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Peptides

Expeditious Synthesis of Enantiopure, Orthogonally Protected Bis-α-Amino Acids (OPBAAs) and their Use in a Study of Nod1 Stimulation

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Abstract: A convenient approach towards the synthesis of orthogonally protected chiral bis-α-amino acids (OPBAAs) is described. The key transformations include: (1) a highly stereoselective conjugation (alkylation) of the Schöllkopf bis-lactim ethers and oxazolidinyl alkyl halides to build a backbone skeleton; and (2) our orthogonal protection strategy. A series of enantiopure OPBAAs bearing a variety of alkyl chain as a spacer; two stereogenic centers; and three protecting groups were prepared as examples. These versatile molecules were applied to the synthesis of biologically interesting di- or tri-peptide analogues, including chiral iE-*meso*-DAP and A-iE-*meso*-DAP, for the study of Nod1 activation in the innate immune response.

Introduction

Many natural or synthetic molecules containing a bis-α-amino acid (BAA) moiety exhibit an increased structural stability compared to a disulfide-linked bis-α-amino acid[1] and show interesting biological activities. For example, the dimer of HP5b (Figure 1),[2] isolated from natural human leukocytes, exhibits hematopoietic inhibition and stimulates hematopoiesis,[3] although the instability of the disulfide linkage limits its biological application. Fortunately, a molecule surrogate, SK&F 107647, containing a 2,7-diaminosuberic acid (DAS) moiety as a spacer, has a greatly improved molecular stability and biological activity (Figure 1).[4] Furthermore, its analog bearing the shorter spacer 2,5-diaminoadipic acid (DAA) instead of DAS exhibits 1000 times more potency than SK&F 107647 for induction of colony-stimulating activity.[3]

Bacterial cell wall peptidoglycan (PGN) consists of linear polysaccharide chains with alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) moieties, which are cross-linked by short peptide chains to support bacterial cell shapes and growth.[5] In the PGN of mycobacteria, gram-negative bacteria, and some gram-positive bacteria, a type of BAA called (2*S*, 6*R*)-2,6-diaminopimelic acid (*meso*-DAP) is responsible for peptide cross-linking, the final stage of cell wall biosynthesis catalyzed by transpeptidases.[6] Interestingly, PGN fragments containing chiral *meso*-DAP such as γ-D-Glu-*meso*-DAP (iE-*meso*-DAP) or L-Ala-γ-D-Glu-*meso*-DAP (A-iE-*meso*-DAP) (Figure 1) have been found to stimulate innate immune responses through recognition with the human intracellular nucleotide-binding oligomerization domain-containing protein 1 (Nod1), to induce the activation of the NF-κB pathway and the production of inflammatory cytokines.[7] In addition, a BAA-containing peptide, heptanoyl-γ-D-Glu-*meso*-DAP-D-Ala (FK-565), is a synthetic immunostimulant which enhances host defense against microbial infection, and shows antiviral activities.[8] Chiral BAAs are therefore important building blocks, but their direct purification from mixtures of stereomers or clean isolation from natural sources is extremely difficult.[9] Thus, synthetic chemistry approaches are the only means by which pure samples can be obtained for biological study.

Structurally, chiral OPBAAs consist of 1) two stereocenters; 2) three protecting groups (one for a carboxyl group and two for amino groups); and 3) a varied and flexible alkyl spacer between two α-amino acid moieties (Figure 1). To date, several methods toward the preparation of BAAs have been reported,[10] for example, using Grubbs' cross metathesis to combine two α-amino acids, but several problems exist among some of

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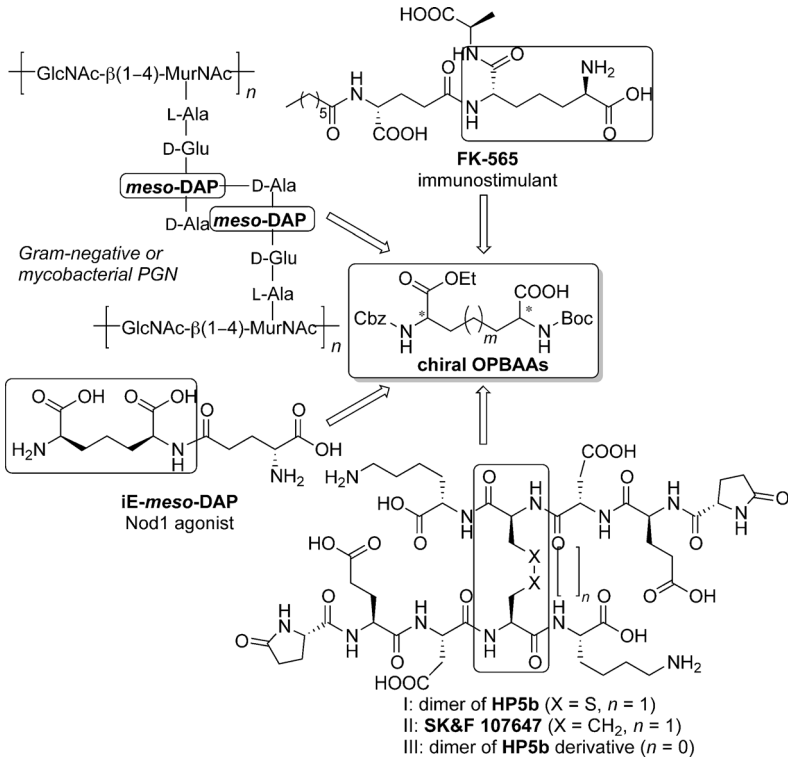


Figure 1. Bioactive and natural molecules containing bis-α-amino acids and the general structure of OPBAAs.

these synthetic routes such as length of the alkyl spacer, lack of an orthogonal protecting group strategy, lack of stereoselectivity, and contamination by homo-coupling side-products. Herein, we report an asymmetric synthetic method and an orthogonal protection strategy for the preparation of various enantiopure OPBAAs bearing a variety of alkyl chains between two stereocenters. These versatile OPBAAs will be utilized as building blocks in the synthesis of biologically interesting di- or tri-peptide analogues for the study of human Nod1 activation in the innate immune response through the NF-κB pathway.

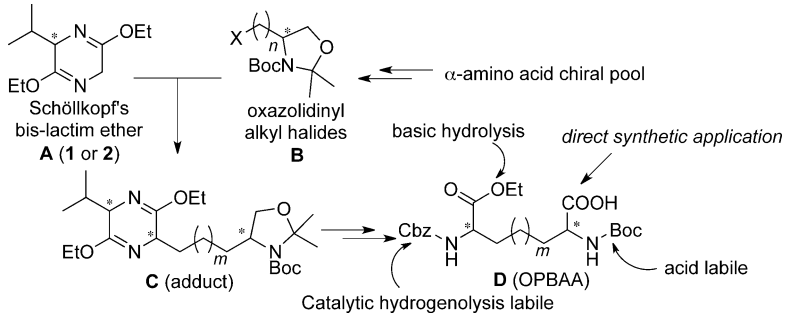
Scheme 1 depicts our synthetic strategy. We propose generation of one desired stereocenter via the chiral Schöllkopf bis-lactim ether method,[11] wherein two enantiopure templates **A** are easily prepared from L- or D-valine.[12] The other stereocenter (in the chiral oxazolidinyl alkyl halides **B**) can be directly prepared from the available α-amino acid chiral pools.[13] Our orthogonal protection strategy is as follows: one carboxyl group will be protected as an ethyl ester, which can be easily deprotected under basic hydrolysis; the neighboring amino group will be protected by the benzyloxycarbonyl (Cbz) group, which can be easily removed via catalytic hydrogenation; and the other amino group will be protected by the acid-labile *tert*-butoxycarbonyl (Boc) group. Notably, there is one free carboxylic acid, which can directly undergo further chemical transformation.

