-7.44 (m, 2H), 5.29 (s, 1H), 4.62-4.49 (m, 4H), 4.95-4.27 (m, 7H), 3.78-3.66 (m, 6H), 3.51 (d, *J* =11.9 Hz, 1H), 3.23-3.21 (m, 2H), 2.91-2.87 (m, 1H), 2.27-1.98 (m, 2H), 1.91-1.90 (m, 1H), 1.43-1.41 (m, 6H), 0.45-0.42 (m, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.2, 174.6, 174.5, 173.9, 173.7, 173.5, 139, 129.5, 129.5, 128.7, 128.6, 125.6, 91.3, 78.1, 77.6, 70.1, 69.5, 61.0, 59.6, 54.5, 54.3, 54.1, 49.5, 42, 34.6, 34.2, 31.4, 27.9, 22.0, 21.9, 21.8, 20.0, 20.0, 17.3, 16.9; HRMS (ESI-TOF) *m/z* calcd for $C_{31}H_{41}F_3N_8O_{11}$ + H^+: 759.2920 [*M*+H]$^+$; found: 759.2932.

Compound 13. *N*-methylpropargylamine (8.4 μL, 0.099 mmol), $CuSO_{4(aq)}$ (1.0 M, 3.3 μL, 0.0033 mmol) and sodium ascorbate$_{(aq)}$ (1.0 M, 9.9 μL, 0.0099 mmol) was added to the solution of **8** (20 mg, 0.033 mmol) in MeCN/H_2O (0.33 mL, v/v = 1:1). The reaction mixture was stirred at 80 °C. After 24 h, the reaction mixture was concentrated to give the residue, followed the reaction which was carried out as described above in **11**. The final product **13** (10 mg, 0.017 mmol, 43% over two steps) was obtained; ^{1}H NMR (600 MHz, D_2O) δ 8.62-8.41 (m, 1H), 5.27 (s, 1H), 4.74-4.71 (m, 1H), 5.05-4.89 (m, 2H), 4.40-3.91 (m, 9H), 3.64-3.62 (m, 1H), 3.40-3.39 (m, 1H), 3.08-2.90 (m, 2H), 2.86-2.83 (m, 3H), 2.36-2.12 (m, 3H), 2.12-2.09 (m, 2H), 1.99 (s, 3H), 1.93-1.87 (m, 2H), 1.42 (s, 3H), 1.41 (s, 3H), 0.76-0.74 (m, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.5, 178.3, 177.6, 177.4, 173.9, 173.6, 149.7, 94.7, 91.3, 89.6, 77.5, 69.5, 69.2, 68.4, 67.6, 64.4, 62.9, 59.8, 54.5, 54.4, 52, 50.7, 49.4, 49.3, 41.5, 41.4, 31.5, 31.4, 27.8, 27.6, 23.2, 22.0, 21.8, 19.5, 17.4, 17.3, 16.9, 16.8, 16.7, 16.5; HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{38}N_8O_{10}$ + H^+: 587.2784 [*M*+H]$^+$; found: 587.2795.

Compound 14. The reaction was carried out as described above in **13**. The final product **14** (13 mg, 0.020 mmol, 48% over two steps) was obtained from 1-methoxy-3-(propyl-2-yn-1-yl)benzene; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.60β) δ 8.54 (s, 1H), 7.43-7.38 (m, 3H), 7.01 (d, *J* = 7.3 Hz, 1H), 5.25 (d, *J* = 3.4 Hz, 1H), 4.11-4.09 (m, 1H), 3.87-3.84 (m, 4H), 3.72-3.68 (m, 1H), 3.59-3.54 (m, 1H), 3.36-3.33 (m, 1H), 2.18-2.07 (m, 2H), 2.04-1.98 (m, 1H), 1.92 (s, 3H), 1.81-1.76 (m, 1H), 1.36-1.33 (m, 3H), 0.61-0.57 (m, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.3, 174.8, 173.81, 159.5, 130.7, 118.6, 114.8, 111.1, 91.4, 78.0, 77.5, 69.6, 61.2, 59.8, 55.5, 54.3, 54.2, 50.5, 49.7, 31.3, 27.8, 22.1, 21.9, 17.3, 16.8, 16.1; HRMS (ESI-TOF) *m/z* calcd for $C_{28}H_{40}N_7O_{11}$ + H^+: 650.2780 [*M*+H]$^+$; found: 650.2790.

