Synthesis and biological evaluation of a chitobiose based peptide N-glycanase inhibitor library

Martin D. Witte\textsuperscript{a}, Daniëlle Horst\textsuperscript{b}, Emmanuel J. H. J. Wiertz\textsuperscript{b}, Gijs A. van der Marel\textsuperscript{a*} and Herman S. Overkleeft\textsuperscript{a*}.

\textsuperscript{a}Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. Email: marel\_g@chem.leidenuniv.nl, h.s.overkleeft@chem.leidenuniv.nl

\textsuperscript{b}Department of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
**General Procedures:**

All reagents were commercial grade and were used as received unless stated otherwise. TTBP was synthesized as described. Diethyl ether (Et₂O), ethyl acetate (EtOAc), light petroleum ether (PE) and toluene (Tol) were purchased from Riedel-de Haën. Acetonitrile (MeCN), dichloroethane, dichloromethane (CH₂Cl₂), dimethylformamide (DMF), methanol (MeOH), pyridine (pyr), tetrahydrofuran (THF) were obtained from Biosolve. THF was distilled over LiAlH₄ prior to use. Before use CH₂Cl₂ was refluxed over CaH₂ for 2h and distilled. Trace of water were removed by refluxing n-Butanol over sodium for 2h followed by distilling and storing over 3Å MS. Trifluoromethanesulfonyl anhydride was distilled from P₂O₅. Molecular sieves 3Å were flame dried before use. All reactions were performed under an inert atmosphere of Argon unless stated otherwise. Solvents used for flash chromatography were of pro analysi quality. Flash chromatography was performed on Screening Devices silica gel 60 (0.04 – 0.063 mm). TLC-analysis was conducted on DC-alufolien (Merck, Kieselgel60, F254) with detection by UV-absorption (254 nm) were applicable and by spraying with 20% sulfuric acid in ethanol followed by charring at ~150°C or by spraying with a solution of (NH₄)₆Mo₇O₂₄·H₂O (25 g/l) and (NH₄)₄Ce(SO₄)₄·2H₂O (10g/l) in 10% sulfuric acid in water followed by charring at ~150°C. ¹H and ¹³C NMR spectra were recorded on a Bruker AV- 400 (400/100 MHz), Bruker AV500 (500/125 MHz) and a Bruker DMX-600 (600/150 MHz) spectrometer. Chemical shifts (δ) are given in ppm relative to the chloroform residual solvent peak or tetramethysilane as internal standard. Coupling constants are given in Hz. All given ¹³C spectra are proton decoupled. High resolution mass spectra were recorded by direct injection (2 µL of a 2 µM solution in
water/acetonitrile; 50/50; v/v and 0.1% formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution $R = 60000$ at $m/z$ 400 (mass range $m/z = 150-2000$) and dioctylphthalate ($m/z = 391.28428$) as a “lock mass”. The high resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan). LC-MS analysis was performed on Finnigan Surveyor HPLC system with a Gemini C18 50 × 4.60 mm column (detection at 200-600 nm), coupled to a Finnigan LCQ Advantage Max mass spectrometer with ESI. LC/MS analysis was performed on a LTQ equipped with an analytical Alltima C$_{18}$ column (Alltech, 4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H$_2$O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument or on a Jasco HPLC-system (detection simultaneously at 214 nm and 254 nm) equipped with an analytical Alltima C$_{18}$ column (Alltech, 4.6 mmD × 250 mmL, 5μ particle size) in combination with buffers A: H$_2$O, B: MeCN and C: 0.5% aq. TFA and coupled to a Perkin Elmer Sciex API 165 mass instrument. For RP-HPLC purifications a Gilson HPLC system (PerSeptive Biosystems, inc.) system equipped with a Phenomenex Gemini 5μm 250×10 mm column and a GX281 fraction collector or a BioCAD “Vision” automated HPLC system (PerSeptive Biosystems, inc.) equipped with a semi-preparative Alltima C$_{18}$ column was used. The applied buffers were A: 0.1% TFA in H$_2$O, B: MeCN. FT-IR-spectra were recorded on a Paragon-PE 1000.