Results and Discussion

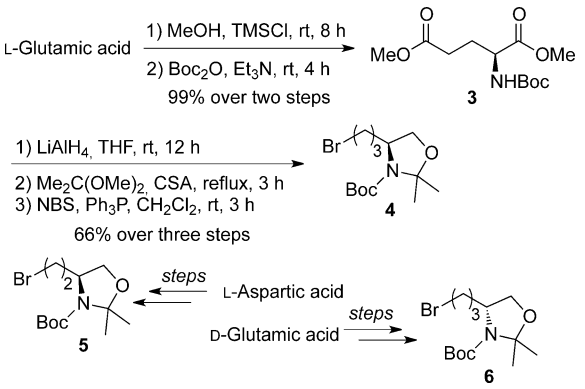
The synthesis of orthogonally protected *meso*-DAP (**D**, $n=3$, Scheme 1) was first pursued, as our model study to examine the feasible conditions of transformations. Following Chen's protocol with slight modifications,[14] Schöllkopf bis-lactim ether **A** (3*S*)-3,6-dihydro-2,5-diethoxy-3-isopropyl-pyrazine (**1**) was prepared from L-valine in 60% overall yield over four steps. Likewise, the enantiomer, (3*R*)-3,6-dihydro-2,5-diethoxy-3-isopropyl-pyrazine (**2**), was also prepared from D-valine.

As illustrated in Scheme 2, L-glutamic acid was converted into the protected glutamic acid **3** in 99% yield over two steps via esterification in the presence of chlorotrimethylsilane (TMSCl) in methanol and *N*-Boc protection under basic conditions.[15] After the reduction with lithium aluminum hydride and *N*,*O*-ketalization with 2,2-dimethoxypropane of compound **3**, a halogenation of the corresponding primary alcohol was performed under Appel's conditions[16] to give oxazolidinyl alkyl bromide **4** in a good yield (66%). Similarly, **5** and **6** were also prepared from the corresponding L-aspartic acid and D-glutamic acid, respectively.

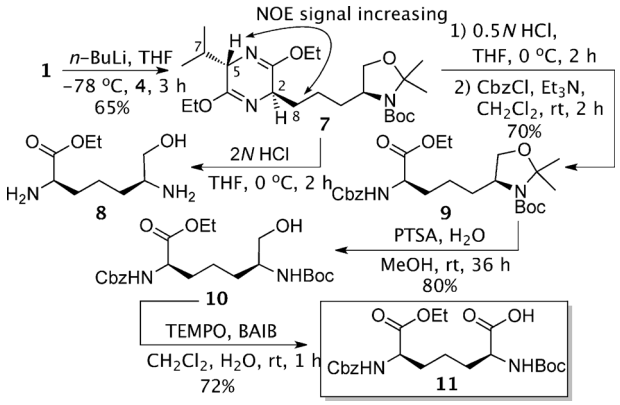
Next, the diastereoselective alkylation of lithiated **1** with **4** (Scheme 3) was carried out to generate the adduct **7** (65%). The stereochemistry of the newly created stereocenter (C2) of **7** was confirmed by 2D-NOSEY NMR experiments.[17] Selective hydrolysis of **7** under various acidic conditions was investigated, and the optimized conditions were found to be treatment with 0.5 N HCl in THF at 0 °C for 2 h, which gave a single product, followed by *N*-Cbz protection to give **9** in 70% yield over two steps. Harsher condi-



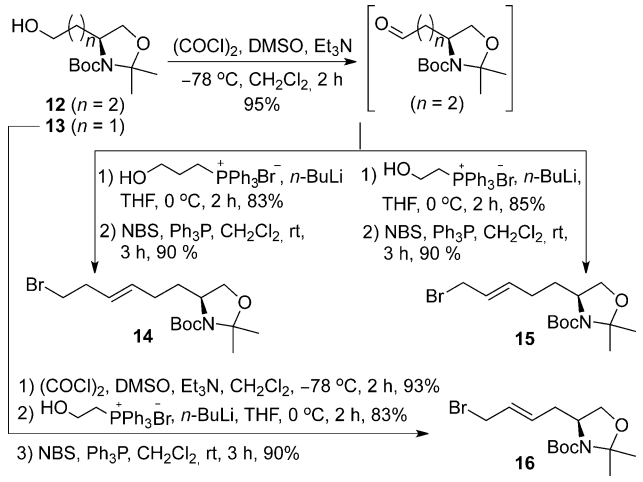
Scheme 1. Strategy for the general synthesis of OPBAAs.



Scheme 2. Synthesis of oxazolidinyl alkyl halides **4**–**6**.



Scheme 3. Synthesis of orthogonally protected *meso*-DAP **11**.



Scheme 4. Preparation of oxazolidinyl alkyl bromides **14**–**16**.

Table 1. Correlations between substrates **A**/**B**, intermediates **C** and products **D**.

Entry	A/B	C (adduct)	Yield (%)[a]	D (OPBAA)	Yield (%)[b]
1	**1/5**	**17**	55	**24**	34
2	**1/4**	**7**	65	**11**	40
3	**1/6**	**21**	62	**29**	36
4	**2/4**	**22**	71	–[c]	–
5	**2/6**	**23**	61	**28**	42
6	**1/16**	**18**	60	**25**	37
7	**1/15**	**19**	58	**26**	45
8	**1/14**	**20**	60	**27**	45

[a] Two steps (coupling and hydrogenation) for entries 6–8. [b] Calculated yields from **C** (adduct). [c] Not prepared.

tions resulted in complete deprotection to give **8** and/or poor selectivity.

Selective deprotection of the oxazolidine ring in **8** was performed over 36 h in the presence of PTSA at room temperature to generate alcohol **10** (80%) without affecting the *N*-Boc moiety.[18] TEMPO oxidation gave acid **11** (72%), our first OPBAA example. This model study and feasible reaction conditions encouraged us to apply this strategy to prepare various interesting OPBAAs.

In order to vary the spacer in the oxazolidinyl alkyl halide (Scheme 4), alcohols **12** ($n=2$) and **13** ($n=1$) prepared from L-glutamic acid and L-aspartic acid, respectively, were oxidized under Swern conditions and then condensed with various triphenyl phosphonium ylides in a Wittig reaction, followed by bromination to generate the corresponding oxazolidinyl alkyl bromides **14**–**16** with high yields (ca. 90%).

With two chiral templates **1** and **2** and six oxazolidinyl alkyl bromides **4**–**6** and **14**–**16** in hand, a series of chiral adducts **C** (eight examples) and chiral OPBAAs (seven examples), as shown in Table 1, were successfully prepared based on the conditions previously discovered in Scheme 3. Notably, when **14**–**16** were used, hydrogenation of an unsaturated spacer in adducts **C** was performed after conjugation of **A** and **B** (see entries 6–8, Table 1). All chemical structures of adducts and OPBAAs are shown in Figure 2 and Figure 3; the variety in structure and high yields obtained emphasizes the feasibility and flexibility of our synthetic approach.

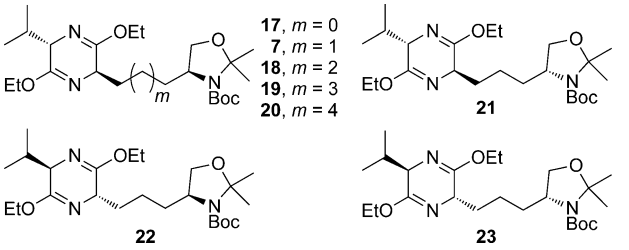


Figure 2. Structures of all synthetic adducts **C** in this study.