Compound 18. A mixture of **15** (1.5 eq), dipeptide **S1** (1.0 eq), HBTU (2.0 eq), DIPEA (3.0 eq) was stirred in CH_2Cl_2 at rt. After 8 h, the reaction was washed with 1.0 N $HCl_{(aq)}$, sat. $NaHCO_{3(aq)}$, and water, subsequently. The organic layer was collected, dried over magnesium sulphate, and purified by cc (CH_2Cl_2/MeOH = 70:1, silica gel) to give a fully-protected MDP. The MDP was dissolved in the solution of CH_2Cl_2/TFA (v/v = 3:1), and stirred at 0 ºC. After 30 min, the reaction mixture was concentrated to get residue. A resuspension of the residue, $Pd(OH)_2$ /C (20% w/w), and AcOH was stirred under hydrogen gas (80 psi). After 16 h, the reaction mixture was filtered through a pad of celite, and the filtrate was concentrated. Subsequently, the crude product was purified by cc to afford the final compound **18** (180 mg, 36% over 3 steps); ^{1}H NMR (600 MHz, CD_3OD) (Anomers-1.00α : 0.30β) δ 5.16 (d, *J* = 3.2 Hz, 1H), 4.45-4.35 (m, 3H), 4.19-4.16 (m, 2H), 3.85 (dd, *J* = 10.4, 3.2 Hz, 1H), 3.82-3.67 (m, 4H), 3.50-3.44 (m, 1H), 2.36-2.35 (m, 2H), 2.22-2.14 (m, 1H), 1.96-1.93 (m, 4H), 1.41-1.37 (m, 6H), 1.27 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 176.6, 176.3, 174.8, 174.5, 173.74, 173.71, 173.2, 172.1, 171.7, 96.0, 91.0, 81.4, 78.6, 76.9, 76.7, 76.5, 71.8, 70.4, 70.1, 61.3, 61.2, 61.1, 61.0, 56.7, 54.2, 52.22, 52.20, 49.0, 48.9, 30.8, 26.6, 26.3, 21.7, 21.5, 20.0, 18.3, 18.2, 17.0, 16.9, 13.1; HRMS (ESI-TOF) *m/z* calcd for $C_{21}H_{35}N_3O_{12}$ + Na^+: 544.2113 [*M*+Na]$^+$; found: 544.2121.

Compound 19. The reaction was carried out as described above in **18**. The final product, **19** (160 mg, 29% over 3 steps) was obtained from **S16**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.50β) δ 5.15 (d, *J* = 3.5 Hz, 1H), 4.44-4.41 (m, 1H), 4.31-4.25 (m, 2H), 4.22-4.13 (m, 4H), 3.95 (dd, *J*

= 10.5, 3.5 Hz, 1H), 3.88-3.67 (m, 3H), 3.59-3.45 (m, 2H), 2.49-2.41 (m, 2H), 2.26-2.20 (m, 1H), 2.01-1.96 (m, 4H), 1.43-1.41 (m, 3H), 1.38-1.36 (m, 3H), 1.24 (q, *J* = 14.7, 7.3 Hz, 6H); ^{13}C NMR (150 MHz, D_2O) δ 175.7, 175.4, 175.04, 175.02, 174.9, 174.2, 173.9, 172.9, 94.9, 90.9, 82.5, 79.5, 78.0, 77.7, 75.7, 71.4, 68.9, 68.7, 62.7, 61.8, 60.6, 60.4, 56.1, 53.7, 51.9, 49.6, 49.5, 46.1, 46.0, 30.1, 25.94, 25.90, 25.6, 22.2, 21.9, 18.6, 16.7, 13.3, 13.2; HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{39}N_3O_{12}$ + Na^+: 572.2426 [*M*+Na]$^+$; found: 572.2428.

