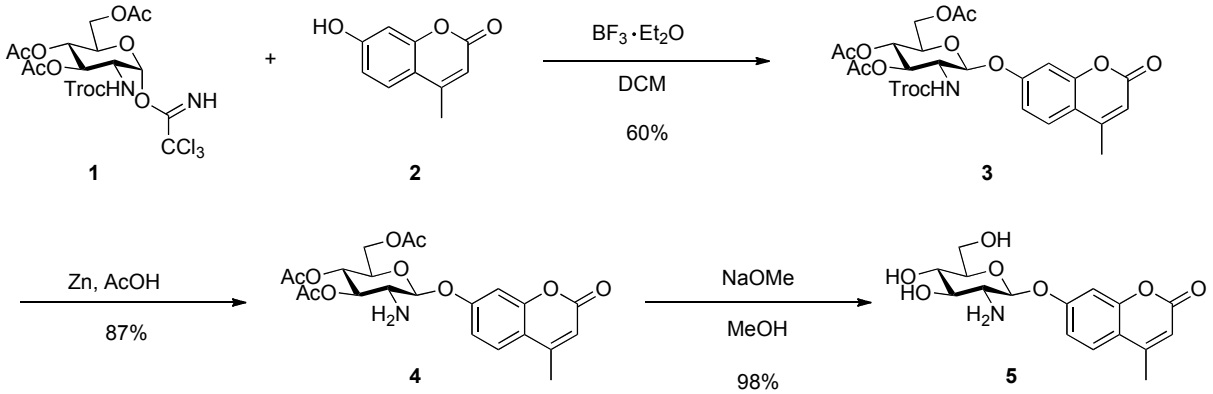
# Supplementary Materials

## Muropeptide rescue in *Bacillus subtilis* involves sequential hydrolysis by exo-β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase

Silke Litzinger[1], Amanda Duckworth[1], Katja Nitzsche[1], Christian Risinger[2], Valentin Wittman[2] and Christoph Mayer[1*]

**Synthesis of 4-Methylumbelliferyl-2-amino-2-deoxyglucopyranoside 5**




**Scheme 1.** Synthesis of 4-Methylumbelliferyl-2-amino-2-deoxyglucopyranoside **5**

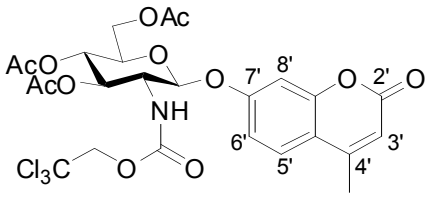
4-Methylumbelliferyl glycoside **4** (3, 5, 6) was synthesized by an improved procedure (Scheme 1). Troc protected trichloroacetimidate **1** (2) and 4-Methylumbelliferone **2** were reacted under $BF_3$ catalysis. The obtained glycoside **3** was then deprotected in two steps to yield the desired compound **5**.

**Experimental Section**

General Methods: Cation exchange resin Amberlite IR-120 ($H^+$) was pre-washed with dry MeOH before use. Analytical thin layer chromatography (TLC) was performed on Merck Silica Gel 60 F245 aluminium sheets (thickness 0.2 mm). Compound spots were visualized by quenching of fluorescence and/or by charring after treatment with cerium reagent (5 g molybdatophosphoric acid, 2.5 g ceric sulphate tetrahydrate, 25 mL sulphuric acid and 225 mL water), ethanolic ninhydrin (3% v/v), and ethanolic sulphuric acid (15 % v/v), respectively. Flash column chromatography (FC) was performed on Macherey-Nagel Silica Gel 60 (0.04-0.063 mm; 230-400 mesh ASTM). $^1H$ and $^{13}C$ NMR spectra were recorded at 293 K on Bruker AC 250, Bruker Avance III 400 or Bruker Avance DRX 600 spectrometers. Resonance assignments were made by the aid of COSY, HSQC and HMBC when necessary. $^1H$ chemical shifts are referenced to residual protic solvent ($CDCl_3$, $\delta_H$ = 7.26 ppm; $CD_3OD$, $\delta_H$ = 3.31 ppm; DMSO-$d_6$, $\delta_H$ = 2.50 ppm ). $^{13}C$ chemical shifts are referenced to the solvent signal ($CDCl_3$, $\delta_C$ = 39.5 ppm; $CD_3OD$, $\delta_C$ = 49.2 ppm; DMSO-$d_6$, $\delta_C$ = 77.0 ppm). ESI-IT mass spectra were recorded on a Bruker Daltonics Esquire 3000 plus instrument. MALDI-TOF mass spectra were recorded on a Bruker Biflex III spectrometer in positive, linear mode with a delayed extraction MALDI source and a pulsed nitrogen laser (337 nm). Combustion

elemental analysis was performed on an elementar CHNS vario EL analyzer. RP-HPLC was performed on a LC-20 prominence system from Shimadzu. Used column: Nucleosil 100-5 C-18 (semipreperative: 8 x 250 mm, flow 3 mL $min^{-1}$) from Knauer.Eluent: gradient of water with 0.1% formic acid (eluent A) in acetonitrile with 0.1% formic acid (eluent B).