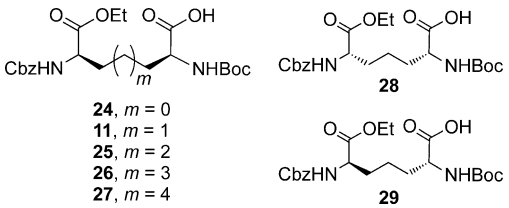
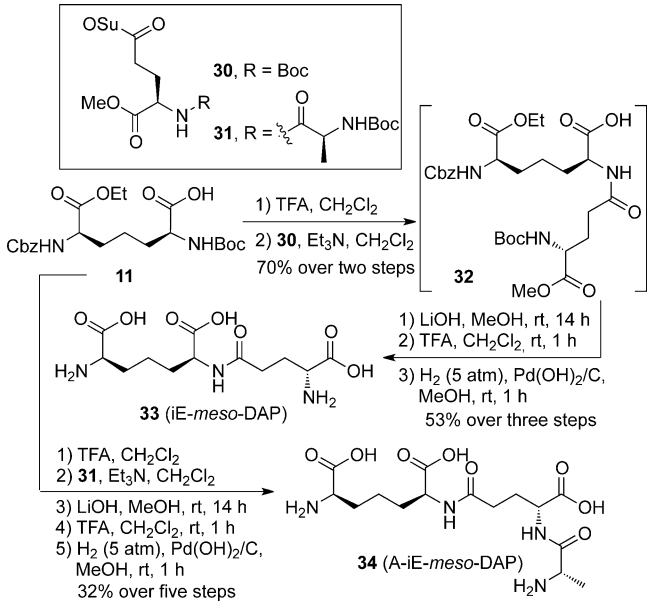


Figure 3. Structures of all OPBAAs **D** in this study.

To exemplify the utility of these chiral OPBAAs, biologically interesting small peptides containing a chiral OPBAA were designed, synthesized, and evaluated towards human Nod1 stimulation.

Currently, peptidoglycan fragments possessing the iE-*meso*-DAP moiety, such as dipeptide (iE-*meso*-DAP) or tripeptide (A-iE-*meso*-DAP), have been reported as human Nod1 agonists. Our chemistry and various OPBAAs allowed us not only to prepare bioactive peptides including **33** (iE-*meso*-DAP) and **34** (A-iE-*meso*-DAP) but also to examine whether small peptides with a different spacer length in OPBAA affect human Nod1 stimulation activity or not.

As illustrated in Scheme 5, the activated glutamic acid derivatives **30** and **31** were prepared from D-Glu(OBn)-OH.[19] *N*-Boc deprotection of OPBAA **11** with trifluoroacetic acid (TFA), followed by conjugation to **30**, basic hydrolysis of the methyl ester, *N*-Boc deprotection of the adduct **32**, and hydrogenolysis, gave the desired molecule **33** in an overall 37% yield over five steps. Likewise, a series of dipeptides were prepared using our various OPBAAs or intermediates, and all structures are shown in Figure 4. In addition, tripeptide **34** was also prepared from **11** by using **31** instead of **30**, and the overall yield was 32% over five steps (Scheme 5). Notably, the purification of dipeptide **36** and tripeptide **40** through silica gel column chromatography was easier than that of corresponding **33** and **34**, as the former pair possessed a primary alcohol group instead of a carboxylic acid. These molecules were selected for synthesis and testing in order to understand the effect of the moiety (R = CH_2OH or COOH) on agonist behavior.



Scheme 5. Preparation of bioactive peptides **33** (iE-*meso*-DAP) and **34** (A-iE-*meso*-DAP).

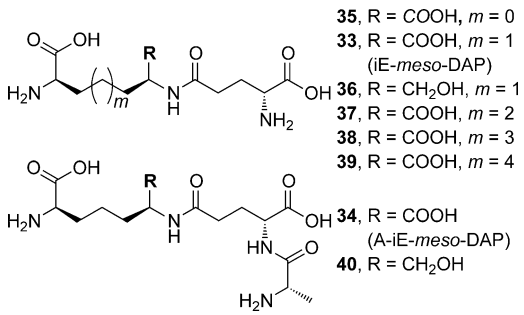


Figure 4. Structures of six dipeptides and two tripeptides.

Biological Evaluation

Eight enantiopure di- or tri-peptides **33**–**40**, prepared from the OPBAAs **11**, **24**–**27**, as well as commercially available mix iE-DAP **41** (a mixture of γ-D-Glu-D-mDAP and γ-D-Glu-L-mDAP, used for comparison purposes) were evaluated for their capacity to stimulate human Nod1. As shown in Figure 5 for dipeptide-based molecules toward human Nod1 agonist study, enantiopure iE-*meso*-DAP (**33**, m = 1) was found to be the most potent Nod1 dipeptid ligand tested, more potent than mix iE-DAP (**41**). This suggests that the chirality of DAP profoundly affects human Nod1 stimulating activity, and is consistent with previously reported results.[7b,20] Surprisingly, only the molecule containing the *meso*-DAP typed BAA such as **33** (*m* = 1) is human Nod1 agonist (Figure 5). By contrast, compounds containing shorter (*m* = 0) or longer (*m* = 2, 3, or 4) spacers were inactive toward the human Nod1 agonist study, even at the concentrations up to 10 μg mL^{-1} (Figure 5). These results suggest that subtle structural modifications on the backbone of BAA apparently influence the human Nod1 stimulation activity.

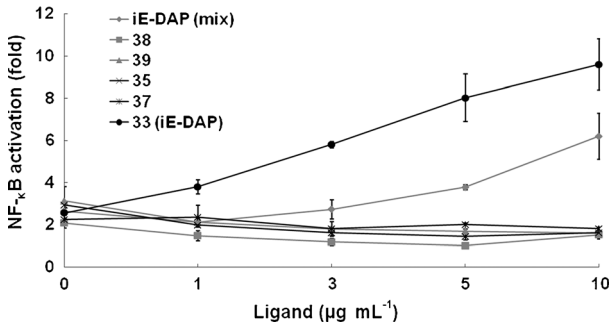


Figure 5. Stimulation of human Nod1 by dipeptide-based molecules including mix iE-DAP (reference), **33**, **35**, **37**, **38**, and **39**. HEK293T cells were transfected with human Nod1 plasmid and κB reporter. The indicated amount of each compound was added to the cells for 24 h, and the ability of each compound to activate NF-κB was determined by luciferase reporter assay. The basal NF-κB activity under Nod1 transfection without ligand stimulation was 2.61 ± 0.37 (mean ± SE) fold on average.

Next, dipeptides **33** and **36** and tripeptides **34** and **40**, sharing the common *meso*-DAP typed BAA, were chosen to evaluate their ability of human Nod1-dependent NF-κB activation, and the results are shown in Figure 6. The enantiopure tripeptide **34** (A-iE-*meso*-DAP) showed a clear trend in potency and was a more active Nod1 agonist than others, even at lower concentrations (0.1 μg mL^{-1}). At a concentration of 1 μg mL^{-1},

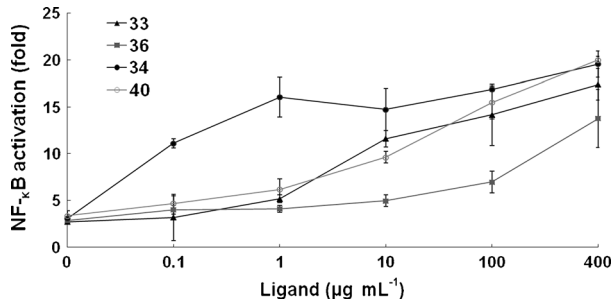


Figure 6. Stimulation of human Nod1 by **33**, **34**, **36**, and **40**. HEK293T cells were transfected with human Nod1 plasmid and κB reporter. The indicated amount of each compound was added to the cells, and the ability of each compound to activate NF-κB was determined by luciferase reporter assay. The basal NF-κB activity under Nod1 transfection without ligand stimulation was 3.24 ± 0.54 (mean ± SE) fold on average.

34 exhibited three times the potency of **33**. Interestingly, when the internal carboxylic acid (R = COOH) in DAP was changed to the hydroxymethyl group ($R = CH_2OH$), the activity decreased (**33** vs. **36** and **34** vs. **40**), implying that the internal carboxylic acid contributes more to human Nod1 recognition than the hydroxymethyl group. Notably, the activity trend of tripeptide **40** was similar to that of dipeptide **33** in the concentration range from 0.1 to 100 μg mL^{-1} (Figure 6), indicating that the additional L-Ala residue in tripeptide **40** could compensate for the loss of stimulation activity caused from the functional group conversion from R = COOH to CH_2OH. From a synthetic chemistry point of view, tripeptide **40** is easier to prepare than dipeptide **33**, a finding that establishes it as the molecular template of choice for the exploration of new tripeptide-based human Nod1 agonists.