Compound 20. Lithium hydroxide (2.0 N) was added to the solution of **19** in THF. After stirring for 1 h, the mixture was neutralized with Dowex, filtered, and concentrated. The crude product was purified by cc to afford **20** (29 mg, 34%) as a white solid; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.60β) δ 5.14 (d, *J* = 3.5 Hz, 1H), 4.31-4.19 (m, 3H), 3.94 (dd, *J* = 10.5, 3.5 Hz, 1H), 3.92-3.68 (m, 3H), 3.58-3.45 (m, 2H), 2.30-2.23 (m, 2H), 2.13-2.05 (m, 1H), 1.95 (s, 3H), 1.91-1.85 (m, 1H), 1.43-1.41 (m, 3H), 1.38-1.36 (m, 3H). ^{13}C NMR (150 MHz, D_2O) δ 179.8, 177.9, 175.7, 174.2, 174.0, 94.9, 90.9, 82.3, 79.5, 78.0, 77.8, 75.6, 71.5, 69.0, 60.4, 56.0, 54.4, 53.6, 49.6, 32.1, 27.8, 21.9, 18.6, 16.9; HRMS (ESI-TOF) *m/z* calcd for $C_{19}H_{31}N_3O_{12}$ + Na^+: 516.1800 [*M*+Na]$^+$; found: 516.1803.

Compound 21. The reaction was carried out as described above in **18**. The final product, **21** (58 mg, 40% over 3 steps) was obtained from **S14**; ^{1}H NMR (600 MHz, CD_3OD) (Anomers-1.00α : 0.20β) δ 5.16 (d, *J* = 3.4 Hz, 1H), 4.39-4.33 (m, 2H), 4.29-4.26 (m, 1H), 4.13 (q, *J* = 7.1 Hz, 2H) 3.87 (dd, *J* = 10.4, 3.2 Hz, 1H), 3.82-3.78 (m, 2H), 3.72 (dd, *J* = 11.9, 5.2 Hz, 1H), 3.63 (dd, *J* = 10.4, 8.9 Hz, 1H), 3.49 (t, *J* = 9.5 Hz, 1H), 2.43-2.40 (m, 2H), 2.25-2.10 (m, 1H), 1.95-1.88 (m, 4H), 1.40-1.38 (m, 6H), 1.25 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 175.2, 174.9, 174.0, 173.0, 172.1, 91.0, 79.0, 76.7, 71.8, 70.2, 60.3, 54.1, 52.3, 49.4, 30.1, 26.5, 21.5, 18.3, 16.3, 13.1; HRMS (ESI-TOF) *m/z* calcd for $C_{21}H_{36}N_4O_{11}$ + Na^+: 543.2273 [*M*+Na]$^+$; found: 543.2289.

Compound 22. The reaction was carried out as described above in **18**. The final product, **22** (94 mg. 31% over 3 steps) was obtained from **S15**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.50β) δ 5.18 (d, *J* = 3.5 Hz, 1H), 4.34-4.19 (m, 4H), 3.98 (dd, *J* = 10.5, 3.5 Hz, 1H), 3.90-3.71 (m, 4H), 3.62-3.50 (m, 2H), 2.35-2.32 (m, 2H), 2.16-2.06 (m, 1H), 2.00-1.94 (m, 4H), 1.46-1.39 (m, 6H); ^{13}C NMR (150 MHz, D_2O) δ 178.6, 177.7, 175.8, 175.6, 174.2, 174.1, 173.99., 173.96, 94.9, 90.9, 82.5, 79.6, 79.5, 78.1, 77.8, 75.7, 71.5, 69.0, 68.8, 60.7, 60.5, 56.1, 54.5, 54.3, 53.7, 49.6, 49.5, 31.52, 31.50, 27.8, 22.2, 22.0, 18.7, 18.6, 16.8, 16.5; HRMS (ESI-TOF) *m/z* calcd for $C_{19}H_{32}N_4O_{11}$ + Na^+: 515.1960 [*M*+Na]$^+$; found: 515.1944.

Compound 23. The reaction was carried out as described above in **18**.The final product, **23** (76 mg, 48% over 3 steps) was obtained from **S17**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.50β) δ 5.15 (d, *J* = 3.4 Hz, 1H), 4.55 (t, *J* = 12.7, 6.4 Hz, 1H), 4.30-4.23 (m, 2H), 3.97 (dd, *J* = 10.4, 3.4Hz, 1H), 3.91-3.69 (m, 4H), 3.59-3.49 (m, 2H), 2.66 (d, *J* = 6.4 Hz, 2H), 1.98 (s, 3H), 1.43-1.38 (m, 6H); ^{13}C NMR (150 MHz, D_2O) δ 177.7, 176.2, 175.9, 174.61, 174.60, 174.2, 173.9, 94.8, 90.94, 90.92, 82.4, 79.6, 78.01, 78.0, 77.72, 77.71, 75.6, 71.4, 68.8, 68.7, 60.6, 60.5, 56.1, 53.7, 51.0, 49.70, 49.69, 38.3, 22.2, 22.0, 18.7, 18.4; HRMS (ESI-TOF) *m/z* calcd for $C_{18}H_{30}N_4O_{11}$ + Na^+: 501.1803 [*M*+Na]$^+$; found: 501.1792.