**4-Methylumbelliferyl-3,4,6-tri-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino) -β-D-glucopyranoside (3)**

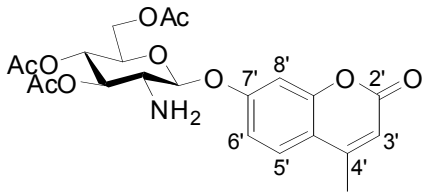



$C_{25}H_{26}Cl_3NO_{12}$

638.83 g/mol

Compound **1** (1.875 g, 3 mmol, 1.0 eq) and 4-methylumbelliferone **2** (792 mg, 4.5 mmol, 1.5 eq) were dissolved at 0 °C in dry $CH_2Cl_2$ (30 mL). $BF_3{\cdot}OEt_2$ (36 µL, 0.3 mmol, 0.1 eq) was added and the mixture was stirred for 20 h at RT. The mixture was diluted with $CH_2Cl_2$ (120 mL), washed with saturated $NaHCO_3$ (2 x 120 mL), with water (2 x 120 mL) and with brine (1 x 120 mL), dried ($MgSO_4$), and the solvent was evaporated. Purification by FC (petroleum ether-EtOAc 1:2) yielded **7** (1.154 g, 60%) as a white solid. $R_f = 0.31$ (petroleum ether-EtOAc 1:2); $^1$H NMR (400.1 MHz, $CDCl_3$): $\delta$ = 7.46 (d, $J$ = 8.6 Hz, 1 H; H-5'), 6.94 (s, 1 H; H-8') , 6.89 (d, $J$ = 8.6 Hz, 1 H; H-6'), 6.16 (s, 1 H; H-3'), 5.62 (d, $J$ = 8.7 Hz, 1 H; NH), 5.44 (dd, $J$ = 10.0, 9.6 Hz, 1 H; H-3), 5.35 (d, $J$ = 8.0 Hz, 1 H; H-1), 5.14 (dd, $J$ = 9.6, 9.4 Hz, 1 H; H-4), 4.77-4.59 (m, 2 H; $Cl_3CCH_2$), 4.31 (dd, $J$ = 12.0, 6.8 Hz, 1 H; H-6a), 4.18 (dd, $J$ = 12.0, 1.5 Hz, 1 H; H-6b), 4.03-3.90 (m, 2 H; H-2, H-5), 2.37 (s, 1 H; Me), 2.12 (s, 3 H; $C(O)CH_3$), 2.08 (s, 3 H; $C(O)CH_3$), 2.07 (s, 3 H; $C(O)CH_3$) ppm; $^{13}$C-NMR (100.0 MHz, $CDCl_3$): $\delta$ = 170.6 ($C(O)CH_3$), 170.5 ($C(O)CH_3$), 169.5 ($C(O)CH_3$), 160.9, 159.3, 154.7, 154.1, 152.3 (quaternary C´s), 125.6 (C-5'), 115.3 (quaternary C), 114.0 (C-6'), 113.0 (C-3'), 103.7 (C-8'), 98.2 (C-1), 95.4 ($Cl_3CCH_2$), 74.4 ($Cl_3CCH_2$), 72.4 (C-5), 71.5 (C-3), 68.4 (C-4), 61.9 (C-6), 56.0 (C-2), 20.7 ($C(O)CH_3$), 20.6 ($C(O)CH_3$), 20.6 ($C(O)CH_3$), 18.6 ($CH_3$') ppm; (MALDI-TOF-MS, pos. Mode, CHCA): $m/z$ $[M+Na]^+$ calcd : 660.1, found: 660.9; m/z $[M+K]^+$ calcd: 676.1, found: 676.9; Anal. Calcd for $C_{25}H_{26}Cl_3NO_{12}$: C, 47.00; H, 4.10; N, 2.19. Found: C, 46.96; H, 4.28; N, 2.32.

