Membrane Association Dictates Ligand Specificity for the Innate Immune Receptor NOD2

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I. Synthesis of 6-amino Peptidoglycan Derivatives

All chemicals were purchased from Sigma Aldrich, Alfa Aesar or Invivogen and used without further purification. Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates pre-coated with silica gel (250 µm, Sorbent Technologies). Flash chromatography was carried out on silica gel (60 Å, 40-63 µm), purchased from Sorbent Technologies. Preparative HPLC was performed on an Agilent Series 1100 using a Phenomenex Luna 5 µm C18 column (250 x 10.00 mm), or using a Maxi-Clean 800mg C18 column. NMR spectra were recorded on a Bruker AV 400 MHz and AV III 600 MHz spectrometers. IR spectra were obtained on an IR100 with an ATR Probe. Mass spectra (ESI) were obtained at the Mass Spectroscopy Facility at the Department of Chemistry, University of Delaware, (Shimadzu LCMS 2020). High Resolution Masses were obtained at the Mass Spectroscopy Facility at the Department of Chemistry, University of Delaware, (Thermo Q-Exactive Orbitrap). Synthesis of all compounds can be found in Supporting Information.

\[ \text{Scheme S1.} \]

\( N-((2R,3R,4R,5S,6R)-2-(benzyloxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide (4): \) Synthesized from N-acetylglucosamine according to exact literature precedent\(^{[S1]}\).

\( N-((2S,4aR,6R,7R,8R,8aS)-6-(benzyloxy)-8-hydroxy-2-phenylhexahydropyran[3,2-d][1,3]dioxin-7-yl)acetamide (5): \) 4 (16.0 g, 51.39 mmol) was dissolved in 125mL of anhydrous N,N-dimethylformamide, and benzaldehyde dimethyl acetal (23.1 mL, 154.2 mmol) and a catalytic amount of p-toluenesulfonic acid (1.77 g, 10.28 mmol) were added. The solution was stirred for 1.5 hrs at 60 °C and under vacuum. A second addition of benzaldehyde dimethyl acetal (23.1 mL, 154.2 mmol) was added and stirring continued for another 1.5 hrs at 60 °C and under vacuum. The dark brown solution was then cooled to room temperature and quenched...
with 315 mL of an aqueous saturated sodium bicarbonate solution and allowed to stir for 2 hrs. The white precipitate was filtered from the solution and washed with copious amounts of water followed by diethyl ether. White powder, mixture of anomers (18.8 g, 92%). NMR data agreed with the literature.\footnote{S2}

\((R)-2-(((2S,4aR,6R,7R,8R,8aS)-7-acetamido-6-(benzyl)-2-phenylhexahydropyrano[3,2-d][1,3]dioxin-8-yl)oxy)propanoic acid (6): 5 (4.68 g, 11.7 mmol) was co-evaporated with toluene (3x) and subsequently dissolved in 40 mL of anhydrous N,N-dimethylformamide with stirring at room temperature under \(N_2\) gas. Sodium hydride (60% in oil, 0.937 g, 23.4 mmol) was added and \(H_2\) gas evolved. The solution was stirred for 30 mins under \(N_2\) gas and then (S)-\((-\))-2-chloroproprionic acid (4.31 mL, 46.9 mmol) was added dropwise as \(H_2\) gas evolved. The solution was stirred an additional 30 mins under \(N_2\) gas and then sodium hydride (60% in oil, 0.937 g, 23.4 mmol) was added and \(H_2\) gas evolved. The solution was then allowed to continue stirring for 16 hrs while under \(N_2\) gas. The reaction was slowly quenched with water and then filtered. The filtrate was acidified (pH: 4-5) with aqueous 1N HCl. A precipitate was observed and the mixture was filtered to yield a white powder (3.90 g, 71% yield). NMR data agreed with the literature.\footnote{S3,S4} \([\alpha]_D^{20} +117^\circ \) (c 0.51, methanol); \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \(\delta\) 12.87 (s, 1H, COOH), 7.97 (d, \(J = 6.3\) Hz, 1H, NH-Acetate), 7.47 – 7.33 (m, 9H, Phenyls), 7.30 (hept, \(J = 3.9\) Hz, 1H, Phenyls), 5.70 (s, 1H, Acetal-\(CH\)), 5.04 (d, \(J = 3.5\) Hz, 1H, H\(_1\)), 4.70 (d, \(J = 12.4\) Hz, 1H, benzyl methylene’), 4.49 (d, \(J = 12.4\) Hz, 1H, benzyl methylene’’), 4.29 (q, \(J = 6.9\) Hz, 1H, CH-Lac), 4.15 (dd, \(J = 9.8, 4.3\) Hz, 1H, H\(_6\)‘), 3.83 – 3.66 (m, 5H, H\(_5\), H\(_4\), H\(_3\), H\(_2\)‘), 1.85 (s, 3H, Acetate-\(CH_3\)), 1.28 (d, \(J = 6.9\) Hz, 3H, CH\(_3\)-Propionic). \(^1\)C NMR (151 MHz, DMSO-\(d_6\)) \(\delta\) 175.70, 169.82, 138.05, 129.26, 128.73, 128.62, 128.08, 126.30, 100.73, 97.29, 82.01, 75.55, 75.53, 69.44, 68.34, 63.35, 53.98, 23.08, 19.15. HRMS. Calc. for C\(_{25}\)H\(_{30}\)O\(_8\)N [M+H]\(^+\): 472.19659; found 472.19808.

\((R)-methyl 2-(((2R,3R,4R,5S,6R)-3-acetamido-2-(benzyl)-5-hydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-4-yl)oxy)propanoate (7): Synthesized from 6 according to literature precedent.\footnote{S5}

\((R)-methyl 2-(((2R,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyl)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanoate (8): 7 (5.50g, 13.8 mmol) dissolved in 60 mL
of anhydrous dichloromethane. Solution cooled to 0 °C. Pyridine (5.60 mL, 69.0 mmol) added to solution. Methane sulfonyl chloride (1.50 mL, 19.4 mmol) added dropwise and reaction allowed to stir for 3 hrs. Reaction determined complete by TLC (7% methanol/dichloromethane) and reaction quenched with 4.5 mL water. Organic layer washed with 1N hydrochloride, saturated sodium bicarbonate, brine and dried with sodium sulfate. Organic layer concentrated, co-evaporated with toluene (3x) and subsequently dissolved in 30 mL of anhydrous dimethylformamide. Sodium azide (5.38 g, 82.8 mmol) added to solution and reaction refluxed at 70 °C for 14.5 hours. The reaction was cooled to RT and diluted with ethyl acetate. The organic layer was washed with water, 1 N hydrochloride, saturated sodium bicarbonate, brine and dried with sodium sulfate. The organic layer was evaporated and purified by column chromatography (Gradient: 1% methanol/dichloromethane to 7% methanol dichloromethane). White powder (4.77g, 82% yield). NMR data agreed with the literature\[551\].

Scheme S2.

(R)-methyl 2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(aminomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanoate (20): 8 (50.0 mg, 0.118 mmol) dissolved in 4 mL methanol, 3 mL water and 0.5 mL acetic acid with stirring at room temperature. 10% Palladium on carbon (15 mg, 0.014 mmol) was added and the reaction was degassed and stirred under H2 gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (5% acetonitrile in water with 0.1% trifluoroacetic acid – isocratic flow for 5 mins then 20 min gradient to 100% acetonitrile with 0.1% trifluoroacetic acid). White powder (21.6 mg, 60% yield). (Anomers – 1.00α : 0.21β – for simplicity 0.21β-H set to 1.00 β-H). 1H NMR (400 MHz, Methanol-d4) δ 5.34 (d, J = 3.2 Hz, 1H, α H-1), 4.68 – 4.61 (m, 2H, α CH-Lac, β H-1), 4.58 (q, J = 6.9 Hz, 1H, β CH-Lac), 4.00 – 3.91 (m, 2H, α H-5, β H-5), 3.73-3.74 (m, 6H, α and β methyl ester), 3.71 (d, J = 3.2 Hz, 1H, α 2-H), 3.64 (dd, J = 10.8, 8.5 Hz, 1H, α 3-H), 3.58 – 3.44 (m, 2H, β H-3, β H-2), 3.40 – 3.32 (m, 4H, α 4-H, α 6-H', β 4-H, β 6-H'), 3.02 (dd, J = 12.9, 8.5 Hz, 2H, α 6-H", β 6-H") 2.01 (d, J = 1.7 Hz, 6H, α and β acetyl) 1.41 (d, J = 7.0 Hz,
3H, α CH₃-Lac), 1.38 (d, J = 6.9 Hz, 1H, β CH₃-Lac). ¹³C NMR (101 MHz, Methanol-d₄) δ 177.00, 176.08, 174.74, 173.55, 97.40, 91.96, 82.07, 78.89, 76.93, 74.31, 73.95, 73.40, 69.23, 57.87, 55.49, 52.80, 52.61, 41.81, 23.07, 22.79, 19.23.