**Potassium fluoroacetate (5)**
Ethyl fluoroacetate, was synthesized as described in literature. Ethyl fluoroacetate (0.107 g, 1 mmol) was dissolved in absolute EtOH, cooled to 0°C and potassium hydroxide (56.1 mg, 1 mmol) was added. The reaction was stirred overnight after which petroleum ether was added. The solution was cooled to -20°C and filtered giving title compound 5 (66%, 77 mg, 0.66 mmol) as a white solid. $^1$H-NMR (400 MHz, D$_2$O) $\delta$ 4.75 (d, $J = 48.3$, 2H). $^{13}$C-NMR (101 MHz, D$_2$O) $\delta$ ppm 79.51 (d, $J = 176$). FT-IR: $v_{\text{max}}$ (neat)/cm$^{-1}$ 3381, 1606, 1423, 1398, 1337, 1009.

2-(2,6-dimethylbenzoyloxy)acetic acid (7)

Tert-butyl bromoacetate (0.437 ml, 3 mmol), dimethylbenzoic acid (0.496 g, 3.3 mmol) and potassium fluoride (0.261 g, 4.5 mmol) were dissolved in DMF. After overnight stirring, Et$_2$O was added after which the solution was extracted with NaHCO$_3$ (sat. aq.). The organic layer was dried, concentrated in vacuo and subsequently dissolved in TFA/H$_2$O (95/5). The solution was concentrated after 1h, coevaporated with toluene to remove residual traces of TFA and applied to silica gel chromatography (0→10% EA/Tol) giving title compound 7 (81%, 0.506 g, 2.43 mmol) as a white solid. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ ppm 7.22 (dd, $J = 15.2$ Hz, 7.6 Hz, 1H), 7.05 (d, $J = 7.6$ Hz), 4.89 (s, 2H), 2.37 (s, 6H). $^{13}$C-NMR (101 MHz, CDCl$_3$) $\delta$ ppm 173.68, 169.10, 135.70, 132.14, 129.86, 127.71, 60.40, 19.79. FT-IR: $v_{\text{max}}$ (neat)/cm$^{-1}$ 2955.7, 1720.7, 1597.4, 1462.9, 1424.3, 1306.1, 1281.6, 1240.1, 1165.5, 1113.7, 1082.7, 1029.1, 1003.3.

3-C-(3,6-di-O-benzyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl) propene (26)
Known 3-C-(3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-1-propene\(^2\) (0.512 g, 1 mmol) was coevaporated 3× toluene before being dissolved in freshly distilled CH\(_2\)Cl\(_2\). Activated molsieves (4 Å) and triethylsilane (0.533 mL, 3.3 mmol) were added. The mixture was stirred at room temperature for 15 min, cooled to -78°C followed by the addition of trifluoromethanesulfonic acid (0.265 mL, 3 mmol). TLC-analysis showed complete conversion after 45 min after which the reaction was quenched with MeOH/Et\(_3\)N, extracted with NaHCO\(_3\) and concentrated. Silica gel purification (0→30% EA/PE) afforded acceptor 26 (70%, 0.359 g, 0.70 mmol) as an oil. 

\(^1\)H-NMR (400 MHz, CDCl\(_3\)) δ ppm 7.80-7.75 (m, 1H), 7.70-7.63 (m, 3H), 7.38-7.23 (m, 5H), 7.05-6.98 (m, 2H), 6.95-6.90 (m, 3H), 5.72 (dt, \(J = 16.9, 16.8, 7.0\) Hz, 1H), 4.89 (d, \(J = 17.2\) Hz, 1H), 4.83 (d, \(J = 10.2\) Hz, 1H), 4.76 (d, \(J = 12.2\) Hz, 1H), 4.64 (d, \(J = 12.1\) Hz, 1H), 4.58 (d, \(J = 12.1\) Hz, 1H), 4.52 (d, \(J = 12.2\) Hz, 1H), 4.30-4.18 (m, 2H), 4.08 (t, \(J = 10.0\) Hz, 1H), 3.82-3.71 (m, 3H), 3.61 (td, \(J = 9.1, 4.4, 4.4\) Hz, 1H), 3.04 (s, 1H), 2.27-2.13 (m, 2H). \(^{13}\)C-NMR (101 MHz, CDCl\(_3\)) δ ppm 168.14, 167.78, 138.24, 137.69, 133.78, 133.72, 133.31, 131.47, 131.44, 128.36, 127.98, 127.70, 127.65, 127.22, 123.24, 123.13, 116.93, 79.56, 77.55, 74.43, 74.23, 73.59, 70.62, 55.30, 36.92. FT-IR: \(v_{\text{max}}\) (neat)/cm\(^{-1}\) 3473.9, 2869.8, 1774.6, 1709.7, 1383.9, 1201.4, 1076.3, 913.3. ESI/MS: \(m/z = 514.13\) (M+H\(^+\)).