Conclusions

A concise, stereoselective approach for the preparation of structurally diverse OPBAAs bearing two stereocenters, three orthogonally protecting groups, and a spacer of different length between two α-amino acid moieties has been developed. Using these versatile and enantiopure building blocks, a series of small peptides inspired from peptidoglycan fragments were synthesized and then studied for their ability to activate human Nod1 receptor in the innate immune response through the NF-κB pathway. Preliminary structure–activity relationship (SAR) results showed that only the dipeptides containing a specific length of BAAs are recognized as a potent human Nod1 agonist. By contrast, shorter or longer spacers than DAP abolished the molecular recognition with the human Nod1 receptor. When the internal carboxylic acid was converted into the hydroxymethyl moiety in iE-*meso*-DAP or A-iE-*meso*-DAP, human Nod1 agonistic activity decreased dramatically, indicating that the carboxylic acid group plays an important role in human Nod1 recognition. This chemistry and the chiral building blocks and intermediates described herein will be used to synthesize various peptidoglycan fragments to study their biological functions in the future. Additionally, a more detailed and clearer SAR study of tripeptide analogues toward the human Nod1 receptor will be sought in order to understand the pathogen–host interaction, and the results will be published in due course.

Experimental Section

General Information

All solvents and reagents were obtained commercially and used without further purification. ^{1}H NMR spectra were recorded on a Bruker AVANCE 600 spectrometer in deuterated solvents such as chloroform-*d* (δ = 7.24), [D$_4$]MeOH (δ = 3.31), and deuterium oxide (δ = 4.80) at ambient temperature. ^{13}C NMR spectra were obtained with a Bruker AVANCE 600 spectrometer and were assigned according to chloroform-*d* (δ = 77.23 ppm of central line). Chemical shifts are given in ppm (δ) and coupling constants (*J*) are given in Hz. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and dd (double of doublets). High resolution mass spectra were obtained on a Bruker Daltonics BioTOF III spectrometer (ESI-MS). Optical rotations were measured with a PerkinElmer Model 341 polarimeter. Flash column chromatography was carried out using Merck Kieselgel Si60 (40–63 μm). Reactions were monitored by analytical thin-layer chromatography (TLC) in silica gel 60 F254 plates and visualized by exposure to ultraviolet light at 254 nm and/or immersion in a staining solution (*p*-anisaldehyde or acidic ninhydrin, phosphomolybdic acid or potassium permanganate) followed by heating on a hot plate. Concentration refers to rotary evaporation.

(*S*)-tert-Butyl 4-(3-((2*R*,5*S*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)propyl)-2,2-dimethyloxazolidine-3-carboxylate (7)

To a solution of compound **1** (100 mg, 0.47 mmol) in THF (4 mL) *n*-butyllithium (201 μL, 0.52 mmol, 2.5 M in hexanes) was added dropwise at −78 °C. After stirring for 30 min at −78 °C, a solution of **4** (166 mg, 0.52 mmol) in THF (2 mL) was added dropwise and the resulting mixture was stirred for 3 h. The mixture was quenched with saturated aqueous solution of ammonium chloride, and evaporated under vacuum. The residue was extracted with EtOAc/H_2O, and the layers were separated. The organic phase was washed with saturated aqueous solution of sodium bicarbonate, brine, and dried over anhydrous $MgSO_4$. The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography (10% EtOAc in hexanes, silica gel) to give **7** as a light yellow oil (138 mg, 0.31 mmol, 65%). $[\alpha]_D^{25} = +6.5$ (c = 8.9, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): δ = 4.03–4.11 (4H, m), 3.94 (1H, s, br), 3.67–3.85 (4H, m), 2.21–2.23 (1H, m), 1.74–1.90 (4H, m), 1.50–1.60 (4H, m), 1.41–1.43 (13H, m), 1.22 (6H, t, J = 7.2 Hz), 0.99 (3H, d, J = 7.2 Hz), 0.64 ppm (3H, d, J = 7.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 163.4, 163.3, 163.3, 163.2, 152.3, 152.0, 93.7, 93.2, 80.1, 79.5, 67.2, 66.7, 61.0, 60.8, 60.7, 57.9, 57.5, 55.5, 55.3, 34.2, 34.0, 33.8, 32.7, 32.1, 31.9, 28.6, 27.7, 27.0, 24.8, 23.5, 19.2, 16.8, 14.5 ppm; HRMS (ESI): *m/z* calcd for $C_{24}H_{43}N_3O_5 + H^+$: 454.3275 [M+H$^+$]; found: 454.3271.

(*S*)-tert-Butyl 4-((*R*)-4-(((benzyloxy)carbonyl)amino)-5-ethoxy-5-oxopentyl)-2,2-dimethyloxazolidine-3-carboxylate (9)

To a solution of compound **7** (100 mg, 0.22 mmol) in THF (1.5 mL) was added 0.5 M HCl aqueous solution (0.5 mL) and reacted at 0 °C for 2 h. After the reaction was completed (checked by TLC), aqueous sat. $NaHCO_3$ was added until pH > 8. Benzyl chloroformate was

added to the mixture and reacted at room temperature for 2 h. The residue was extracted with EtOAc. The combined organic phases were washed with saturated sodium bicarbonate aqueous solution, brine, dried over anhydrous $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (10% EtOAc in hexanes, silica gel) to give **9** as a colorless oil (73 mg, 0.15 mmol, 70%). $[\alpha]_D^{25} = +3.6$ ($c = 4.3$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta = 7.28$–7.33 (5H, m), 5.47–5.31 (1H, m), 5.07 (2H, s), 4.16–4.32 (3H, m), 3.64–3.87 (3H, m), 1.68–1.83 (4H, m), 1.50–1.60 (4H, m), 1.42–1.43 (11H, m), 1.35–1.26 ppm (5H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 172.5$, 156.1, 156.0, 152.5, 151.9, 136.5, 136.4, 129.2, 128.7, 128.4, 128.3, 127.1, 125.5, 93.9, 93.4, 80.3, 79.7, 67.2, 67.1, 67.0, 61.6, 61.6, 57.3, 57.2, 54.0, 33.5, 32.8, 32.4, 28.6, 28.5, 27.8, 27.0, 24. 7, 23.4, 22.1, 22.0, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{25}H_{38}N_2O_7 + H^+$: 479.2752 [M+H$^+$]; found: 479.2758.

(2*R*,6*S*)-Ethyl 2-(((benzyloxy)carbonyl)amino)-6-((tert-butoxycarbonyl)amino)-7-hydroxyheptanoate (10)

To a solution of **9** (1.0 g, 2.09 mmol) in MeOH (10 mL) was added *p*-toluenesulfonic acid monohydrate (39 mg, 0.21 mmol) and several drops of H_2O. After stirring for 36 h at room temperature, MeOH was removed under vacuum. The residue was purified by flash column chromatography (50% EtOAc in hexanes, silica gel) to give **10** as a colorless oil (733 mg, 1.67 mmol, 80%). $[\alpha]_D^{25} = -9.7$ ($c = 1.0$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta = 7.29$–7.34 (5H, m), 5.41 (1H, d, $J = 7.2$ Hz), 5.08 (2H, s), 4.83 (1H, d, $J = 7.2$ Hz), 4.34–4.36 (1H, m), 4.16–4.19 (2H, m), 3.50–3.58 (3H, m), 1.81–1.83 (1H, m), 1.61–1.65 (2H, m), 1.34–1.39 (12H, m), 1.24–1.27 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 172.5$, 156.1, 156.0, 152.5, 151.9, 136.5, 136.4, 129.2, 128.7, 128.4, 128.3, 127.1, 125.5, 93.9, 93.4, 80.3, 79.7, 67.2, 67.1, 67.0, 61.63, 61.55, 57.3, 57.2, 54.0, 33.5, 32.8, 32.4, 28.6, 28.5, 27.8, 27.0, 24.7, 23.4, 22.1, 22.0, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{22}H_{34}N_2O_7 + H^+$: 439.2439 [M+H$^+$]; found: 439.2443.