HRMS. Calc. for C₁₂H₂₃O₇N₂ [M+H]+: 307.14998; found 307.15091.

Scheme S3.

(S)-methyl 2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyloxy)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanoate (10): 8 (187.0 mg, 0.444 mmol) dissolved in 14 mL methanol. Subsequently 4 mL of 0.5M of potassium hydroxide was added and the reaction was monitored by TLC (7% methanol/dichloromethane) until complete. The solvent was then evaporated and the white solid was co-evaporated with toluene (3x) and subsequently dissolved in 6 mL of anhydrous N,N-dimethylformamide with stirring at room temperature under N₂ gas. 1-Hydroxybenzotriazole hydrate (wetted with not less than 20 wt % water) (90.0 mg, 0.533 mmol), 2,4,6-Collidine (176.0 µL, 1.33 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (127.7 mg, 0.666 mmol) were subsequently added and the reaction was allowed to stir for 2 mins at which point L-alanine methyl ester hydrochloride (123.9 mg, 0.888 mmol) was added. The reaction stirred 19 hrs and was quenched with 6 mL water and then diluted with ethyl acetate. The phases were separated and the organic phase was washed with 1 N hydrochloride, saturated sodium bicarbonate, brine and dried with sodium sulfate. The organic phase was evaporated and purified by column chromatography (5% methanol/dichloromethane) to yield a white powder (129.8 mg, 59% yield). ¹H NMR (600 MHz, Chloroform-d) δ 7.41 – 7.13 (m, 5H, Phenyl), 7.01 (d, J = 7.3 Hz, 1H, NH-Ala), 6.17 (d, J = 8.7 Hz, 1H, NH-Acetyl), 4.83 (d, J = 3.4 Hz, 1H, 1-H), 4.66 (d, J = 11.8 Hz, 1H, benzyl methylene’), 4.44 – 4.35 (m, 2H, benzyl methylene”, CH-Ala), 4.16 (td, J = 9.2, 3.7 Hz, 1H, 2-H), 4.08 (q, J = 6.6 Hz, 1H, CH-Lac), 3.76 – 3.71 (m, 1H, 4-H), 3.62 (s, 3H, methyl ester), 3.51 – 3.42 (m, 3H, 3-H, 6-H’, 6-H”), 3.38 (dd, J = 13.1, 6.0 Hz, 1H, 5-H), 1.82 (s, 3H, acetyl), 1.35
(d, J = 7.2 Hz, 3H, CH$_3$-Ala), 1.31 (d, J = 6.7 Hz, 3H, CH$_3$-Lac). $^{13}$C NMR (151 MHz, Chloroform-$d$): δ 173.25, 172.92, 170.43, 136.83, 128.63, 128.26, 128.25, 96.95, 80.17, 77.31, 71.57, 70.46, 69.92, 52.44, 52.38, 51.48, 47.99, 23.27, 18.96, 17.51. LRMS (ESI-Pos) for C$_{22}$H$_{31}$N$_5$O$_8$ (493.22): 494.30 [M+H]$^+$. 

(S)-methyl 2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanoate (19): 10 (20.0 mg, 0.041 mmol) dissolved in 1.6 mL methanol, 1.2 mL water and 0.2 mL acetic acid with stirring at room temperature. 10% Palladium on carbon (10 mg, 0.009 mmol) was added and the reaction was degassed and stirred under H$_2$ gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (5% acetonitrile in water with 0.1% trifluoroacetic acid – isocratic flow for 5 mins then 20 min gradient to 100% acetonitrile with 0.1% trifluoroacetic acid). White powder (quantitative yield). (Anomers – 1.00α : 0.15β – for simplicity 0.15 β-H set to 1.00 β-H). $^1$H NMR (400 MHz, methanol-$d_4$) δ 5.18 (d, J = 3.3 Hz, 1H, α-1-H), 4.62 (d, J = 8.4 Hz, 1H, β-1-H), 4.43 – 4.32 (m, 3H, β and α CH-Ala, α CH-Lac), 4.31 – 4.24 (m, 1H, β CH-Lac), 4.02 (td, J = 9.4, 8.8, 3.0 Hz, 1H, α-5-H), 3.92 (dd, J = 10.6, 3.3 Hz, 1H, α-2-H), 3.73 (s, 6H, α and β methyl ester), 3.65 (dd, J = 10.5, 8.8 Hz, 1H, α-3-H), 3.57 – 3.49 (m, 1H, β-5-H), 3.48 – 3.40 (m, 1H, β-3-H), 3.38 – 3.32 (m, 4H, α-4-H, α-6-H”, β-4-H, β-6-H”), 3.05 (dd, J = 12.8, 8.6 Hz, 2H, β-6-H”, α-6-H”), 1.96 (d, J = 3.8 Hz, 6H, α and β acetyl), 1.41 (d, J = 7.3 Hz, 6H, α and β CH$_3$-Ala), 1.39 (d, J = 6.8 Hz, 6H, α and β CH$_3$-Lac). $^{13}$C NMR (101 MHz, methanol-$d_4$) δ 175.98, 175.76, 174.58, 174.52, 174.35, 173.55, 97.36, 92.49, 83.01, 80.00, 78.71, 78.49, 73.42, 73.21, 72.71, 69.10, 57.76, 55.22, 52.88, 49.35, 42.05, 23.08, 22.82, 19.51, 17.37, 17.32. HRMS. Calc. for C$_{15}$H$_{28}$O$_8$N$_3$ [M+H]$^+$: 378.18709; found 378.18837.

Scheme S4.

(S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyloxy)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanoic acid (11): 10 (30.0 mg, 0.061
mmol) dissolved in 1.5 mL methanol and 1.5 mL 1,4-dioxane. The pH was raised to 10 with 0.5M potassium hydroxide and stirred for 1 hr. The pH was monitored and maintained at 10 and the reaction was determined complete by mass-spectrometry. Upon completion the solution was evaporated to yield a white powder (quantitative). 1H NMR (400 MHz, methanol-d4) δ 7.34 (ddt, J = 21.9, 14.0, 7.0 Hz, 5H, phenyl), 4.84 (d, J = 3.6 Hz, 1H, 1-H), 4.75 (d, J = 12.0 Hz, 1H benzyl methylene”), 4.54 (d, J = 12.0 Hz, 1H, benzyl methylene”), 4.26 (q, J = 6.7 Hz, 1H, CH-Ala), 4.17 (q, J = 7.1 Hz, 1H, CH-Lac), 4.03 (dd, J = 10.6, 3.6 Hz, 1H, 2-H), 3.82 (dd, J = 8.8, 6.0, 2.2 Hz, 1H, 5-H), 3.64 (dd, J = 10.4, 8.8 Hz 1H, 3-H), 3.55 – 3.40 (m, 3H, 4-H, 6-H”, 6-H’), 1.91 (s, 3H, Acetyl), 1.37 (d, J = 6.5 Hz, 3H, CH3-Ala), 1.35 (d, J = 7.0 Hz, 3H, CH3-Lac). 13C NMR (101 MHz, methanol-d4) δ 179.58, 174.77, 173.28, 138.59, 129.47, 129.46, 129.01, 97.43, 80.54, 78.64, 73.29, 72.06, 70.43, 54.63, 52.65, 51.52, 22.85, 19.52, 19.35. LRMS (ESI-Pos) for C21H29N5O8 (479.20): 480.25 [M+H]+.