Compound 24. The reaction was carried out as described above in **18**. The final product, **24** (20 mg, 13% over 3 steps) was obtained from **S2**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.50β) δ 5.16 (d, *J* = 3.5 Hz, 0.6H), 4.66 (d, *J* = 8.5 Hz, 0.3H), 4.41 (dd, *J* = 9.2, 5.3 Hz, 1H), 4.33 – 4.20 (m, 2H), 4.16 (t, *J* = 6.5 Hz, 2H), 3.98 – 3.44 (m, 6H), 2.43 – 2.37 (m, 2H), 2.23 – 2.15 (m, 1H), 2.05 – 1.93 (m, 4H), 1.66 – 1.59 (m, 2H), 1.42 (dd, *J* = 7.3, 4.3 Hz, 3H), 1.39 – 1.31 (m, 5H), 0.89 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.03, 177.99, 175.7, 175.4, 174.9, 174.2, 173.9, 173.2, 94.9, 90.9, 82.4, 79.4, 78.0, 77.7, 75.7, 71.4, 68.9, 68.7, 66.3, 60.6, 60.4, 56.1, 53.7, 52.3, 49.54, 49.50, 30.8, 29.7, 25.94, 25.91, 22.2, 22.0, 21.9, 18.6, 18.4, 16.77, 16.76, 12.8; HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{39}N_3O_{12}$ + Na^+: 572.2426 [*M*+Na]$^+$; found: 572.2425.

Compound 25. The reaction was carried out as described above in **18**. The final product, **25** (20 mg, 14% over 3 steps) was obtained from **S9**;

^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.47β) δ 5.16 (d, *J* = 3.5 Hz, 0.64H), 4.66 (d, *J* = 8.5 Hz, 0.30H), 4.46 – 4.42 (m, 3H), 4.32 – 4.22 (m, 2H), 3.95 – 3.48 (m, 8H), 2.36 – 2.33 (m, 2H), 2.21 – 2.19 (m, 1H), 2.02 – 1.97 (m, 4H), 1.43 (dd, *J* = 7.3, 4.4 Hz, 3H), 1.37 (t, *J* = 6.1 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 179.62, 179.58, 175.7, 175.4, 175.0, 174.2, 174.0, 172.7, 94.9, 90.8, 82.4, 79.4, 78.0, 77.7, 75.7, 71.5, 69.0, 68.7, 65.6, 60.4, 56.1, 53.7, 52.49, 52.47, 49.54, 49.50, 41.8, 32.1, 26.5, 26.4, 22.2, 21.9, 18.6, 16.8, 16.5; HRMS (ESI-TOF) *m/z* calcd for $C_{21}H_{34}ClN_3O_{12}$ + H^+: 556.1904 [*M*+H]$^+$; found: 556.1905.

Compound 26. The reaction was carried out as described above in **18**. The final product, **26** (40 mg, 35% over 3 steps) was obtained from **S7**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.48β) δ 5.16 (d, *J* = 3.5 Hz, 0.63H), 4.66 (d, *J* = 8.5 Hz, 0.30H), 4.35 – 4.22 (m, 3H), 3.95 – 3.49 (m, 6H), 2.38 – 2.28 (m, 2H), 2.21 – 2.10 (m, 1H), 2.06 – 1.92 (m, 4H), 1.85 – 1.75 (m, 2H), 1.74 – 1.61 (m, 2H), 1.53 – 1.45 (m, 3H), 1.42 (dd, *J* = 7.2, 4.1 Hz, 3H), 1.40 – 1.24 (m, 6H). ^{13}C NMR (150 MHz, D_2O) δ 179.5, 179.4, 175.6, 175.3, 174.9, 174.2, 173.9, 172.8, 94.9, 90.8, 82.3, 79.3, 78.0, 77.6, 75.7, 75.3, 71.5, 69.0, 68.7, 60.6, 60.4, 56.1, 53.7, 53.0, 52.89, 52.87, 49.5, 49.4, 49.0, 32.4, 31.9, 30.69, 30.65, 30.61, 26.6, 26.4, 26.3, 24.7, 22.9, 22.8, 22.2, 21.9, 18.59, 18.57, 16.8, 16.5; HRMS (ESI-TOF) *m/z* calcd for $C_{25}H_{41}N_3O_{12}$ + H^+: 576.2763 [*M*+H]$^+$; found: 576.2761.