**4-Methylumbelliferyl-3,4,6-tri-*O*-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (4).**

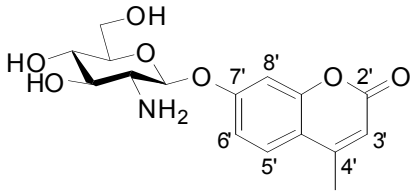



$C_{22}H_{25}NO_{10}$

463.43 g/mol

Freshly activated Zn dust (3.5 g) was added to a solution of N-Troc derivative 3 (1.50 g, 2.35 mmol, 1.0 eq) in AcOH (85 mL). The reaction vessel was then sonicated for 24 h in a classic ultrasonic cleaning bath below rt until the disappearance of starting material, as determined by TLC. The mixture was filtered through Celite, the filtrate was quenched with $H_2O$ (80 mL), and washed with $CH_2Cl_2$ (3 x 80 mL). The combined organic phases were washed with saturated $NaHCO_3$ (80 mL) and with water (80 mL), dried ($MgSO_4$), and the solvent was evaporated. Purification by FC (EtOAc) yielded 8 (946 mg, 2.04 mmol, 87%) as a white solid. $R_f$ = 0.31 (EtOAc); $^1$H NMR (250 MHz, $CDCl_3$): $\delta$ = 7.47 (d, $J$ = 8.6 Hz, 1 H; H-5'), 6.94 (s, 1 H; H-8') , 6.91 (d, $J$ = 8.6 Hz, 1 H; H-6'), 6.13 (d, $J$ = 1.2 Hz, 1 H; H-3'), 5.25-4.90 (m, 3 H; H-1, H-3. H-4,), 4.27 (dd, $J$ = 12.3, 5.5 Hz, 1 H; H-6a), 4.10 (dd, $J$ = 12.3 ,2.0 Hz, 1H; H-6b), 3.95-3.83 (m, 1H; H-5) 3.21 (t, $J$ = 8.6 Hz, 1 H; H-2), 2.36 (s, 3H; Me), 2.07 (s, 3H; $C(O)CH_3$), 2.06 (s, 3 H; $C(O)CH_3$), 2.01 (s, 3 H; $C(O)CH_3$) ppm; (MALDI-TOF-MS, pos. Mode, CHCA): $m/z$ [$M$+Na]$^+$ calcd: 486.1, found: 486.1; Anal. Calcd for $C_{22}H_{25}NO_{10}$: C, 57.02; H, 5.44; N, 3.02. Found: C, 56.99; H, 5.58; N, 2.75.

**4-Methylumbelliferyl-2-amino-2-deoxy-β-D-glucopyranoside (5)**




$C_{16}H_{19}NO_7$

337.32 g/mol

To a solution of 4 (32 mg, 0.069 mmol) in MeOH (1 mL) was added a solution of sodium methylate (0.5 M in MeOH, 0.15 eq). The mixture was stirred for 90 min at rt. After neutralization with acidic ion exchanger resin (Amberlite IR-120 ($H^+$)), the mixture was filtered and lyophilized to yield deacetylated glycoside 9 (23 mg, 0.068 mmol, 98%). The glycoside was further purified by semi-preparative RP-HPLC before using it in biological assays.

$R_f$ = 0.38 (MeCN-$H_2O$ 4:1); RP-HPLC (semi-preparative column) (5–80% B in 20 min): $t_R$ 10.7 min; $^1$H NMR (600 MHz, MeOD): $\delta$ = 8.46 (br s; $NH_2$), 7.75 (d, $J$ = 8.0 Hz, 1 H; H-5'), 7.17-7.12 (m, 2 H; H-6', H-8'), 6.24 (s, 1 H; H-3'), 5.18 (d, $J$ = 8.0 Hz, 1 H; H-1), 3.92 (dd, $J$ = 12.3, 2.0 Hz, 1 H; H-6a), 3.74 (dd, $J$ = 12.3, 5.8 Hz, 1 H; H-6b), 3.59-3.40 (m, 3 H; H-3, H-4, H-5), 3.08 (t, $J$ = 9.5 Hz, 1 H; H-2), 2.47 (s, 3 H; Me) ppm; $^{13}$C-NMR (150 MHz, $CDCl_3$): δ = 163.4, 162.0, 156.2, 155.6 (quaternary C's), 127.5 (C-5'), 116.3 (quaternary C), 115.1 (C-6'), 113.1 (C-3'), 105.2 (C-8'), 102. 6 (C-1), 78.8 (C-5), 77.6 (C-3), 71.6 (C-4), 62.6 (C-6), 58.4 (C-2), 18.9 (Me) ppm; (ESI-MS, pos. Mode): $m/z$ $[M+H]^+$ calc. : 338.1, found: 338.1.