(S)-2-((R)-2-(((S),3R,4R,5R,6R)-3-acetamido-6-(aminomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanoic acid (18): 11 (29.0 mg, 0.060 mmol) dissolved in 1.6 mL methanol, 1.2 mL water and 0.2 mL acetic acid with stirring at room temperature. 10% Palladium on carbon (10 mg, 0.009 mmol) was added and the reaction was degassed and stirred under H2 gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (5% acetonitrile in water with 0.1% trifluoroacetic acid – isocratic flow for 5 mins then 20 min gradient to 100% acetonitrile with 0.1% trifluoroacetic acid). White powder (18.5 mg, 84% yield). (Anomers – 1.00α : 0.51β – for simplicity 0.51 β-H set to 1.00 β-H). 1H NMR (400 MHz, Methanol-d4) δ 5.16 (d, J = 3.0 Hz, 1H, α 1-H), 4.61 (d, J = 8.3 Hz, 1H, β 1-H), 4.34 (tt, J = 14.7, 7.1 Hz, 4H, β and α CH-Ala, β and α CH-Lac), 4.02 (td, J = 10.0, 2.7 Hz, 1H, α 5-H), 3.94 (dd, J = 10.6, 3.0 Hz, 1H, α 2-H), 3.70 (dt, J = 26.3, 9.7 Hz, 2H, α 3-H, β 2-H, β 5-H), 3.56 – 3.44 (m, 1H, β 3-H, 3.37 (q, J = 10.8, 10.4 Hz, 2H, β 6-H’, β 4-H, α 6-H’, α 4-H), 3.05 (dd, J = 12.8, 8.6 Hz, 2H, α 6-H”, β 6-H”), 1.96 (d, J = 5.5 Hz, 6H, β and α Acetyl), 1.43 (d, J = 7.2 Hz, 6H, , β and α CH3-Ala), 1.39 (d, J = 6.5 Hz, 6H, β and α CH3-Lac). 13C NMR (101 MHz, Methanol-d4) δ 175.88, 175.79, 175.72, 175.62, 174.42, 173.58, 173.44, 172.57, 172.72, 172.04, 78.63, 78.51, 73.42, 73.18, 72.83, 69.07, 57.76, 55.17, 49.14, 42.05, 23.08, 22.83, 19.46, 17.68, 17.55. HRMS. Calc. for C14H26O8N3 [M+H]+: 364.17144; found 364.17281.
Scheme S5.

(S)-benzyl 4-(((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyloxy)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoate (15): 11 (31.0 mg, 0.065 mmol) dissolved in 1 mL of anhydrous DMF with stirring at room temperature under N\textsubscript{2} gas. 1-Hydroxybenzotriazole hydrate (wetted with not less than 20 wt % water) (13.1 mg, 0.078 mmol), 2,4,6-collidine (25.6 µL, 0.194 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (18.6 mg, 0.097 mmol) were subsequently added and the reaction was allowed to stir for 2 mins at which point 1-γ-benzyl Isoglutamine perchlorate\textsuperscript{[S6]} (30.6 mg, 0.091 mmol) was added. The reaction stirred for 13.5 hrs and was quenched with 2 mL water and then diluted with ethyl acetate. The phases were separated and the organic phase was washed with 1N hydrochloride, saturated sodium bicarbonate, brine and was dried with sodium sulfate. The organic phase was evaporated and purified by column chromatography (10% methanol/dichloromethane) to yield a white powder (38.1 mg, 84% yield). NMR data agreed with the literature\textsuperscript{[S5]}.

(S)-4-(((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(aminomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoic acid (3): Synthesized from 15 according to literature precedent\textsuperscript{[S5]}. HRMS. Calc. for C\textsubscript{19}H\textsubscript{34}O\textsubscript{10}N\textsubscript{5}[M+H]\textsuperscript{+}: 492.23002; found 492.23190.
**Scheme S6.**

(R)-benzyl 4-((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyloxy)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoate (14): 11 (31.0 mg, 0.065 mmol) dissolved in 1 mL of anhydrous DMF with stirring at room temperature under N₂ gas. 1-Hydroxybenzotriazole hydrate (wetted with not less than 20 wt % water) (13.1 mg, 0.078 mmol), 2,4,6-collidine (25.6 µL, 0.194 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (18.6 mg, 0.097 mmol) were subsequently added and the reaction was allowed to stir for 2 mins at which point d-γ-benzyl Isoglutamine perchlorate[S6] (30.6 mg, 0.091 mmol) was added. The reaction stirred for 16 hrs and was quenched with 2 mL water and then diluted with ethyl acetate. The phases were separated and the organic phase was washed with 1 N hydrochloride, saturated sodium bicarbonate, brine and was dried with sodium sulfate. The organic phase was evaporated and purified by column chromatography (10% methanol/dichloromethane) to yield a white powder (37.2 mg, 82% yield). NMR data agreed with the literature[S5].

(R)-4-((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(aminomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoic acid (2): Synthesized from 15.1 according to literature precedent[S5]. HRMS. Calc. for C₁₉H₃₄O₁₀N₅ [M+H]^+: 492.23002; found 492.23165.

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**Scheme S7.**
(S)-methyl 4-((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyloxy)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoate (13): 11 (31.0 mg, 0.065 mmol) dissolved in 1 mL of anhydrous DMF with stirring at room temperature under N₂ gas. 1-Hydroxybenzotriazole hydrate (wetted with not less than 20 wt % water) (13.1 mg, 0.078 mmol), 2,4,6-collidine (25.6 µL, 0.194 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (18.6 mg, 0.097 mmol) were subsequently added and the reaction was allowed to stir for 2 mins at which point L-γ-methyl isoglutamine hydrochloride (25.4 mg, 0.129 mmol) was added. The reaction stirred for 16 hrs and was quenched with 2 mL water and then diluted with ethyl acetate. The phases were separated and the organic phase was washed with 1 N hydrochloride, saturated sodium bicarbonate, brine and was dried with sodium sulfate. The organic phase was evaporated and purified by column chromatography (10% methanol/dichloromethane) to yield a white powder (29.3 mg, 73% yield). ¹H NMR (600 MHz, Methanol-d₄) δ 7.39 (d, J = 7.3 Hz, 2H, Aromatic), 7.35 (t, J = 7.5 Hz, 2H, Aromatic), 7.30 (t, J = 7.2 Hz, 1H, Aromatic), 4.89 (d, J = 3.5 Hz, 1H, 1-H), 4.74 (d, J = 11.9 Hz, 1H, benzyl methylene’), 4.54 (d, J = 11.9 Hz, 1H, benzyl methylene”), 4.37 (dd, J = 9.1, 4.9 Hz, 1H, CH-isoglutamine), 4.34 – 4.26 (m, 2H, CH-Lac, CH-alanine), 4.00 (dd, J = 10.6, 3.6 Hz, 1H, 2-H), 3.81 (ddd, J = 8.9, 6.2, 2.1 Hz, 1H, 5-H), 3.65 (s, 3H, methyl ester), 3.62 (dd, J = 10.4, 8.9 Hz, 1H, 3-H), 3.55 – 3.41 (m, 3H, 4-H, 6-H, 6’-H), 2.42 – 2.36 (m, 2H, isoglutamine methylene), 2.15 – 2.07 (m, 1H, isoglutamine methylene’), 1.97 – 1.85 (m, 4H, isoglutamine methylene”, acetyl), 1.39 (d, J = 7.2, 3H, CH₂-alanine), 1.37 (d, J = 6.6, 3H, CH₃-Lac). ¹³C NMR (151 MHz, Methanol-d₄) δ 176.10, 175.84, 174.94, 174.91, 173.38, 173.38, 138.61, 129.48, 129.45, 129.02, 97.46, 80.54, 78.41, 73.27, 72.13, 70.56, 54.75, 53.44, 52.62, 52.19, 50.46, 31.01, 28.43, 22.81, 19.64, 17.85. LRMS (ESI-Pos) for C₂₇H₃₉N₇O₁₀ (621.28): 622.25 [M+H]⁺.

(S)-methyl 4-((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(aminomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoate (17): 13 (24.0 mg, 0.039 mmol) dissolved in 1.6 mL methanol, 1.2 mL water and 0.2 mL acetic acid with stirring at room temperature. 10% Palladium on carbon (7 mg, 0.006 mmol) was added and the reaction was degassed and stirred under H₂ gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (5% acetonitrile in water with 0.1% trifluoroacetic acid – isocratic flow for 5 mins then 20 min.
1.00\% acetonitrile with 0.1\% trifluoroacetic acid). White powder (quantitative yield). (Anomers - 1.00α : 0.48β – for simplicity 0.48 β-H set to 1.00 β-H). 1H NMR (600 MHz, methanol-d4) δ 5.19 (d, J = 3.2 Hz, 1H, α 1-H), 4.66 – 4.62 (m, 1H, β 1-H), 4.38 (p, J = 6.0 Hz, 4H, β and α CH-isoglutamine, β and α CH-Lac), 4.31 (q, J = 7.2 Hz, 2H, β and α CH-alanine), 4.04 – 3.98 (m, 1H, α 5-H), 3.93 (dd, J = 10.6, 3.2 Hz, 1H, α 2-H), 3.76 – 3.70 (m, 1H, β 2-H), 3.67 (d, J = 7.3 Hz, 7H, β and α methyl ester, α 3-H), 3.61 – 3.45 (m, 2H, β 5-H, β 3-H), 3.40 – 3.32 (m, 4H, β and α 4-H, β and α 6-H’), 3.06 (dd, J = 12.9, 8.1 Hz, 2H, β and α 6-H”), 2.47 – 2.42 (m, 4H, β and α isoglutamine methylene), 2.15 (dt, J = 13.5, 7.2 Hz, 2H, β and α isoglutamine methylene’), 1.96 (t, J = 7.8 Hz, 8H, β and α isoglutamine methylene”, β and α acetyl), 1.39 (m, J = 6.5 Hz, 12H, β and α CH3-Ala, β and α CH3-Lac). 13C NMR (151 MHz, methanol-d4) δ 176.22, 175.94, 175.05, 175.01, 173.54, 92.52, 87.56, 80.50, 79.75, 77.99, 73.41, 73.06, 69.07, 64.71, 60.24, 55.14, 53.55, 52.24, 50.58, 45.13, 42.05, 31.05, 28.31, 22.84, 22.57, 19.65, 19.24, 17.82. HRMS. Calc. for C20H36O10N5 [M+H]^+: 506.24567; found 506.24801.