(2*S*,6*R*)-6-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-7-ethoxy-7-oxoheptanoic acid (11)

To the solution of the **10** (50 mg, 0.11 mmol) in dichloromethane (1 mL) and water (5 mL) was added (diacetoxyiodo)benzene (177 mg, 0.55 mmol) and 2,2,6,6-tetramethyl-1-piperidinyloxy (31 mg, 0.02 mmol). After stirring at room temperature for 1 h, the reaction was quenched with 10% aqueous $Na_2S_2O_3$. The organic layer was washed with 10% aqueous $Na_2S_2O_3$, brine, dried over anhydrous $MgSO_4$, and concentrated. The residue was purified by flash column chromatography (5% MeOH and 0.3% formic acid in dichloromethane, silica gel) to give **11** as a pale yellow oil (35 mg, 0.08 mmol, 72%). $[\alpha]_D^{25} = +2.1$ ($c = 1.1$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 7.28$–7.33 (5H, m), 5.46–5.50 (1H, m), 5.12–5.17 (1H, m), 5.06–5.11 (2H, m), 4.09–4.32 (4H, m), 1.81–1.86 (2H, m), 1.67–1.76 (2H, m), 1.41 (11H, s, br), 1.12–1.25 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 176.0$, 172.5, 156.3, 156.0, 136.4, 128.7, 128.4, 128.4, 80.5, 67.3, 61.8, 53.8, 53.1, 32.3, 32.0, 28.5, 21.2, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{22}H_{32}N_2O_8 + H^+$: 453.2231 [M+H$^+$]; found: 453.2233.

(*S*)-tert-Butyl 4-(2-((2*R*,5*S*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)ethyl)-2,2-dimethyloxazolidine-3-carboxylate (17)

The title compound **17** was synthesized by the procedure as described for the preparation of compound **7**, except that **5** was used as an alkylating agent, in 55% yield as a colorless oil. $[\alpha]_D^{25} = +12.8$ ($c = 1.1$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta = 4.04$–4.12 (4H, m), 3.94 (1H, s, br), 3.64–3.88 (4H, m), 2.15–2.21 (1H, m), 1.65–1.84 (4H, m), 1.51–1.58 (4H, m), 1.38–1.47 (11H, m), 1.20 (6H, t, $J = 7.2$ Hz), 0.96 (3H, d, $J = 7.2$ Hz), 0.64 ppm (3H, d, $J = 7.2$ Hz); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 163.4$, 163.2, 162.9, 152.3, 152.0, 93.8, 93.3, 79.9, 79.4, 67.4, 67.1, 61.0, 60.9, 60.7, 60.6, 57.7, 57.4, 55.4, 55.1, 32.1, 30.9, 28.6, 27.7, 26.9, 24.7, 23.4, 19.2, 16.8, 14.5 ppm; HRMS (ESI): *m/z* calcd for $C_{23}H_{41}N_3O_5 + H^+$: 440.3119 [M+H$^+$]; found: 440.3121.

(*S*)-tert-Butyl 4-(4-((2*R*,5*S*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)butyl)-2,2-dimethyloxazolidine-3-carboxylate (18)

The title compound **18** was synthesized by the procedure as described for the preparation of compound **7** except for using **16** as an alkylating agent and the adduct was subsequently hydrogenated [20% Pd(OH$_2$)/C, MeOH, rt, 1 h] in 60% yield as a colorless oil. $[\alpha]_D^{25} = +27.1$ ($c = 0.9$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta = 4.01$–4.11 (4H, m), 3.90 (1H, s, br), 3.65–3.90 (4H, m), 2.19–2.21 (1H, m), 1.65–1.70 (4H, m), 1.46–1.56 (4H, m), 1.35–1.46 (11H, m), 1.10–1.32 (10H, m), 0.99 (3H, d, $J = 7.2$ Hz), 0.64 ppm (3H, d, $J = 7.2$ Hz); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 163.4$, 163.1, 152.3, 151.9, 93.7, 93.1, 80.0, 79.4, 67.1, 66.8, 60.8, 60.6, 60.5, 57.9, 57.4, 55.4, 34.3, 34.2, 33.6, 33.0, 31.9, 30.4, 28.6, 27.7, 26.9, 26.5, 26.3, 24.7, 24.6, 23.4, 19.2, 16.7, 14.5, 14.5 ppm; HRMS (ESI): *m/z* calcd for $C_{25}H_{47}N_3O_5 + H^+$: 468.3432 [M+H$^+$]; found: 468.3431.

(*S*)-tert-Butyl 4-(5-((2*R*,5*S*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)pentyl)-2,2-dimethyloxazolidine-3-carboxylate (19)

The title compound **19** was synthesized by the procedure as described for the preparation of compound **7** except for using **15** as an alkylating agent and the adduct was subsequently hydrogenated [20% Pd(OH$_2$)/C, MeOH, rt, 1 h] in 58% yield as a colorless oil. $[\alpha]_D^{25} = +9.6$ ($c = 3.2$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta = 4.03$–4.14 (4H, m), 3.94 (1H, s, br), 3.66–3.88 (4H, m), 2.23–2.25 (1H, m), 1.66–1.72 (4H, m), 1.50–1.55 (4H, m), 1.43–1.45 (11H, m), 1.17–1.30 (12H, m), 1.00 (3H, d, $J = 7.2$ Hz), 0.67 ppm (3H, d, $J = 7.2$ Hz); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 163.6$, 163.2, 152.4, 152.1, 93.8, 93.2, 80.1, 79.5, 67.3, 66.9, 60.9, 60.7, 60.6, 58.0, 57.6, 55.6, 34.3, 33.8, 33.9, 31.9, 29.7, 28.7, 27.8, 27.0, 26.6, 26.5, 24.8, 24.7, 23.5, 19.3, 16.8, 14.6, 14.6 ppm; HRMS (ESI): *m/z* calcd for $C_{26}H_{47}N_3O_5 + H^+$: 482.3588 [M+H$^+$]; found: 482.3592.

(*S*)-tert-Butyl 4-(6-((2*R*,5*S*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)hexyl)-2,2-dimethyloxazolidine-3-carboxylate (20)

The title compound **20** was synthesized by the procedure as described for the preparation of compound **7** except for using **14** as an alkylating agent and the adduct was subsequently hydrogenated [20% Pd(OH$_2$)/C, MeOH, rt, 1 h] in 60% yield as a colorless oil. $[\alpha]_D^{25} = +10.5$ ($c = 2.2$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta = 4.02$–4.15 (4H, m), 3.94–3.97 (1H, m), 3.66–3.88 (4H, m), 2.24–2.25 (1H, m), 1.67–1.73 (4H, m), 1.51–1.55 (4H, m), 1.43–1.45 (11H, m), 1.18–1.28 (14H, m), 1.01 (3H, d, $J = 7.2$ Hz), 0.67 ppm (3H, d, $J = 7.2$ Hz); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta = 163.6$, 163.2, 152.4, 152.1, 93.8, 93.2, 80.1, 79.5, 67.3, 66.9, 60.8, 60.7, 60.6, 58.0, 57.6, 55.7, 55.6, 52.5, 34.4, 34.3, 33.8, 33.1, 31.9, 31.9, 29.7, 29.6, 28.7, 27.8, 27.0, 26.6, 26.5, 24.8, 24.6, 23.5, 19.3, 16.8, 14.6,

14.6 ppm; HRMS (ESI): *m/z* calcd for $C_{27}H_{49}N_3O_5+H^+$: 496.3745 [*M*+H$^+$]; found: 496.3737.