Compound 27. The reaction was carried out as described above in **18**. The final product, **27** (32 mg, 21% over 3 steps) was obtained from **S8**. ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.51β) 7.36 – 7.23 (m, 5H), δ 5.15 (d, *J* = 3.5 Hz, 0.63H), 4.64 (d, *J* = 8.4 Hz, 0.32H), 4.43 – 4.37 (m, 1H), 4.38 – 4.32 (m, 2H), 4.31 – 4.18 (m, 2H), 3.98 – 3.41 (m, 6H), 2.94 (t, *J* = 6.4 Hz, 2H), 2.25 (t, *J* = 7.4 Hz, 2H), 2.06 – 1.97 (m, 1H), 1.93 (d, *J* = 4.7 Hz, 3H), 1.88 – 1.74 (m, 1H), 1.36 (dd, *J* = 6.2, 5.6 Hz, 6H); ^{13}C NMR (150 MHz, D_2O) δ 176.91, 176.88, 175.5, 175.3, 174.6, 174.2, 173.9, 172.6, 138.1, 129.0, 128.6, 126.7, 94.9, 90.9, 82.3, 79.4, 78.0, 77.6, 75.7, 71.4, 69.0, 68.7, 66.4, 60.6, 60.5, 56.1, 53.6, 51.9, 49.43, 49.39, 34.1, 29.9, 25.7, 22.2, 21.9, 18.6, 16.8. HRMS (ESI-TOF) *m/z* calcd for $C_{27}H_{39}N_3O_{12}$ + H^+: 598.2607 [*M*+H]$^+$; found: 598.2617.

Compound 28. The reaction was carried out as described above in **18**. The final product, 28 (50 mg, 40% over 3 steps) was obtained from **S10**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.42β) δ 5.14 (d, *J* = 3.5 Hz, 0.64H), 4.66 (d, *J* = 8.5 Hz, 0.27H), 4.32 – 4.07 (m, 3H), 4.00 – 3.44 (m, 6H), 3.25 – 3.12 (m, 2H), 2.48 – 2.40 (m, 2H), 2.18 – 2.09 (m, 1H), 2.00 – 1.89 (m, 4H), 1.49 – 1.43 (m, 2H), 1.41 (dd, *J* = 7.2, 4.1 Hz, 3H), 1.37 (dd, *J* = 6.8, 4.4 Hz, 3H), 1.31 – 1.23 (m, 2H), 0.86 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.8, 175.8, 175.6, 175.0, 174.9, 174.2, 173.9, 173.1, 173.0, 94.9, 90.9, 82.5, 79.6, 78.1, 77.7, 75.7, 71.4, 68.9, 68.6, 60.6, 60.4, 56.1, 54.3, 53.7, 53.6, 53.55, 53.51, 49.7, 42.5, 39.2, 39.1, 31.5, 30.4, 26.8, 26.78, 22.1, 21.9, 19.33, 19.28, 18.6, 17.7, 16.6, 16.2, 12.9, 12.1; HRMS (ESI-TOF) *m/z* calcd for $C_{23}H_{40}N_4O_{11}$ + H^+: 549.2766 [*M*+H]$^+$; found: 549.2770.

Compound 29. The reaction was carried out as described above in **18**. The final product, **29** (45 mg, 34% over 3 steps) was obtained from **S11**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.50β) δ 5.15 (d, *J* = 3.5 Hz, 0.62H), 4.67 (d, *J* = 8.5 Hz, 0.31H), 4.32 – 4.07 (m, 3H), 4.01 – 3.44 (m, 6H), 3.25 – 3.11 (m, 2H), 2.41 – 2.30 (m, 2H), 2.16 – 2.05 (m, 1H), 2.00 –

1.87 (m, 3H), 1.52 – 1.45 (m, 2H), 1.42 (dd, *J* = 7.2, 3.8 Hz, 3H), 1.37 (dd, *J* = 6.8, 4.6 Hz, 3H), 1.31 – 1.19 (m, 6H), 0.88 – 0.81 (m, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.8, 175.8, 175.6, 174.94, 174.91, 174.2, 173.9, 172.9, 94.9, 90.9, 82.5, 79.6, 78.1, 77.7, 75.7, 71.4, 68.9, 68.7, 60.6, 60.4, 56.1, 53.8, 53.6, 53.5, 49.7, 49.0, 39.4, 31.6, 30.6, 28.2, 26.8, 25.63, 25.56, 22.1, 21.9, 21.8, 18.62, 18.61, 16.58, 16.56, 16.5, 13.3; HRMS (ESI-TOF) *m/z* calcd for $C_{25}H_{44}N_4O_{11}$ + H^+: 577.3079 [*M*+H]$^+$; found: 577.3088.