**Scheme S8.**

(R)-methyl 4-((S)-2-((R)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(azidomethyl)-2-(benzyloxy)-5-hydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoate (12): 11 (25.0 mg, 0.052 mmol) dissolved in 1 mL of anhydrous DMF with stirring at room temperature under N2 gas. 1-Hydroxybenzotriazole hydrate (wetted with not less than 20 wt % water) (10.6 mg, 0.063 mmol), 2,4,6-collidine (20.7 µL, 0.157 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (15.0 mg, 0.078 mmol) were subsequently added and the reaction was allowed to stir for 2 mins at which point D-γ-methyl isoglutamine hydrochloride (20.5 mg, 0.104 mmol) was added. The reaction stirred for 14 hrs and was quenched with 2 mL water and then diluted with ethyl acetate. The phases were separated and the organic phase was washed with 1 N hydrochloride, saturated sodium bicarbonate, brine and was dried with sodium...
sulfate. The organic phase was evaporated and purified by column chromatography (10% methanol/dichloromethane) to yield a white powder (26.2 mg, 81% yield). $^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 7.39 (d, $J = 7.2$ Hz, 2H, Aromatic), 7.35 (t, $J = 7.4$ Hz, 2H, Aromatic), 7.30 (t, $J = 7.2$ Hz, 1H, Aromatic), 4.88 (d, $J = 3.6$ Hz, 1H, 1-H), 4.74 (d, $J = 12.0$ Hz, 1H, benzyl methylene’), 4.55 (d, $J = 12.0$ Hz, 1H, benzyl methylene’’), 4.35 (dd, $J = 9.5$, 4.7 Hz, 1H, CH-isoglutamine), 4.31 (q, $J = 6.7$ Hz, 1H, CH-Lac), 4.26 (q, $J = 7.1$ Hz, 1H, CH-alanine), 3.99 (dd, $J = 10.6$, 3.6 Hz, 1H, 2-H), 3.80 (ddd, $J = 8.9$, 6.1, 2.2 Hz, 1H, 5-H), 3.66 (s, 3H, methyl ester), 3.62 (dd, $J = 10.5$, 8.8 Hz, 1H, 3-H), 3.54 – 3.39 (m, 3H, 4-H, 6-H, 6’-H), 2.40 (t, $J = 7.5$ Hz, 2H, isoglutamine methylene), 2.20 (ddd, $J = 12.7$, 7.9, 4.8 Hz, 1H, isoglutamine methylene’), 1.91 (s, 3H, acetyl), 1.89 – 1.83 (m, 1H, isoglutamine methylene’’), 1.38 (d, $J = 7.1$ Hz, 3H, CH$_3$-alanine), 1.36 (d, $J = 6.8$ Hz, 3H, CH$_3$-Lac). $^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 176.18, 176.10, 175.19, 174.81, 173.39, 138.58, 129.50, 129.49, 129.05, 97.46, 80.54, 78.40, 73.27, 72.10, 70.56, 54.75, 53.54, 52.62, 52.20, 50.76, 31.11, 28.01, 22.80, 19.58, 17.79. LRMS (ESI-Pos) for C$_{27}$H$_{32}$N$_7$O$_{10}$ (621.28): 622.25 [M+H]$^+$.

(R)-methyl 4-((S)-2-(((2S,3R,4R,5R,6R)-3-acetamido-6-(aminomethyl)-2,5-dihydroxytetrahydro-2H-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoate (16): 12 (28.0 mg, 0.045 mmol) dissolved in 1.6 mL methanol, 1.2 mL water and 0.2 mL acetic acid with stirring at room temperature. 10% Palladium on carbon (10 mg, 0.009 mmol) was added and the reaction was degassed and stirred under H$_2$ gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (95% water with 0.1% trifluoroacetic acid/ 5% acetonitrile with 0.1% trifluoroacetic acid to 100% acetonitrile with 0.1% trifluoroacetic acid – isocratic for 5 mins then gradient for 20 mins). White powder (quantitative yield). (Anomers – 1.00α : 0.12β – for simplicity 0.12 β-H set to 1.00 β-H). 1H NMR (400 MHz, Methanol-$d_4$) $\delta$ 5.21 (d, $J = 3.1$ Hz, 1H, α 1-H), 4.69 – 4.60 (d, $J = 8.1$ Hz, 1H, β 1-H), 4.33 (ddt, $J = 32.5$, 14.2, 6.9 Hz, 6H, β and α CH-isoglutamine, β and α CH-Lac, β and α CH-alanine), 4.04 – 3.98 (m, 1H, α 5-H), 3.90 (dd, $J = 10.6$, 3.2 Hz, 1H, α 2-H), 3.77 (dd, $J = 18.0$, 8.0 Hz, 1H, β 2-H), 3.67 (s, 7H, β and α methyl ester, α 3-H), 3.52 (dt, $J = 28.9$, 4.6 Hz, 2H, β 5-H, β 3-H), 3.41 – 3.33 (m, 4H, β and α 4-H, β and α 6-H’), 3.06 (dd, $J = 12.8$, 8.4 Hz, 2H, β and α 6-H’’), 2.44 (t, $J = 7.4$ Hz, 4H, β and α isoglutamine methylene), 2.23 (td, $J= 13.1$, 10.9, 5.3 Hz, 2H, β and α isoglutamine methylene’), 1.97 (d, $J = 3.6$ Hz, 6H, β and
α acetyl), 1.93 – 1.87 (m, 2H, β and α isoglutamine methylene”), 1.39 (t, J = 6.8 Hz, 12H, β and α CH₃-lac, CH₃-alanine). ¹³C NMR (101 MHz, methanol- d₄) δ 176.40, 176.25, 175.43, 174.83, 173.53, 92.44, 79.62, 77.93, 73.15, 69.07, 55.20, 53.75, 52.24, 50.79, 42.00, 31.14, 27.86, 22.84, 19.68, 17.67. HRMS. Calc. for C₂₀H₃₆O₁₀N₅ [M+H]+: 506.24567; found 506.24796.

Scheme S9.

N-((2S,3R,4R,5S,6R)-6-(azidomethyl)-2-(benzyloxy)-4,5-dihydroxytetrahydro-2H-pyran-3-yl)acetamide (21.1): Synthesized according to literature precedent[57].

N-((2S,3R,4R,5S,6R)-6-(aminomethyl)-2,4,5-trihydroxytetrahydro-2H-pyran-3-yl)acetamide (21): 21.1 (32.0 mg, 0.095 mmol) dissolved in 4.9 mL methanol, 6.5 mL water and 0.2 mL acetic acid with stirring at room temperature. 10% Palladium on carbon (20 mg, 0.019 mmol) was added and the reaction was degassed and stirred under H₂ gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (95% water with 0.1% trifluoroacetic acid/ 5% acetonitrile with 0.1% trifluoroacetic acid – isocratic for 5 mins then gradient for 20 mins). White powder (quantitative yield). ¹H NMR (600 MHz, Methanol- d₄) (Anomers – α 1.00 : β 0.37 – for simplicity 0.37 β-H set to 1.00 β-H ) δ 5.13 (d, J = 3.3 Hz, 1H, α 1-H), 4.65 (d, J = 8.3 Hz, 1H, β 1-H), 4.03 – 3.98 (m, 1H α 5-H), 3.86 (dd, J = 10.7, 3.3 Hz, 1H, α 1-H), 3.75 – 3.69 (m, 1H, α 3-H), 3.65 – 3.58 (m, 1H, β 2-H), 3.53 – 3.46 (m, 2H, β 3-H, β 5-H), 3.41 – 3.33 (m, 2H, α 6-H”, β 6-H”), 3.27 – 3.20 (m, 2H, α 4-H, β 4-H), 3.04 (dd, J = 13.0, 8.5 Hz, 2H, α 6-H”, β 6-H”), 2.00 (s, 3H, β acetyl), 1.99 (s, 3H, α acetyl). ¹³C NMR (151 MHz, Methanol- d₄) δ 172.90, 172.41, 95.83, 91.24, 74.01, 72.72, 72.33, 72.03, 70.73, 67.45, 57.32, 54.36, 40.74, 40.71, 21.52, 21.21. HRMS. Calc. for C₈H₁₇O₅N₂ [M+H]+: 221.11320; found 221.11402.
Scheme S10.