(*R*)-tert-Butyl 4-(3-((2*R*,5*S*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)propyl)-2,2-dimethyloxazolidine-3-carboxylate (21)

The title compound **21** was synthesized by the procedure as described for the preparation of compound **7** except for using **6** as an alkylating agent in 62% yield as a colorless oil. $[\alpha]_D^{25}=+1.1$ ($c=3.2$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta=4.02$–4.13 (4H, m), 3.94 (1H, s, br), 3.67–3.87 (4H, m), 2.22–2.24 (1H, m), 1.76–1.81 (1H, m), 1.66–1.68 (1H, m), 1.50–1.55 (4H, m), 1.42–1.44 (13H, m), 1.21–1.25 (8H, m), 0.99 (3H, d, $J=7.2$ Hz), 0.67 ppm (3H, br); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=163.5$, 163.4, 163.3, 152.3, 152.0, 93.8, 93.3, 80.1, 79.5, 67.4, 66.8, 61.0, 60.8, 60.7, 60.6 57.9, 57.6, 55.7, 55.4, 34.3, 33.9, 32.8, 32.1, 31.9, 28.7, 27.7, 27.0, 24.8, 23.5, 21.6, 16.8, 14.6, 14.5 ppm; HRMS (ESI): *m/z* calcd for $C_{24}H_{43}N_3O_5+H^+$: 454.3275 [*M*+H$^+$]; found: 454.3278.

(*S*)-tert-Butyl 4-(3-((2*S*,5*R*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)propyl)-2,2-dimethyloxazolidine-3-carboxylate (22)

The title compound **22** was synthesized by the procedure as described for the preparation of compound **7** except for using **4** as an alkylating agent in 71% yield as a colorless oil. $[\alpha]_D^{25}=+4.3$ ($c=5.3$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta=4.01$–4.12 (4H, m), 3.93 (1H, s, br), 3.66–3.86 (4H, m), 2.21–2.23 (1H, m), 1.64–1.90 (4H, m), 1.48–1.53 (4H, m), 1.40–1.43 (13H, m), 1.19–1.24 (8H, m), 0.99 (3H, d, $J=7.2$ Hz), 0.65 ppm (3H, s, br); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=163.4$, 163.4, 163.2, 162.7, 152.3, 152.0, 93.7, 93.2, 80.1, 79.5, 67.3, 66.8, 61.01, 60.96, 60.8, 60.7, 60.5, 57.9, 57.6, 55.6, 55.3, 34.3, 33.9, 32.8, 32.1, 31.9, 28.6, 27.7, 26.9, 24.8, 23.5, 21.6, 19.8, 19.3, 17.8, 17.7, 16.8, 14.6 ppm; HRMS (ESI): *m/z* calcd for $C_{24}H_{43}N_3O_5+H^+$: 454.3275 [*M*+H$^+$]; found: 454.3269.

(*R*)-tert-Butyl 4-(3-((2*S*,5*R*)-3,6-diethoxy-5-isopropyl-2,5-dihydropyrazin-2-yl)propyl)-2,2-dimethyloxazolidine-3-carboxylate (23)

The title compound **23** was synthesized by the procedure as described for the preparation of compound **7** except for using **6** as an alkylating agent in 61% yield as a colorless oil. $[\alpha]_D^{25}=-5.7$ ($c=2.8$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta=4.02$–4.13 (4H, m), 3.93 (1H, s, br), 3.65–3.86 (4H, m), 2.21–2.23 (1H, m), 1.71–1.77 (3H, m), 1.49–1.53 (4H, m), 1.41–1.44 (13H, m), 1.22 (8H, t, $J=7.2$ Hz), 0.98 (3H, d, $J=7.2$ Hz), 0.66 ppm (3H, br); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=163.5$, 163.4, 163.3, 152.3, 152.0, 93.7, 93.2, 80.1, 79.5, 67.3, 66.8, 61.0, 60.8, 60.7, 57.9, 57.5, 55.5, 55.4, 34.2, 34.0, 33.8, 32.7, 32.1, 31.9, 28.6, 27.7, 27.0, 24.8, 23.5, 21.6, 19.3, 16.8, 14.6, 14.5 ppm; HRMS (ESI): *m/z* calcd for $C_{24}H_{43}N_3O_5+H^+$: 454.3275 [*M*+H$^+$]; found: 454.3271.

(2*S*,5*R*)-5-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-6-ethoxy-6-oxohexanoic acid (24)

The title compound **24** was synthesized by the procedure as described for the preparation of compound **11** except for using **17** as a starting material over four steps in 34% yield as a pale yellow oil. $[\alpha]_D^{25}=+3.9$ ($c=1.7$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta=7.28$–7.33 (5H, m), 5.56 (1H, s), 5.20 (1H, s), 5.08 (2H, s), 4.28–4.35 (2H, m), 4.11–4.19 (2H, m), 1.91 (2H, s, br), 1.69–1.76 (2H, m), 1.42 (9H, s), 1.17–1.26 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=175.7$, 175.4, 172.9, 172.4, 172.1, 156.8, 156.4, 155.9, 136.3, 136.0, 128.7, 128.4, 128.3, 82.0, 80.5, 67.7, 67.3, 61.9, 61.8, 54.5, 54.2, 53.9, 53.7, 53.2, 53.0, 52.8, 29.9, 28.7, 28.6, 28.5, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{21}H_{30}N_2O_8+H^+$: 439.2075 [*M*+H$^+$]; found: 439.2079.

(2*S*,7*R*)-7-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-8-ethoxy-8-oxooctanoic acid (25)

The title compound **25** was synthesized by the procedure as described for the preparation of compound **11** except for using **18** as a starting material over four steps in 37% yield as a pale yellow oil. $[\alpha]_D^{25}=+2.0$ ($c=2.2$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): $\delta=7.28$–7.33 (5H, m), 5.41–5.43 (1H, m), 5.08–5.12 (1H, m), 5.06 (2H, s), 4.09–4.33 (4H, m), 1.74–1.85 (2H, m), 1.60–1.66 (2H, m), 1.41 (9H, s), 1.31–1.38 (4H, m), 1.17–1.25 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=176.5$, 172.7, 156.2, 155. 9, 136.4, 128.7, 128.4, 128.3, 80.4, 67.2, 61.7, 53.9, 53.4, 32.7, 32.4, 28.5, 24.9, 24.9, 14.3 ppm; HRMS calcd for $C_{23}H_{34}N_2O_8+H^+$: 467.2388 [*M*+H$^+$]; found: 467.2386.

(2*S*,8*R*)-8-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-9-ethoxy-9-oxononanoic acid (26)

The title compound **26** was synthesized by the procedure as described for the preparation of compound **11** except for using **19** as a starting material over four steps in 45% yield as a pale yellow oil. $[\alpha]_D^{25}=+2.4$ ($c=3.3$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta=7.27$–7.33 (5H, m), 5.38–5.39 (1H, m), 5.10–5.12 (1H, m), 5.08 (2H, s), 4.09–4.35 (3H, m), 3.62–3.71 (1H, m), 1.78–1.80 (2H, m), 1.62–1.63 (2H, m), 1.42 (9H, s), 1.30–1.34 (6H, m), 1.18–1.26 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=176.9$, 176.6, 176.33, 173.3, 172.8, 172.5, 157.0, 156.7, 156.2, 155.8, 136.4, 136.4, 136.1, 128.7, 128.4, 128.3, 128.2, 81.7, 80.3, 67.7, 67.2, 61.7, 61.6, 54.8, 54.6, 54.04, 53.99, 53.5, 52.6, 52.5, 32.7, 32.6, 32.4, 29.9, 28.8, 28.5, 25.2, 25.1, 25.1, 14.4 ppm; HRMS (ESI): *m/z* calcd for $C_{24}H_{36}N_2O_8+H^+$: 481.2544 [*M*+H$^+$]; found: 481.2549.

(2*S*,9*R*)-9-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-10-ethoxy-10-oxodecanoic acid (27)

The title compound **27** was synthesized by the procedure as described for the preparation of compound **11** except for using **20** as a starting material over four steps in 45% yield as a pale yellow oil. $[\alpha]_D^{25}=+1.7$ ($c=2.9$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta=7.27$–7.33 (5H, m), 5.34–5.35 (1H, m), 5.08 (2H, s), 5.02–5.03 (1H, m), 4.09–4.33 (4H, m), 1.77–1.79 (2H, m), 1.61–1.63 (2H, m), 1.42 (9H, s), 1.31–1.35 (8H, m), 1.18–1.27 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): $\delta=176.8$, 176.6, 173.4, 172.9, 172.5, 156.7, 156.5, 156.2, 155.8, 136.4, 128.7, 128.4, 128.3, 128.2, 81.5, 80.3, 67.6, 67.2, 61.7, 54.8, 54.1, 53.5, 52.6, 32.8, 32.5, 29.9, 29.0, 28.5, 25.3, 25.7, 25.0, 14.4 ppm; HRMS (ESI): *m/z* calcd for $C_{25}H_{38}N_2O_8+H^+$: 495.2701 [*M*+H$^+$]; found: 495.2703.