**Imidate (39)**

3-O-benzyl-4,6-O-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranose\(^3\) (2.88 g, 5.9 mmol) was dissolved in anhydrous CH\(_2\)Cl\(_2\). K\(_2\)CO\(_3\) (3.017 g, 21.83 mmol) and trichloroacetonitrile (2.97 mL, 29.5 mmol) were added. After overnight stirring, the
mixture was filtrate, concentrated in vacuo and purified by silicagel column chromatography. $^1$H-NMR (400 MHz, CDCl$_3$) $\delta$ ppm 8.58 (s, 1H), 7.78-7.66 (m, 4H), 7.55-7.51 (m, 2H), 7.44-7.37 (m, 3H), 7.03-6.99 (m, 2H), 6.95-6.86 (m, 3H), 6.50 (d, $J = 8.3$ Hz, 1H), 5.64 (s, 1H), 4.82 (d, $J = 12.4$ Hz, 1H), 4.60-4.45 (m, 4H), 3.94-3.80 (m, 3H). $^{13}$C-NMR (101 MHz, CDCl$_3$) $\delta$ ppm 167.37, 160.77, 137.74, 137.12, 133.95, 131.37, 129.07, 128.28, 128.03, 127.43, 126.06, 123.38, 101.44, 94.32, 82.60, 74.36, 74.18, 68.51, 66.88, 54.71. FT-IR: $v_{\text{max}}$ (neat)/cm$^{-1}$ 1776.4, 1714.1, 1677.8, 1453.7, 1383.8, 1294.2, 1112.9, 1053.4, 1023.1.

**(2S,3S)-3-N-(O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranosylcarbamoyl) oxirane-2-carboxylic acid ethyl ester (49)**

Known epoxide$^2$ (166 mg, 0.199 mmol) was dissolved in EtOH/Dioxane (2/1 v/v, 5 mL). Pd(OH)$_2$ on charcoal (25 mg) was added followed by stirring under H$_2$ atm for 1h. The solution was filtered, concentrated, redissolved in pyridine (5 mL) and cooled to 0°C before being treated with Ac$_2$O (3 mL). The mixture was stirred overnight, concentrated, redissolved in CH$_2$Cl$_2$, washed with H$_2$O, dried (Na$_2$SO$_4$) and concentrated in vacuo. RP-HPLC (linear gradient 22 → 32% B in 10.00 min) gave title compound 49 (26%, 39.69 mg, 51.2 $\mu$mol) as a white solid. $^1$H-NMR (400 MHz, CDCl$_3$/MeOD) $\delta$ ppm 5.27 (dd, $J = 10.2$, 9.5 Hz, 1H), 5.09 (dd, $J = 10.5$, 8.4 Hz, 1H), 5.07-4.98 (m, 2H), 4.67 (d, $J = 8.3$ Hz, 1H), 4.43 (dd, $J = 12.1$, 1.4 Hz, 1H), 4.42 (dd, $J = 12.4$, 4.4 Hz, 1H), 4.28 (m, 2H), 4.07 (dd, $J = 11.9$, 4.7 Hz, 1H), 4.05-3.98 (m, 2H), 3.78-3.69 (m, 4H), 3.62 (d, $J = 1.7$ Hz, 1H), 3.48 (d, $J = 1.7$ Hz, 1H), 2.14 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.02 (s, 6H),
1.94 (s, 3H), 1.91 (s, 3H), 1.32 (t, $J = 7.1, 7.1$ Hz, 3H). $^{13}$C-NMR (101 MHz, CDCl$_3$/MeOD) $\delta$ ppm 172.29, 171.55, 171.22, 170.66, 170.58, 170.51, 169.60, 166.97, 166.63, 100.23, 78.23, 75.23, 74.22, 72.57, 71.88, 71.21, 68.09, 62.14, 62.09, 61.53, 54.40, 52.94, 52.37, 51.97, 22.11, 22.08, 20.29, 20.17, 20.11, 20.04, 19.97, 13.47. FT-IR: $v_{\text{max}}$ (neat)/cm$^{-1}$ 3288.1, 1738.9, 1665.5, 1541.9, 1435.1, 1372.0, 1228.2, 1046.1, 902.1, 600.8. LC/MS: $R_t$ 4.700 min; linear gradient 10→90% B in 13.5 min; ESI/MS: $m/z = 776.5$ (M+H$^+$). HRMS: (M + H$^+$) calc. for C$_{32}$H$_{45}$N$_3$O$_{19}$ 776.27200, found 776.27207.