(S)-1-amino-5-methoxy-1,5-dioxopentan-2-aminium chloride (**L-γ-methyl Isoglutamine hydrochloride**): L-isoglutamine (0.490g, 3.35 mmol) dissolved in 30 mL of anhydrous methanol and chlorotrimethylsilane (0.935 mL, 7.37 mmol) added. Reaction stirred 24 hours at room temperature under N$_2$ gas. Solvent was then evaporated to yield a white powder (quantitative yield). $^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 3.96 (t, $J = 6.3$ Hz, 1H, methyne), 3.70 (s, 3H, methyl ester), 2.54 (td, $J = 7.9$, 4.0 Hz, 2H, methylene), 2.23 – 2.08 (m, 2H, methylene). $^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 174.15, 171.77, 53.55, 52.46, 30.05, 27.56. LRMS (ESI-Pos) for C$_6$H$_{12}$N$_2$O$_3$ (160.08): 161.10 [M+H]$^+$. 

![Scheme S10](image)

Scheme S11.

(R)-1-amino-5-methoxy-1,5-dioxopentan-2-aminium chloride (**D-γ-methyl isoglutamine hydrochloride**): D-isoglutamine (0.490g, 3.35 mmol) dissolved in 30 mL of anhydrous methanol and chlorotrimethylsilane (0.935 mL, 7.37 mmol) added. Reaction stirred 24 hours at room temperature under N$_2$ gas. Solvent was then evaporated to yield a white powder (quantitative yield). $^1$H NMR (600 MHz, Methanol-$d_4$) $\delta$ 3.96 (t, $J = 6.4$ Hz, 1H, methyne), 3.70 (s, 3H, methyl ester), 2.54 (td, $J = 7.8$, 4.0 Hz, 2H, methylene), 2.22 – 2.11 (m, 2H, methylene). $^{13}$C NMR (151 MHz, Methanol-$d_4$) $\delta$ 174.16, 171.77, 53.55, 52.46, 30.05, 27.56. LRMS (ESI-Pos) for C$_6$H$_{12}$N$_2$O$_3$ (160.08): 161.10 [M+H]$^+$. 

![Scheme S11](image)

Scheme S12.

(R)-benzyl 5-amino-4-((S)-2-((tert-butoxycarbonyl)amino)propanamido)-5-oxopentanoate (**22.1**): Synthesized according to literature precedent.$^{[S5]}$

![Scheme S12](image)
(R)-5-amino-4-((S)-2-aminopropanamido)-5-oxopentanoic acid (22): 22.1 (17.2 mg, 0.042 mmol) dissolved in 0.4 mL trifluoroacetic acid and stirred for 30 mins. Solvent evaporated to yield a light yellow oil, which was subsequently treated with ether to precipitate a white product. The product was filtered and dissolved in 2.0 mL water, 1.5 mL methanol and 0.25 mL acetic acid. 10% Palladium on carbon (9.2 mg, 0.009 mmol) was added and the reaction was degassed and stirred under H₂ gas for 16 hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified by HPLC (95% water with 0.1% trifluoroacetic acid/ 5% acetonitrile with 0.1% trifluoroacetic acid to 100% acetonitrile with 0.1% trifluoroacetic acid – isocratic for 5 mins then gradient for 20 mins). White powder (6.1 mg, 66% yield). ¹H NMR (600 MHz, Methanol-d₄) δ 4.42 (dd, J = 9.2, 4.9 Hz, 1H, isoglutamine methyne), 3.97 (q, J = 7.0 Hz, 1H, alanine methyne), 2.40 (t, J = 7.5 Hz, 2H, isoglutamine methylene), 2.15 (ddt, J = 12.8, 7.8, 5.0 Hz, 1H, isoglutamine methylene), 1.94 (ddt, J = 14.3, 9.2, 7.2 Hz, 1H, isoglutamine methylene), 1.52 (d, J = 7.1 Hz, 3H, methyl alanine). ¹³C NMR (151 MHz, methanol-d₄) δ 176.21, 175.94, 171.15, 53.85, 50.33, 31.17, 28.43, 17.61. HRMS. Calc. for C₈H₁₆O₄N₃ [M+H]⁺: 218.11353; found 218.11404.

Scheme S13.

2-Acetamido-2-deoxy-3-O-[(R)-1-(methoxycarbonyl)ethyl]-D-glucopyranose (23): 7 (0.030g, 0.075mmols) was dissolved in 2.85mLs of MeOH then 2.15mL of water was added. 20% Pd(OH)₂ (0.026g, 0.037mmols, 0.5eq) was carefully added. The reaction was evacuated and filled three times with H₂. The reaction was then left to stir under H₂ for 36hrs. The reaction was monitored by mass-spectrometry and filtered through celite upon completion. Purified using a Maxi-Clean 800mg C18 column (95% water/ 5% acetonitrile) to yield a white powder (15.4mg, 67% yield). (Anomers – α 1.00 : β 0.5 – for simplicity 0.5 β-H set to 1.00 β-H ). ¹H NMR (400 MHz, Methanol-d₄) δ 8.13 (d, J = 5.7 Hz, 1H, NH-Acetate), 5.32 (d, J = 3.0 Hz, 1H, α 1-H), 4.68
(q, J = 7.0 Hz, 1H, α CH-Lac), 4.64 – 4.56 (m, 2H, β CH-Lac, β 1-H), 3.83 (dd, J = 11.9, 2.4 Hz, 1H, β 6-H’), 3.77 (s, 4H, α methyl ester, α H-6’), 3.74 (m, 4H, β methyl ester, α H-5), 3.72 – 3.58 (m, 4H, α 2-H, α 3-H, α 6-H”, β 6-H”), 3.55 – 3.46 (m, 2H, β 2-H, α 4-H), 3.46 – 3.39 (m, 2H, β H-5, β H-3), 3.26 (ddd, J = 9.7, 5.7, 2.4 Hz, 1H, β 4-H), 2.01 (d, J = 1.1 Hz, 6H, β and α acetyl), 1.40 (d, J = 7.0 Hz, 3H, α CH3-Lac), 1.38 (d, J = 6.9 Hz, 3H, β CH3-Lac). 13C NMR (101 MHz, Methanol-d4) δ 175.83, 174.89, 173.28, 172.04, 95.94, 90.43, 81.32, 77.95, 76.49, 75.40, 71.99, 71.44, 71.07, 61.14, 61.01, 56.57, 54.23, 51.36, 51.17, 21.68, 21.44, 17.96, 17.87. HRMS. Calc. for C12H22O8N [M+H]+: 308.13399; found 308.13365

II. Protein Expression and Purification

**Human WT Flag-tagged NOD2 protein.** Flag-tagged human WT NOD2 expressed as previously described[8] with minor adaptions to the purification. All steps were performed at 4 °C. A pellet from a 50 mL culture was resuspended in 1 mL of cold lysis buffer (50 mM TrisHCl buffer, pH 7.4, containing 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 10% glycerol and protease tablet (Promega)). The cells were lysed by repeated passage through a 20 gauge needle (approximately 15 times) and incubated on ice for 1 hour. The soluble cell lysate was isolated by centrifugation at 15,000 rpm for 15 min and then incubated on a rotator with 100 µL ANTI-FLAG agarose resin (Sigma Aldrich) equilibrated with TBS buffer (50 mM TrisHCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM DTT) for 2 hours. In order to remove non-specifically bound proteins, the following washes were performed: overnight wash with 1 mL of TBS followed by several short washes (15 minutes) using a step gradient of NaCl (300 and 600 mM) in TBS buffer, pH 7.4 before returning to TBS buffer, 150 mM NaCl by three additional 1 mL washes of TBS. The FLAG-tagged human WT NOD2 protein was eluted from the resin by incubating with 1.2 mL of 0.2 mg/mL 3x FLAG peptide in TBS buffer for two hours at 4 °C. The supernatant containing FLAG-tagged human WT NOD2 was collected by centrifugation at 5000 rpm for 30 seconds. Protein was immediately analyzed by SDS-PAGE gel to assess purity and concentration. Purified protein was kept at 4°C and was used within three days of purification.