(2*R*,6*S*)-6-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-7-ethoxy-7-oxoheptanoic acid (28)

The title compound **28** was synthesized by the procedure as described for the preparation of compound **11** except for using **23** as a starting material over four steps in 42% yield as a pale yellow oil. $[\alpha]_D^{25}=-1.6$ ($c=10.8$, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$): $\delta=7.28$–7.33 (5H, m), 5.51–5.52 (1H, m), 5.14–5.19 (1H, m), 5.05–5.12 (2H, m), 4.09–4.32 (4H, m), 1.83–1.86 (2H, m), 1.67–1.76 (2H, m), 1.41

(11H, s, br), 1.16–1.25 ppm (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 176.1, 175.6, 172.6, 172.2, 157.0, 156.3, 155.9, 155.7, 136.3, 135.9, 128.6, 128.3, 128.3, 128.1, 81.9, 80.3, 67.7, 67.2, 61.7, 61.6, 54.7, 54.4, 53.8, 53.2, 53.1, 32.10, 32.05, 31.9, 31.8, 28.4, 28.3, 21.4, 21.26, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{22}H_{32}N_2O_8+H^+$: 453.2231 [*M*+H$^+$]; found: 453.2234.

(2*R*,6*R*)-6-(((Benzyloxy)carbonyl)amino)-2-((tert-butoxycarbonyl)amino)-7-ethoxy-7-oxoheptanoic acid (29)

The title compound **29** was synthesized by the procedure as described for the preparation of compound **11** except for using **21** as a starting material over four steps in 36% yield as a pale yellow oil. $[\alpha]_D^{25}=-4.6$ (c = 2.2, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) (two rotamers were observed): δ = 7.28–7.32 (5H, m), 5.52–5.53 (1H, m), 5.21–5.31 (1H, m), 5.05–5.11 (2H, m), 3.62–4.31 (4H, m), 1.67–1.83 (4H, m), 1.39–1.41 (11H, m), 1.13–1.25 (3H, m); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 176.3, 175.4, 173.1, 172.7, 172.6, 172.2, 157.3, 156.5, 156.3, 156.2, 156.0, 136.4, 136.0, 128.6, 128.4, 128.4, 128.2, 81.8, 80.5, 80.3, 67.8, 67.3, 61.8, 61.6, 54.5, 53.8, 53.7, 53.2, 52.7, 32.3, 32.1, 31.9, 31.8, 28.5, 21.4, 21.2, 14.3 ppm; HRMS (ESI): *m/z* calcd for $C_{22}H_{32}N_2O_8+H^+$: 453.2231 [*M*+H$^+$]; found: 453.2233.

(2*R*,6*S*)-2-Amino-6-((*R*)-4-amino-4-carboxybutanamido)heptanedioic acid (33, iE-*meso*-DAP)

To a solution of compound **11** (50 mg, 0.11 mmol) in dichloromethane (3 mL) was added trifluoroacetic acid (1 mL) at 0 °C. After stirring for 1 h, trifluoroacetic acid was removed under vacuum. Triethylamine (33 mg, 0.33 mmol) and compound **39** (47 mg, 0.13 mmol) were added to the residue in dichloromethane (10 mL) at 0 °C. After stirring for 16 h at room temperature, the mixture was washed with aqueous 1 M HCl, aqueous sat. $NaHCO_3$, brine, dried over anhydrous $MgSO_4$, and concentrated. The residue was purified by flash column chromatography (50% EtOAc in hexanes, silica gel) to give **32** as a white solid (48 mg, 0.08 mmol, 70%). To a solution of **32** (50 mg, 0.09 mmol) in MeOH (4 mL) was added lithium hydroxide (21 mg, 0.9 mmol) and 5 drops of H_2O. After stirring at room temperature for 14 h, trifluoroacetic acid (1 mL) was added at 0 °C and stirred at room temperature for 1 h. The mixture was concentrated under vacuum and then purified by flash column chromatography (33% ammonium hydroxide in 1-propanol, silica gel). The purified product was dissolved in MeOH (5 mL), and 20% palladium hydroxide on activated charcoal (9 mg) was added to the mixture. The resulting mixture was stirred under 5 atm pressure of H_2 gas at room temperature for 1 h. The mixture was filtered through Celite, and the filtrate was evaporated. The residue was purified by flash column chromatography (33% ammonium hydroxide in 1-propanol, silica gel) to provide **33** as a white solid. $[\alpha]_D^{25}=-6.5$ (c = 0.8, D_2O); ^{1}H NMR (600 MHz, D_2O): δ = 4.24–4.27 (1H, m), 3.76–3.83 (2H, m), 2.48–2.51 (2H, m), 2.14–2.20 (2H, m), 1.83–1.97 (3H, m), 1.73–1.79 (1H, m), 1.41–1.58 ppm (2H, m); ^{13}C NMR (150 MHz, D_2O): δ = 177.6, 174.4, 174.4, 173.8, 54.5, 54.2, 31.4, 31.1, 30.6, 29.9, 26.2, 21.1 ppm; HRMS (ESI): *m/z* calcd for $C_{12}H_{21}N_3O_7+H^+$: 320.1452 [*M*+H$^+$]; found: 320.1455.

(2*R*,6*S*)-2-Amino-6-((*R*)-4-((*S*)-2-aminopropanamido)-4-carboxybutanamido)heptanedioic acid (34, A-iE-DAP)

The title compound **34** was synthesized by the procedure as described for the preparation of compound **33** except for using **31** as a coupling partner over five steps as a white solid. $[\alpha]_D^{25}=-0.4$ (c = 1.3, D_2O); ^{1}H NMR (600 MHz, D_2O): δ = 4.21–4.23 (1H, m), 4.12–4.18 (2H, m), 3.74–3.76 (1H, m), 2.37–2.40 (2H, m), 2.12–2.21 (1H, m), 1.96–2.07 (1H, m), 1.83–1.93 (3H, m), 1.68–1.77 (1H, m), 1.54 (3H, d, J = 7.14 Hz), 1.42–1.47 ppm (2H, m); ^{13}C NMR (150 MHz, D_2O): δ = 178.9, 177.9, 174.9, 174.7, 170.4, 54.9, 54.9, 54.6, 49.2, 32.3, 31.2, 30.1, 27.4, 21.2, 16.5 ppm; HRMS (ESI): *m/z* calcd for $C_{15}H_{26}N_4O_8+H^+$: 391.1823 [*M*+H$^+$]; found: 391.1825.

(2*R*,5*S*)-2-Amino-5-((*R*)-4-amino-4-carboxybutanamido)hexanedioic acid (35)

The title compound **35** was synthesized by the procedure as described for the preparation of compound **33** except for using **24** as a starting material over five steps as a white solid. $[\alpha]_D^{25}=-8.2$ (c = 3.3, D_2O); 1H NMR (600 MHz, D_2O): δ = 4.18–4.19 (1H, m), 3.74–3.81 (2H, m), 2.48–2.53 (2H, m), 2.12–2.21 (2H, m), 1.69–1.95 ppm (4H, m); ^{13}C NMR (150 MHz, D_2O): δ = 178.3, 178.2, 174.3, 174.3, 174.2, 174.1, 174.0, 54.8, 54.7, 54.7, 54.6, 54.4, 54.3, 54.2, 54.1, 31.6, 31.2, 27.4, 27.3, 27.1, 26.3, 26.2 ppm; HRMS (ESI): *m/z* calcd for $C_{11}H_{19}N_3O_7+H^+$: 306.1296 [*M*+H$^+$]; found: 306.1294.