Compound 30. The reaction was carried out as described above in **18**. The final product, **30** (30 mg, 25% over 3 steps) was obtained from **S13**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.52β) δ 5.15 (d, *J* = 3.5 Hz, 0.62H), 4.66 (d, *J* = 8.5 Hz, 0.32H), 4.31 – 4.17 (m, 3H), 3.99 – 3.44 (m, 7H), 2.48 – 2.36 (m, 2H), 2.15 – 2.05 (m, 1H), 1.99 – 1.90 (m, 4H), 1.84 – 1.74 (m, 2H), 1.74 – 1.63 (m, 2H), 1.61 – 1.53 (m, 1H), 1.43 – 1.39 (m, 3H), 1.39 – 1.34 (m, 3H), 1.36 – 1.02 (m, 5H); ^{13}C NMR (150 MHz, D_2O) δ 177.38, 177.36, 175.8, 175.6, 174.94, 174.92, 174.2, 173.9, 171.7, 94.9, 90.9, 82.5, 79.7, 78.1, 77.7, 75.7, 71.4, 68.8, 68.6, 60.6, 60.4, 56.1, 53.7, 53.2, 49.69, 49.66, 49.1, 31.8, 30.4, 26.4, 24.8, 24.4, 24.37, 22.2, 21.9, 18.6, 16.5; HRMS (ESI-TOF) *m/z* calcd for $C_{25}H_{42}N_4O_{11}$ + H^+: 575.2923 [*M*+H]$^+$; found: 575.2938.

Compound 31. The reaction was carried out as described above in **18**. The final product, **31** (40 mg, 15% over 3 steps) was obtained from **S12**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.49β) δ 5.15 (d, *J* = 3.5 Hz, 0.57H), 4.67 (d, *J* = 8.5 Hz, 0.28H), 4.41 (dd, *J* = 9.4, 5.2 Hz, 1H), 4.31 – 4.18 (m, 2H), 4.09 – 3.43 (m, 8H), 2.51 – 2.40 (m, 2H), 2.25 – 2.09 (m, 1H), 2.05 – 1.92 (m, 4H), 1.42 (dd, *J* = 7.3, 4.3 Hz, 3H), 1.37 (dd, *J* = 6.8, 4.8 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 177.07, 177.05, 175.8, 175.6, 175.0, 174.2, 174.0, 173.9, 163.4, 163.1, 162.9, 162.6, 119.2, 117.3, 115.3, 113.4, 94.9, 90.9, 82.5, 79.6, 78.1, 77.7, 75.7, 71.4, 68.8, 68.6, 60.6, 60.4, 56.1, 53.7, 53.0, 49.6, 38.6, 38.4, 38.2, 30.0, 26.1, 22.1, 21.9, 18.6, 16.6; HRMS (ESI-TOF) *m/z* calcd for $C_{22}H_{33}F_5N_4O_{11}$ + H^+: 625.2139 [*M*+H]$^+$; found: 625.2131.

Compound 32. The reaction was carried out as described above in **18**. The final product, **32** (63 mg, 17% over 3 steps) was obtained from **S3**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.53β) δ 5.16 (d, *J* = 3.5 Hz, 0.66H), 4.66 (d, *J* = 8.4 Hz, 0.35H), 4.38 (dd, *J* = 9.0, 5.1 Hz, 1H), 4.35 – 4.21 (m, 2H), 4.20 – 4.09 (m, 2H), 3.98 – 3.43 (m, 6H), 2.34 – 2.25 (m, 2H), 2.20 – 2.10 (m, 1H), 1.69 – 1.60 (m, 2H), 1.42 (dd, *J* = 7.3, 4.1 Hz, 3H), 1.37 (t, *J* = 6.1 Hz, 3H), 1.35 – 1.21 (m, 10H), 0.84 (t, *J* = 6.5 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 179.74, 179.71, 175.5, 175.2, 174.72, 174.69, 174.2, 173.9, 173.2, 94.9, 90.8, 82.3, 79.3, 78.0, 77.6, 75.7, 71.5, 69.1, 68.8, 66.32, 66.29, 60.7, 60.5, 56.1, 53.7, 52.6, 52.6, 49.51, 49.46, 32.4, 31.2, 28.5, 28.4, 27.8, 26.8, 26.7, 25.2, 22.2, 22.1, 22.0, 18.6, 16.9, 13.5; HRMS (ESI-TOF) *m/z* calcd for $C_{27}H_{47}N_3O_{12}$ + Na^+: 628.3052 [*M*+Na]$^+$; found: 628.3057.