**Human GST-tagged NOD2 protein.** GST-tagged NOD2 was generated by cloning WT NOD2 into the pFastBacM30b baculovirus expression vector (EMBL). NOD2 was amplified from the
pBKCMV vector using primers (forward: 5’GTCAATGGATCCATGGGGGAAGAGGGTGG3’ and reverse: 5’ CAATGCGGCCGCTCAAAGCAAGAGTCTG3’), and inserted into the pFastBacM30b vector using restriction sites BamH1 and NotI. Techniques described in the Guide to Baculovirus Expression Vector Systems and Insect Cell Culture Techniques (Invitrogen Life Technologies) were used to generate, isolate, and analyze recombinant vectors and bacmids, as well as to initially transfecet insect cells. Briefly, overexpression of the desired proteins was carried out in Sf21 cells infected with 10% of the desired recombinant baculovirus (P4) and incubated on a shaker at room temperature for two days. Cells were harvested by centrifugation at 800 rpm for 10 minutes, and pellets were stored at -80°C until purification. Purifications were performed at 4 °C. A pellet from a 50 mL culture was re-suspended in 1 mL of cold lysis buffer (50 mM TrisHCl, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 10% glycerol, and protease tablet (Promega); pH 7.4) and lysed by repeated passage through a 20 gauge needle (approximately 15 times) for 1.5 hours. Soluble lysate was isolated by centrifugation at 15,000 rpm for 15 min. The supernatant was applied to 1 mL of Glutathione Sepharose resin (GE Healthcare) on a gravity column equilibrated with TBS buffer (50 mM TrisHCl, 150 mM NaCl, and 1 mM DTT; pH 7.4) and allowed to incubate on the shaker for 3 hours. In order to remove nonspecifically bound protein, the system was washed using a step gradient of NaCl (300 and 600 mM) in TBS buffer, pH 7.4, and finally returned to TBS buffer, pH 7.4. Protein was eluted by incubating overnight with PreScission protease in TBS. The eluate was collected and immediately analyzed on SDS-PAGE gels to assess purity and concentration. Purified protein was kept at 4°C and was used within three days of purification.

Human WT Flag-tagged NOD1 protein. NOD1 cDNA was received from the Podolsky lab. FLAG-tagged NOD1 was generated by cloning WT NOD1 into the pFastBac-CFlag baculovirus expression vector using NdeI and NotI restriction sites. Techniques described in the Guide to Baculovirus Expression Vector Systems and Insect Cell Culture Techniques (Invitrogen Life Technologies) were used to generate, isolate, and analyze recombinant vectors and bacmids, as well as to initially transfet insect cells. Overexpression of the desired proteins was carried out in Sf21 cells infected with 10% of the desired recombinant baculovirus and incubated on a shaker at room temperature for two days. Cells were harvested by centrifugation at 800 rpm for 10 minutes, and pellets were stored at -80°C until purification. Purifications were performed at 4 °C. A pellet from a 50 mL culture was re-suspended in 1 mL of cold lysis buffer (50 mM
TrisHCl, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 10% glycerol, and protease tablet (Promega); pH 7.4) and lysed by repeated passage through a 20 gauge needle (approximately 15 times) for 1.5 hours. Soluble lysate was isolated by centrifugation at 15,000 rpm for 15 min. The soluble cell lysate was incubated with 100 µL ANTI-FLAG agarose resin (Sigma Aldrich) equilibrated with TBS buffer (50 mM TrisHCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM DTT) on a rotator for 2 hours. In order to remove non-specifically bound proteins, the following washes were performed: overnight wash with 1 mL of TBS followed by several short washes (15 minutes) using a step gradient of NaCl (300 and 600 mM) in TBS buffer, pH 7.4 before returning to TBS buffer, 150 mM NaCl by three additional 1 mL washes. The FLAG-tagged human WT NOD2 protein was eluted from the resin by mixing with 1.2 mL of 0.2 mg/mL 3x FLAG peptide in TBS buffer. The supernatant containing FLAG-tagged human WT NOD2 was collected by centrifugation at 5000 rpm for 30 seconds. Protein was immediately analyzed by SDS-PAGE gel to assess purity and concentration. Purified protein was kept at 4°C and was used within three days of purification.

Protein Purity and Concentration. Protein purity and concentration was assessed by SDS-Page gel and imaged on the Bio-rad Gel Doc EZ Imager using Image Lab 5.0. A BSA concentration standard curve was generated using the volume (densometer) feature in Image Lab 5.0. All samples for gel analysis were prepared by mixing 10 µL of sample with 2.5 µL running buffer, heat denaturing at 100°C, followed by quick centrifugation before loading 11 µL on the gel. The ladder (PageRuler Broad Range Unstained Protein Ladder, ThermoScientific) was used as a running and staining control for all gels. Protein concentration was determined after normalization of the gel using volume (integral) values of protein ladder, and then applying integral values of purified protein to the established BSA concentration standard curve.
Figure S1. Gel images of NOD1 and NOD2 purified from insect cells. Lane 1: WT NOD2 (Flag-affinity purified), Lane 2: WT NOD2 (GST-affinity purified), Lane 3: NOD1 (Flag-affinity purified).

III. Surface Plasmon Resonance

All SPR experiments were performed on a BIAcore 3000 instrument. Conditions for preparing the chip, mixed-SAM solutions, coupling and binding assay methods were adapted from prior work with minor changes\(^{[8,10]}\). Briefly, glycerol, a stabilizing agent (cryo-protectant) which can complicate SPR analysis, was successfully removed from the wash and elution steps of the NOD2 purification protocol. Second, an additional purification scheme utilizing a cleavable N-terminal GST-tag was developed to produce tag-less protein for use in these studies. The construct of WT NOD2 was cloned into the pFastBacM30b (European Molecular Biology Laboratory - Heidelberg) vector to yield an N-terminal GST tag followed by a precision protease cleavage site. Third, the composition of our SPR running buffer was adjusted to an MES buffer system at pH: 6.5. The NOD2 and MDP interaction was previously established to be strongest at pH 6.5, and therefore the Biacore 3000 instrument is maintained at this pH\(^{[8]}\). All NOD2 solutions are diluted into the SPR running buffer to give the best match between the running
buffer and analyte buffer. Lastly, we observe a more appropriate lower background signal when our blank lane is untransformed compared to the blank lanes with ethanolamine coupled and switched our method accordingly.

Preparation of Mixed-SAMs. Gold chips (GE Healthcare Lifesciences) were stored at 4 °C and handled only under laminar flow prior to mixed-SAM formation. Thiols stored at -80 °C as 50 mM solutions in ethanol aliquoted into vials at volumes appropriate for approximately 1 – 2 uses. Gold chips were immersed in a 1% mole fraction of degassed ethanol solution (2mM total thiol concentration, 2 mL) of hexa(ethylene glycol)-carboxylic acid ((EG)₆CO₂H)-terminated thiol (SensoPath Technologies) in tri(ethylene glycol) ((EG)₃OH)-terminated thiol (SensoPath Technologies) for 24 – 48 hours. The chip was removed, rinsed with ethanol, dried with nitrogen and mounted onto a cassette following the protocol from the SIA Kit (GE Healthcare Lifesciences).

Immobilization of Ligands on SAMs. Ligand immobilizations were performed on a Biacore 3000 instrument (GE Healthcare) using a 10 μL min⁻¹ flow rate, aqueous solutions of 0.1 M N-hydroxysuccinimide (NHS) and 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), 1X phosphate-buffered saline (PBS) (prepared from 10X Lonza BioWhittaker without Ca²⁺ and Mg²⁺, pH 7.4, filtered and degassed) and 2.3 mM ligand solutions (prepared by dilution of a 20mM ligand stock into 1X PBS buffer that has been raised to pH 8.5 with an aqueous saturated sodium bicarbonate solution). Chips contain four lanes and were prepared immediately before use; 6-amino MDP-(LD) and an untransformed lane was included in every chip to act as a positive control and blank lane, respectively. Additionally, two new ligands were immobilized to separate lanes on each chip. Chip surfaces were equilibrated with the 1X PBS running buffer followed by transformation of the surface carboxylic acid groups into NHS esters by flowing a mixture of 0.05 M NHS and 0.20 M EDC over the surface for 7 minutes (the mixture is prepared by the automated robotics of the instrument using the DILUTE command) after which the system was returned to 1X PBS running buffer for 1.5 minutes. Ligand solutions were applied to separate flow cells for seven minutes to form amide bonds by displacement of the NHS esters. The system was returned to 1X PBS running buffer for 1.5 minutes and then the surface was deactivated by washing with 1X PBS, pH 8.5. Following coupling the system was returned to
1X PBS running buffer for 23 minutes. Afterwards buffer was passed at a high rate of flow over the SAM surface using the UNCLOG command.