(2*R*,6*S*)-2-Amino-6-((*R*)-4-amino-4-carboxybutanamido)-7-hydroxyheptanoic acid (36)

The title compound **36** was synthesized by the procedure as described for the preparation of compound **33** except for using **10** as a starting material over five steps as a white solid. $[\alpha]_D^{25}=-12.5$ (c = 2.5, D_2O); ^{1}H NMR (600 MHz, D_2O): δ = 3.87–3.95 (1H, m), 3.76 (1H, t, J = 6.1 Hz), 3.72 (1H, t, J = 6.3 Hz), 3.59 (1H, dd, J = 4.7, 11.6 Hz), 3.49 (1H, dd, J = 6.7, 11.6 Hz), 2.42–2.44 (2H, m), 2.11–2.15 (2H, m), 1.79–1.91 (2H, m), 1.57–1.61 (1H, m), 1.37–1.47 ppm (3H, m); ^{13}C NMR (150 MHz, D_2O): δ = 174.7, 174.6, 173.9, 63.5, 54.6, 54.1, 50.9, 31.6, 30.1, 29.6, 26.4, 20.8 ppm; HRMS (ESI): *m/z* calcd for $C_{12}H_{23}N_3O_6+H^+$: 306.1660 [*M*+H$^+$]; found: 306.1657.

(2*R*,7*S*)-2-Amino-7-((*R*)-4-amino-4-carboxybutanamido)octanedioic acid (37)

The title compound **37** was synthesized by the procedure as described for the preparation of compound **33** except for using **25** as a starting material over five steps as a white solid. $[\alpha]_D^{25}=-2.5$ (c = 0.2, D_2O); ^{1}H NMR (600 MHz, D_2O): δ = 4.15–4.17 (1H, m), 3.73–3.81 (2H, m), 2.46–2.49 (2H, m), 2.13–2.21 (2H, m), 1.80–1.91 (3H, m), 1.69–1.72 (1H, m), 1.35–1.45 ppm (4H, m); ^{13}C NMR (150 MHz, D_2O): δ = 179.1, 174.9, 174.1, 174.0, 55.0, 54.9, 54.6, 54.1, 31.2, 31.0, 30.9, 30.2, 30.2, 26.3, 24.9, 24.8, 24.0, 23.9 ppm; HRMS (ESI): *m/z* calcd for $C_{13}H_{23}N_3O_7+H^+$: 334.1609 [*M*+H$^+$]; found: 334.1608.

(2*R*,8*S*)-2-Amino-8-((*R*)-4-amino-4-carboxybutanamido)nonanedioic acid (38)

The title compound **38** was synthesized by the procedure as described for the preparation of compound **33** except for using **26** as a starting material over five steps as a white solid. $[\alpha]_D^{25}=-2.3$ (c = 1.0, D_2O); ^{1}H NMR (600 MHz, D_2O): δ = 4.13–4.15 (1H, m), 3.72–3.81 (2H, m), 2.45–2.49 (2H, m), 2.11–2.21 (2H, m), 1.78–1.90 (3H, m), 1.65–1.69 (1H, m), 1.32–1.48 ppm (6H, m); ^{13}C NMR (150 MHz, D_2O): δ = 179.4, 175.0, 174.1, 174.0, 55.2, 54.8, 54.1, 31.2, 31.1, 30.3, 27.9, 26.3, 24.8, 24.0 ppm; HRMS (ESI): *m/z* calcd for $C_{14}H_{25}N_3O_7+H^+$: 348.1765 [*M*+H$^+$]; found: 348.1767.

(2*R*,9*S*)-2-Amino-9-((*R*)-4-amino-4-carboxybutanamido)decanedioic acid (39)

The title compound **39** was synthesized by the procedure as described for the preparation of compound **33** except for using **27** as

a starting material over five steps as a white solid. $[\alpha]_D^{25} = -2.0$ ($c =$ 1.5, D_2O); ^1H NMR (600 MHz, D_2O): $\delta = 4.13–4.15$ (1H, m), 3.74–3.81 (2H, m), 2.47–2.50 (2H, m), 2.11–2.24 (2H, m), 1.78–1.92 (3H, m), 1.65–1.71 (1H, m), 1.32–1.48 ppm (8H, m); ^{13}C NMR (150 MHz, D_2O): $\delta = 179.7$, 175.1, 174.2, 174.1, 55.4, 54.8, 54.2, 31.32, 31.29, 30.3, 28.1, 27.9, 26.5, 25.0, 24.1 ppm; HRMS (ESI): *m/z* calcd for $C_{15}H_{27}N_3O_7 + H^+$: 362.1922 [M+H^+]; found: 362.1921.

(2*R*,6*S*)-2-Amino-6-((*R*)-4-((*S*)-2-aminopropanamido)-4-carboxybutanamido)-7-hydroxyheptanoic acid (40)

The title compound **40** was synthesized by the procedure as described for the preparation of compound **33** except for using **10** as a starting material and **31** as a coupling partner over five steps as a white solid. $[\alpha]_D^{25} = -3.4$ ($c = 1.0$, D_2O); ^1H NMR (600 MHz, D_2O): $\delta = 4.18–4.21$ (1H, m), 4.11–4.14 (1H, m), 3.87–3.91 (1H, m), 3.74–3.76 (1H, m), 3.59–3.62 (1H, m), 3.50–3.53 (1H, m), 2.31–2.41 (2H, m), 2.11–2.19 (1H, m), 1.80–2.02 (3H, m), 1.60–1.68 (1H, m), 1.56 (3H, d, $J = 7.08$ Hz), 1.36–1.51 ppm (3H, m); ^{13}C NMR (150 MHz, D_2O): $\delta = 177.8$, 175.4, 174.7, 170.8, 63.54, 63.50, 54.7, 54.6, 51.0, 49.2, 32.4, 30.1, 29.7, 27.6, 20.9, 16.6 ppm; HRMS (ESI): *m/z* calcd for $C_{15}H_{28}N_4O_7 + H^+$: 377.2031 [M+H^+]; found: 377.2030.

HEK293T Bioassay for Nod1 Stimulation: The NF-κB reporter plasmid containing three κB-binding sites (pGL2-ELMA-luciferase) was provided by Dr. S. L. Hsieh (Yang-Ming University, Taipei, Taiwan). The expression vector pCMV-Flag-Nod1 was a gift from Dr. John Reed (Sanford-Burnham Institute for Medical Research, La Jolla, California, USA). Ligand dependent NF-κB activation was determined by using 5×10^4 HEK293T cells transfected with pCMV-Flag-Nod1, NF-κB reporter plasmid and β-galactosidase expression vector (pSVlacZ) using the Lipofectamine 2000 reagent (Invitrogen) for 24 h. After treatment of the various ligands for another 24 h, cells were lysed in reporter lysis buffer (Promega). Subsequently, the lysates were reacted with commercial luciferase substrate provided in the luciferase assay system kit (Promega) and assayed by using a microplate luminometer (Packard, Meriden, CT, USA). Luciferase activity values were normalized to transfection efficiency monitored by β-galactosidase expression and presented as the folds of luciferase activity of the control group without pCMV-Flag-Nod1 transfection.

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Keywords: amino acids · immunology · innate immunity · Nod1 · Schöllkopf bis-lactim ethers · synthetic methods

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FULL PAPER

Peptides

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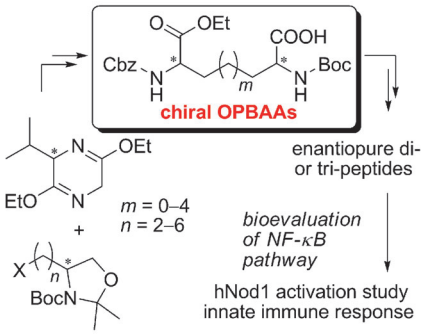
Expeditious Synthesis of Enantiopure, Orthogonally Protected Bis-α-Amino Acids (OPBAAs) and their Use in a Study of Nod1 Stimulation



Give it a nod: A concise preparation of structurally diverse OPBAAs bearing three orthogonally protecting groups and a spacer of varied length has been developed. Using these versatile building blocks, a series of small peptides inspired from peptidoglycan fragments were synthesized and then studied for their ability to activate human Nod1 receptor in the innate immune response through the NF-κB pathway.