Compound 33. The reaction was carried out as described above in **18**. The final product, **33** (124 mg, 43% over 3 steps) was obtained from **S4**; ^{1}H NMR (600 MHz, D_2O) (Anomers-1.00α : 0.36β) δ 5.16 (d, *J* = 3.5 Hz, 0.70H), , 4.66 (d, *J* = 8.4 Hz, 0.25H), 4.52 – 4.44 (m, 1H), 4.39 – 4.20 (m, 2H), 4.17 – 4.03 (m, 2H), 4.00 – 3.41 (m, 6H), 2.39 (t, *J* = 7.4 Hz, 2H), 2.21 – 2.11 (m, 1H), 2.02 – 1.92 (m, 4H), 1.67 – 1.58 (m, 2H), 1.42 (t, *J* = 6.0 Hz, 3H), 1.37 (t, *J* = 5.5 Hz, 3H), 1.35 – 1.18 (m, 18H), 0.86 (t, *J* = 6.7 Hz, 3H); ^{13}C NMR (150 MHz, D_2O) δ 175.9, 175.8, 175.3, 175.1, 174.2, 174.1, 174.0, 173.6, 172.2, 95.0, 90.9, 82.4, 79.6, 78.0, 77.6, 75.8, 71.5, 69.1, 68.9, 68.8, 65.7, 61.7, 60.8, 60.7, 60.4, 56.2, 56.0, 53.6, 53.4, 51.7, 49.6, 49.4, 32.0, 29.9, 29.8, 29.7, 29.5, 28.3, 26.2, 25.8, 22.6, 22.3, 22.1, 18.8, 18.6, 17.2, 16.8, 13.8; HRMS (ESI-TOF) *m/z* calcd for $C_{31}H_{55}N_3O_{12}$ + Na^+: 684.3678 [*M*+Na]$^+$; found: 684.3685.

Compound 34. The reaction was carried out as described above in **18**. The final product, **34** (32 mg, 19% over 3 steps) was obtained from **S5**; ^{1}H NMR (600 MHz, CD_3OD) (Anomers-1.00α : 0.34β) δ 5.14 (d, *J* = 3.4 Hz, 0.70H), 4.55 (d, *J* = 8.3 Hz, 0.24H), 4.48 – 4.32 (m, 3H), 4.17 – 4.06 (m, 2H), 3.93 – 3.40 (m, 6H), 2.38 (t, *J* = 7.3 Hz, 2H), 2.24 – 2.14 (m, 1H), 2.00 – 1.93 (m, 4H), 1.70 – 1.61 (m, 2H), 1.41 – 1.37 (m, 6H), 1.33 – 1.23 (m, 26H), 0.90 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 177.0, 176.3, 176.0, 175.2, 174.6, 173.6, 173.2, 97.5, 92.6, 83.1, 80.4, 78.5, 78.4, 78.1, 73.4, 71.8, 71.6, 66.7, 66.7, 62.9, 62.8, 58.3, 55.7, 53.5, 53.5, 50.5, 50.4, 33.2, 31.6, 31.0, 30.9, 30.9, 30.8, 30.6, 30.5, 29.8, 28.0, 27.8, 27.1, 23.9, 23.3, 23.0, 19.8, 19.8, 18.6, 18.5, 14.6; HRMS (ESI-TOF) *m/z* calcd for $C_{35}H_{63}N_3O_{12}$ + H^+: 718.4485 [*M*+H]$^+$; found: 718.4456.