Equilibrium Analysis of NOD2. Solutions (0, 0.65, 3.25, 6.5, 12.5, 25.0, 32.5, 65.0, 130 nM) of purified WT NOD2 were prepared by dilution of the stocks into the SPR running buffer (10 mM MES, 150 mM NaCl, 3 mM EDTA, 0.005% P20, pH 6.5, filtered and degassed). NOD2 solutions were applied to the chip surfaces in triplicate at a flow rate of 3 uL min⁻¹ for twenty minutes using the KINJECT command, washed with MBS running buffer for ten minutes, 0.5% (w/v) sodium dodecyl sulfate (SDS) for three minutes and then MBS running buffer for ten minutes. The binding of NOD2 to the chip was recorded in resonance units (RU) when the injection reached equilibrium. The similar NLR protein NOD1 (nucleotide-binding oligomerization domain-containing 1) and BSA (Bovine Serum Albumin) were applied to a subset of the ligands as negative protein controls (concentration range 0-260 nM).

Data Analysis. Data analyzed using Scrubber2 (BioLogic Software) and binding curves generated from SigmaPlot (Systate Software). Sensorgrams were double referenced using an untreated flow cell and blank injections to correct for artifacts and non-specific binding\[^{S10,S11}\]. The KD values were obtained by plotting change in resonance units (RU) at equilibrium binding as a function of varying ligand concentration and fitting the resulting points to a simple single site binding model given by

\[
y = \frac{y_{\text{max}}(x)}{K_D + (x)}
\]  

(3-1)

Representative sensorgrams of purified NOD2 applied to ligand surfaces are shown below as response units over time (seconds).
Figure S2. Sensorgrams of WT NOD2 applied to the ligand surfaces of the peptidoglycan library. Sensorgrams processed using Scrubber2 (BioLogic Software).

Figure S3. Binding curves of purified WT NOD2 to the peptidoglycan surfaces as established by SPR. Experimental methods and data analysis are described in Supporting Information.
Figure S4. Sensorgrams of BSA (top) and NOD1 (bottom) applied to a subset of the peptidoglycan library. No specific binding is detected for BSA or NOD1 to these ligands.

IV. Backscattering Interferometry

*Detection of Ligand Binding to Purified Protein.* The interactions of purified NOD2 with the peptidoglycan derivatives were examined by adding increasing concentrations of each peptidoglycan ligand to a fixed concentration (10nM) of either purified NOD2 or NOD1 (Figure S1) in PBS buffer (pH 6.5). Each solution was allowed to incubate for 40 minutes at room temperature to assure the achievement of binding equilibrium. Samples were introduced into the BSI instrument as per standard methods and the resulting changes in phase plotted against ligand concentration. Values observed for NOD1 under otherwise identical conditions were subtracted from the NOD2 values. The resulting curves are shown in Figure S5; fits to a single site binding model are shown. Each experiment was repeated in triplicate giving the range of results shown in Table 1.

*Native Membrane Vesicles.* A cell pellet containing approximately 1x10^8 cells of either HEK293T or HEK293T-Nod2Myc/tet-op was re-suspended in 10 mL of ice-cold buffer (HBSS, 1x EDTA-free, broad-spectrum protease inhibitors). The solution was then probe-sonicated to clarity in an ice bath and transferred to a 220 nm Millipore Ultrafree-MC centrifuge tube filter. The resulting solutions were centrifuged for 2 h at 6,000 g and 4°C. All solution that passed
through the centrifuge tube filter was collected and stored at 4°C for up to four days. Vesicle sizes were determined using a Wyatt Technologies DynaPro dynamic light scattering apparatus, and lipid concentration estimated using FM1-43FX membrane stain (Invitrogen Corporation, Carlsbad CA).

Detection of Ligand Binding to Small Unilamellar Vesicles. Ligand binding to small unilamellar vesicles (SUV’s) was accomplished by incubating either a fixed amount of SUV suspension, or purified protein (NLRs were purified from insect cells as described above), with varying concentrations of ligands for 40 minutes in the dark at room temperature. Solutions were deposited in the channels of a microfluidic chip for analysis using a backscattering interferometer (BSI). Microfluidic chips were passivated prior to the experiment using perfluoro-octyldecyl trichlorisilatrane and rinsed extensively between each sample. The microfluidic devices were maintained at 25°C using a feedback-controlled peltier system. The high-contrast interference fringes produced by each sample were generated using a fiber-coupled HeNe laser as a light source and recorded on a CCD camera. Measurements were analyzed using a combination of in-house software, Microsoft Excel, and OriginPro.

Data Analysis. Spatial changes in interference fringes are measured in near real-time using high-resolution interference fringes and a fast Fourier transform FFT. In the general case, a Fourier transform is defined as

\[ A(x) = F[a(y)] = a(y)e^{-ij2f_y}dy \] (4-1)

Where A(x) is the complex Fourier transform of the function a(x), F[x] denotes the Fourier transform operation, y is a spatial variable, and f is a spatial frequency in the Fourier domain. For a particular set of characteristic frequencies, it is possible to calculate the observed phase change by evaluating the real and imaginary parts of the Fourier transform at a given frequency. As a result, differences in phase changes can be calculated for different ligand-receptor binding pairs. It was observed that only cognate ligand-binding interactions created substantial differences in phase when compared to binding-nonbinding pairs\textsuperscript{[S12]}. For example, the maximal binding phase
change for a particular sample is typically of the magnitude ~0.01, while non-binding phase changes are approximately 10 times lower. By plotting phase changes as a function of varying ligand concentration and fitting the resulting points to a simple single site binding model given by

\[ y = \frac{y_{\text{max}}(x)}{K_D + (x)} \]

where \( y \) is the observed, calculated phase change, \( y_{\text{max}} \) is the observed, maximal, calculated phase change, \( x \) is the ligand concentration and \( K_D \) is the equilibrium dissociation constant. By incubating samples for 8 hours and not removing excess ligand, it can be reasonably implied that the observed binding systems are in a state of dynamic equilibrium.

The interactions of purified NOD2 or NOD2 presented in native membrane vesicles with the peptidoglycan derivatives were examined by adding increasing concentrations of each peptidoglycan ligand to a fixed concentration.
Figure S5. Plots (0-100 nM) of BSI measurements with purified NOD2 and the peptidoglycan library. Lines represent the best fits to the entire concentration range, not just the data points shown.

Additional control experiments for the NOD2 NCM binding experiments are included in Figures S6 and S7.

BSI is a sensor that reports on a universal signal, changes in refractive index, and thus measurements of species of interest need to be performed relative to systems of suitable complexity to account for nonspecific interactions, changes in the bulk solution, etc. In this case,
we observe nonspecific binding of the ligands to the HEK samples, but the final, differential measurement reflects only the specific interactions with Nod2.

**Figure S6.** Example of BSI reference measurements on vesicles derived from the HEK293T cells and BSI response was measured: (A) HEK293T with 2. (B) Nod2Myc/tet-op with 2. (C) Data from A subtracted from B to yield the final binding curve (Figure 4, main text).

**Figure S7.** Additional BSI measurements on vesicles derived from the HEK293T-NOD2myc/Tet-op cells in which NOD2 is expressed. (A) Negative control compounds: iE-DAP (red), muramic acid (green), and 6-amino-glucose (blue). (B,C) Exploration of ligand 18 binding. Panel B is the same as in Figure 5, showing the loss and leveling off of BSI signal with increasing concentrations of 18. Panel C shows an analogous experiment in which 0-75 nM of ligand 18 was introduced in the first part of the experiment, followed by the addition of ligand 2. The x-axis shows total ligand concentration (18 + 2). The maintenance of a stable signal, as opposed to a decrease, suggests that compound 18 induces a change in one or more components of the native-cell membrane vesicles that is not induced by 2.