**Competition assay**

To 9 µL of a solution of yeast PNGase (11.1 ng/µL) in PBS (pH = 7.2, 20 mM sodium phosphate, 150 mM NaCl, BSA (1 µg/µL) was added inhibitor (1 µL) and Bodipy-VAD-Fmk (1µL , 0.5 µM final concentration). The resulting solution was incubated at 37°C for 1h, after which it was quenched by the addition of 4 × SDS-PAGE sample buffer (4 µL) and boiling for 5 min. The proteins were separated on a 10% SDS-PAGE gel and subsequently the fluorescence was measured in the wet gel slabs by using the CY3/Tamra settings ($\lambda_{\text{ex}}$ 532 $\lambda_{\text{em}}$,560) on a Typhoon Variable Mode Imager (Amersham Biosciences). The remaining fluorescence was quantified with ImageQuant followed by analysis with Graphpad Prism.

**In vivo inhibition of human PNGase**

Control and US11-expressing U373 cells (1.5*10$^6$ cells per lane) were collected, washed with PBS and resuspended in Met-/Cys- medium (2 mL) containing 10% FCS and the inhibitors ZL$_3$VS (1.25 µM), ZVAD-Fmk, 2, 49 (30 µM and 100 µM respectively) and
NGT (50 μM) as indicated. The cells were incubated for 45 min at 37°C, pelleted by centrifugation and resuspended in 250 μL Met/Cys medium, again in the presence of the inhibitors. $[^{35}\text{S}]$-labeled Cys/Met (5 μL, 200 μCi/mL) was added and the cells were incubated for 30 minutes at 37°C. The cells were washed with PBS and subsequently lysed in 1 ml cold NP-40 buffer (0.5% NP-40, 50 mM Tris HCl pH 7.5, 5 mM MgCl₂, 10 μM leupeptin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) for 45 min at 4°C. The lysed cells were centrifuged (16.000 g, 15 min) at 4°C in order to obtain post-nuclear lysates. These lysates were precleared twice by the addition of Protein G- and Protein A-sepharose beads (7.5 μL, GE Healthcare), normal rabbit serum (1.5 μL) and normal mouse serum (1.5 μL), followed by incubation for 60 min at 4°C. Next, the class I heavy chains were immunoprecipitated. Specific antibodies (mAb HC10), Protein G- and Protein A-sepharose beads were added to the lysates and samples were incubated o/n at 4°C. Isolated immune complexes were washed thrice with NET buffer containing 0.1% SDS and denatured in reducing sample buffer (50 μL). The samples were separated by SDS-PAGE and visualized by phosphoimaging.

References


$\text{K}^+ \cdot \text{O} - \text{C}_3\text{H}_7\text{F}$

5

ppm (f1)

0.0  1.0  2.0  3.0  4.0  5.0  6.0  7.0  8.0
ppm (f1)
Instrument Method:  C:\Xcalibur\methods\General(TFA)\0020%B10_C=TFA_20ul_+pf 15min.meth
Processing Method:  Vial:  A:23
Injection Volume (µl):  20.00
Sample Weight:  0.00

mdw656 #68  RT: 1.33  AV: 1  NL: 1.19E6
F: + p ESI Full ms [160.00-2000.00]