Compound 35. The reaction was carried out as described above in **18**. The final product, **35** (20 mg, 14% over 3 steps) was obtained from **S6**; ^{1}H NMR (600 MHz, CD_3OD) (Anomers-1.00α : 0.28β) δ 5.16 (d, *J* = 3.4 Hz, 0.78H), 4.55 (d, *J* = 8.0 Hz, 0.22H), 4.48 – 4.31 (m, 3H), 4.20 – 4.05 (m, 2H), 3.94 – 3.40 (m, 6H), 2.44 – 2.31 (m, 2H), 2.26 – 2.12 (m, 1H), 2.06 – 1.89 (m, 4H), 1.72 – 1.59 (m, 2H), 1.39 (t, *J* = 7.8 Hz, 6H), 1.37 – 1.14 (m, 34H), 0.90 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 177.4, 176.2, 175.9, 175.1, 174.6, 173.5, 173.1, 97.4, 92.4, 82.8, 80.0, 78.3, 78.2, 77.9, 73.2, 71.8, 71.5, 66.5, 66.5, 62.7, 62.7, 58.2, 55.6, 53.5, 50.4, 33.1, 31.8, 30.8, 30.7, 30.5, 30.4, 29.7, 28.1, 27.9, 27.7, 27.0, 23.7, 23.1, 22.9, 19.7, 19.6, 18.4, 18.3, 14.4; HRMS (ESI-TOF) *m/z* calcd for $C_{39}H_{71}N_3O_{12}$ + Na^+: 796.4930 [*M*+Na]$^+$; found: 796.4927.Me

Measurement of NF-κB Transcriptional Activity

HEK-Blue™ hNOD2 Cells (Invivogen; San Diego, CA, U.S.) were cultured in accordance with the manufacturer's instructions. HEK-Blue™ hNOD2 Cells was assayed for NF-κB transcriptional activity changes upon incubation (3.6×10^6 cells/mL) with MDP and other NOD2 agonistic compounds (10-1000 nM) for 16 h. Secreted embryonic alkaline phosphatase (SEAP) activity was determined in the supernatant in accordance with the manufacturer's instructions. An amount of 20 µL of SEAP-inducer compound or negative control was added to 180 µL of cells in HEK-Blue™ Detection medium and incubated at 37 °C for 16 h. Absorbance was measured on a M5 microplate reader (Reading, U.K.) at 640 nm.

Statistics

All experiments were performed at least three times, with average values expressed as the mean ± SD. Statistical significance was determined by the Dunnet multiple comparison test. Differences were considered significant for $p < 0.05$ and highly significant for $p < 0.01$.

Computation Method

To understand the binding mode of **1** and **33** in NOD2^{LRR}, molecular docking was performed using AutoDock Vina.[38] We used rabbit NOD2 (rNOD2) crystal structure (PDBID: 5IRN)[34] as macromolecule, and 3D coordinates of **1** and **33** (generated by Chem3D) as ligands. Prior to docking, the polar hydrogens were added to rNOD2^{LRR}. Kollman charges were assigned to rNOD2^{LRR}, and Gasteiger partial charges were applied to ligands (**1** and **33**) using AutoDock Tools-1.5.6.[39] Based on previous studies,[23,35,36] the concave surface of NOD2^{LRR} was considered as binding site. PyMOL (www.pymol.org) and LigPlot+ 2.1[40] was used for interaction study and visualization.

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Keywords: NOD2 • Muramyl dipeptide (MDP) • Peptidoglycan •Structure activity relationship • Innate immunity •Biological activity

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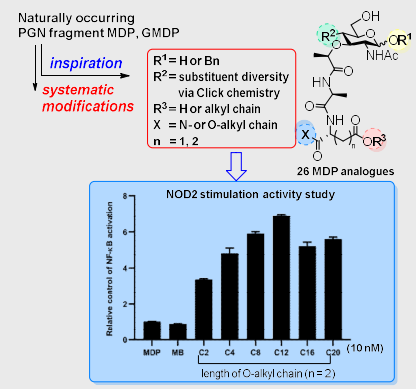
A series of muramyl dipeptide (MDP) analogues with structural modifications at the C4 position of MurNAc and on the D-*iso*-glutamine (*iso*Gln) residue of the peptide part were synthesized and evaluated for the study of human NOD2 stimulation activity in the innate immune response. Interestingly, MDP derivatives with an ester moiety are found to be more potent than reference compound MDP itself or MDP analogues containing an amide moiety.



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Further insights on structural modifications of muramyl dipeptides to study the human NOD2 stimulating activity

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