Binding data for two compounds, MDP-(LD) and N-acetyl muramyl methyl ester, are included in Figure S8. The label free BSI technique does not require the 6-amino modification to the
ligand for immobilization, so select 6-hydroxyl compounds were tested for binding NOD2 in BSI experiments. MDP-(LD) is the current natural minimal fragment known to initiate a NOD2 NF-κB response, and N-acetyl muramyl methyl ester is the 6-hydroxyl version of our proposed minimal binding motif for NOD2. Both ligands bind NOD2 in the NCM environment with low nanomolar affinity (3-30 nM), the same KD range as the 6-amino versions of these compounds.

Figure S8. BSI measurements on N-acetyl muramyl methyl ester (23) and MDP-(LD) (1).
Figure S9. NOD2 western blot analysis of the native-membrane vesicles prepared from HEK293T-NOD2myc/Tet-op cells in which the protein is expressed using a myc antibody.

V. NF-κB Activation Assay

Plasmids. Myc-tagged WT NOD2 in pBKCMV vector was used as previously reported\textsuperscript{[S13]}.

Reporter Assay with transfected Nod2 variants. HEK293T cells (ATCC, Manassas, VA) were cultured in DMEM, 10% FBS (Atlantic Biologicals), 2 mM glutamine, 1% penicillin/streptomycin and grown in a humidified incubator at 37°C and 5% CO2. Cells were transfected for 20 hours with Lipofectamine LTX reagent (Invitrogen), 0.1 ng of NOD2 (WT, R702W, G908R, 1007fsinsC) or NOD1, 10 ng of pGL4.10 NF-κB luciferase reported plasmid and 1.0 ng of Renilla luciferase plasmid. Activity was induced by incubating 20 μM – 200 μM of compounds with transfected cells. After 12 hours of treatment, NF-κB luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s instructions and normalized to Renilla luciferase activity.

Reporter Assay with regulated Nod2 cell lines. HEK293T (control cell line) and HEK293T-NOD2myc/Tet-op cells\textsuperscript{[S13]} were cultured in DMEM, 10% FBS (Atlantic Biologicals), 2 mM glutamine, 1% penicillin/streptomycin and grown in a humidified incubator at 37°C and 5% CO2. Activity was induced by incubating 20 or 200 μM of compounds with transfected cells. After 8 hours of treatment, NF-κB luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s instructions and normalized to Renilla luciferase activity.
Figure S10. Experiments to test the role of lipofectamine for a representative set of compounds (2, 20, 22) and the response of all candidate ligands to the control HEK293T cells: relative NF-κB activation of synthetic peptidoglycan fragments in Hek293T and HEK293T-NOD2Myc/tet-op cells. Relative luciferase activity was measured after eight hours of stimulation with peptidoglycan fragments pre-incubated with or without lipofectamine LTX.
Figure S11. Relative NF-κB activation of the MDP-(LD) components. The dipeptide-(LD) (22) and N-acetyl muramic acid were tested for their ability to stimulate a NOD2-dependent inflammatory response individually and together. The natural ligand, MDP-(LD) (1), as well as the proposed minimal NOD2 binding motif sans the 6-amino modification, N-acetyl muramyl methyl ester (23), and two negative binders (the proposed NOD1 ligand, iE-DAP, and 6-amino glucose), were also tested for their ability to initiate a NOD2-dependent NF-κB response.
VI. $^1$H and $^{13}$C NMR Spectra

$^1$H and $^{13}$C NMR Spectra for 6 (DMSO-d$_6$)
$^1$H and $^{13}$C NMR Spectra for 20 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 10 (Chloroform – d)
$^1$H and $^{13}$C NMR Spectra for 19 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 11 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 18 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 13 (Methanol-d4)
$^1$H and $^{13}$C NMR Spectra for 17 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 12 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 16 (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for 21 (Methanol – d4)
\[
\text{HO} - \text{H}_2\text{N} - \text{Z} - \text{O} - \text{OH} \\
\text{HO} - \text{AcHN} \\
\text{21}
\]
$^1$H and $^{13}$C NMR Spectra for L-$\gamma$-methyl isoglutamine hydrochloride (Methanol – d4)
\(^1\)H and \(^{13}\)C NMR Spectra for \textbf{d-\(\gamma\)-methyl isoglutamine hydrochloride} (Methanol – d4)
$^1$H and $^{13}$C NMR Spectra for **Dipeptide-(LD) (22)** (Methanol – d4)
22: Dipeptide-(LD)
22: Dipeptide-(LD)
$^1$H and $^{13}$C NMR Spectra for 23 (Methanol – d4)
VII. High Resolution Mass Spectrometry

HRMS Spectra for 6

KL-3-89 #55-98 RT: 0.25-0.44 AV: 44 NL: 2.47E8
T: FTMS + p ESI Full ms [100.00-1500.00]
HRMS Spectra for 20

C_{12}H_{23}O_{7}N_{2} = 307.14998
3.02176 ppm
HRMS Spectra for 19

JM3-95 #53-102  RT: 0.24-0.46  AV: 50  NL: 3.57E9
T: FTMS + p ESI Full ms [100.00-1500.00]

C_{15}H_{28}O_8N_3 = 378.18709  3.39414 ppm
HRMS Spectra of 18

JMB-115 #53-101  RT: 0.24-0.46  AV: 49  NL: 1.93E9
T: FTMS + p ESI Full ms [100.00-1500.00]

C_{14}H_{26}O_{8}N_{3} = 364.17144
3.74546 ppm

364.17281

182.59028

159.58736

203.10348

233.11412

276.15650

322.16210

346.16237

378.18840

401.15434

506.24795

564.72905

467.11370

656.30033

616.62060

564.72905
HRMS Spectra for 3

JMI-105 #53-91  RT: 0.24-0.41  AV: 39  NL: 1.45E9
T: FTMS + p ESI Full ms [100.00-1500.00]

C_{19}H_{34}O_{10}N_{5} = 492.23002
3.81536 ppm
HRMS Spectra for 2

JM7-67 #56-102  RT: 0.25-0.46  AV: 47  NL: 1.25E9  
T: FTMS + p ESI Full ms [100.00-1500.00]

C_{19}H_{34}O_{10}N_{5} = 492.23002  
3.31493 ppm
HRMS Spectra for 17

JMI-21 #53-104  RT: 0.24-0.47  AV: 52  NL: 9.63E8
T: FTMS + p ESI Full m/z [100.00-1500.00]

506.24801  C_{20}H_{36}O_{10}N_{5} = 506.24567  4.62894 ppm

203.10334  185.09285  272.60098  253.62744  326.13330  304.15142
304.15142  326.13330  346.16213  363.18859  488.23768  424.17355
424.17355  528.22978  543.22966  577.28488  629.27765  674.20216
674.20216  346.16213  231.11664  424.17355

S70
HRMS Spectra of 16

JM4-9 #55-96 RT: 0.25-0.44 AV: 42 NL: 1.87E9
T: FTMS + p ESI Full ms [100.00-1500.00]

C_{20}H_{36}O_{10}N_{5} = 506.24567
4.51796 ppm
HRMS Spectra of 20

JM6-113 #53-97 RT: 0.24-0.44 AV: 45 NL: 1.62E9
T: FTMS + p ESI Full ms [100.00-1500.00]

221.11402
C₉ H₁₇ O₆ N₂ = 221.11320
3.70409 ppm

185.09284

203.10337

243.09588

276.02766

307.15095

360.22541

423.21028

463.20301

485.18564

509.19638

552.28934

593.16101

644.30099
HRMS Spectra of 22: Dipeptide-(LD)

JM6-71 #62-102  RT: 0.28-0.46  AV: 41  NL: 1.44E9
T: FTMS + p ESI Full ms [100.00-1500.00]

Molecular Formula: C₈H₁₆O₄N₃
Exact Mass: 218.11353
Relative Abundance: 2.34627 ppm

M/z Values:
- 218.11404
- 201.08751
- 173.09256
- 232.12970
- 457.20335
- 155.08189
- 276.02740
- 316.21246
- 674.29641
- 370.05407
- 316.21246
- 628.13884
- 564.49755
- 528.28153
- 498.13803
- 457.20335
- 420.17056
HRMS Spectra of 23

KL-3-131f-7 170426154552  RT: 0.25-0.46  AV: 49  NL: 1.07E9
T: FTMS + p ESI Full ms [100.00-1500.00]

C_{12}H_{22}O_{8}N = 308.13399
-1.12650 ppm

308.1335

290.12320

330.11543

615.26023

559.13079

645.20786

615.2603

645.20786

667.25291

667.25291

134.11760

186.07610

214.57059

276.10776

359.20336

391.22960

445.12044

480.67127

480.67127

519.13917

559.13079

601.24474

667.25291

615.26023

645.20786

559.13079
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