**Table S1. Identity of *Bs*NagZ with a partially purified GlcNAc`ase of *B. subtilis* reported earlier (1, 4)**

| | ***Bs*NagZ (this study)** | **Partially purified GlcNAc`ase (1, 4)** |
|---|---|---|
| Occurrence | produced towards the end of growth (i.e. late log phase, stationary phase) | |
| Compartmentalisation | released into medium<br>mainly found in a sedimentable form (about 70 % in the 30,000 x *g* sediment) with increasing culture age more particulate material (after 15h: about 90 % in the 30,000 x *g* sediment) | |
| NaCl extraction | 80 - 90 % released from cells and from sedimented material with 3 M NaCl at 30,000 x *g* | |
| pH optimum | 5.8-6.2 | 5.9-6.0 |
| most stable at | 4.0- 8.0 | 8.5 |
| pI | 9.37 (calculated) | "seems to be 3.8" |
| MW | [a]70-75 kDa | 75 kDa |
| $K_M$; specific activity (pNP-β-GlcNAc) | [b]171.6 µM<br>8.33 µmol/min mg | 150 µM;<br>14.50 µmol/min mg |
| $K_M$; specific activity (4-β-Mu-GlcNAc) | 109.6 µM;<br>5.39 µmol/min mg | 110 µM;<br>5.26 µmol/min mg |
| $K_M$; Specific activity (GlcNAc-MurNAc) | n.d. | 18 µM;<br>32.6 µmol/min mg |
| 4-Mu-β-GlcN | no substrate | n.d. |
| 4-Mu-β-GalNAc | no substrate | no substrate |

[a] the calculated molecular weight of the cloned protein including $His_6$-tagis 71.3 kDa
[b] kinetic parameters were determined at pH 5.8 in Clark and Lubs buffer (0.1M $KH_2PO_4$/0.1M NaOH). n.d., not determined.

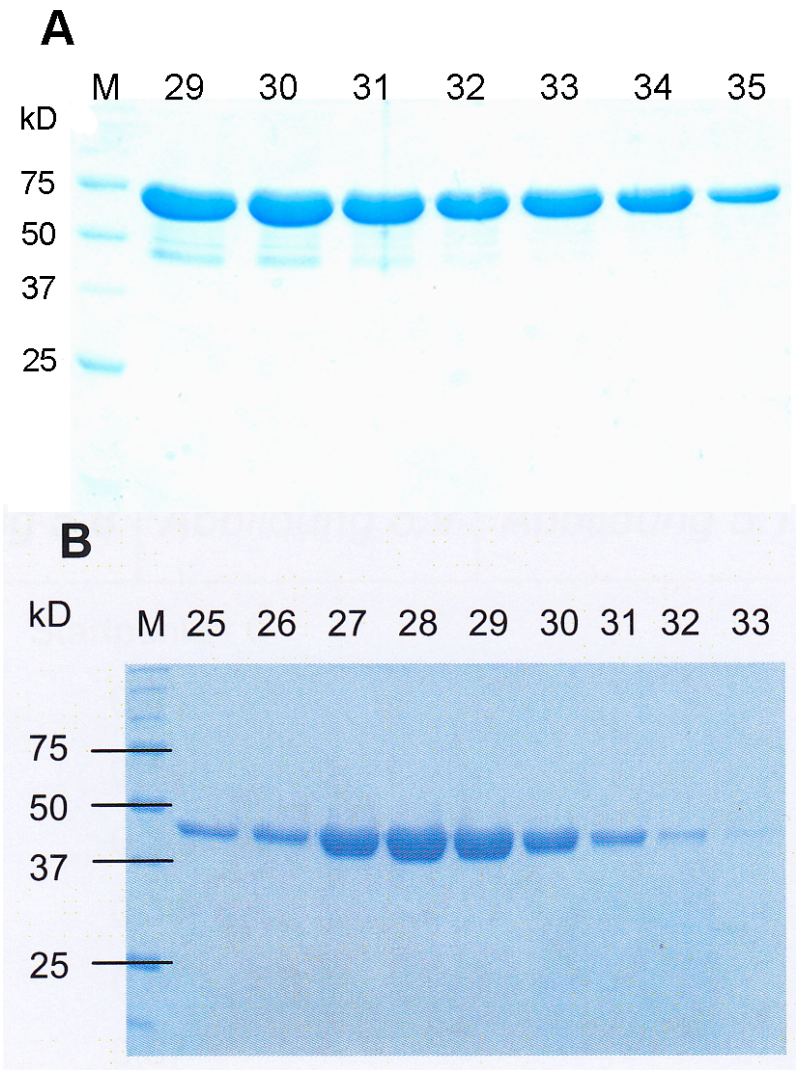



**Figure S1. Purity of recombinant *Bs*NagZ and *Bs*AmiE.** Sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) of elution fractions (numbers) of the Ni-chelate affinity chromatography of *Bs*NagZ (A) and *Bs*AmiE (B). *Bs*NagZ shows a molecular weight of about 70-75 kDa (calculated 71.3 kDa) and *Bs*AmiE of about 40-45 kDa (calculated 47.9 kDa). The molecular weight marker is indicated (M).

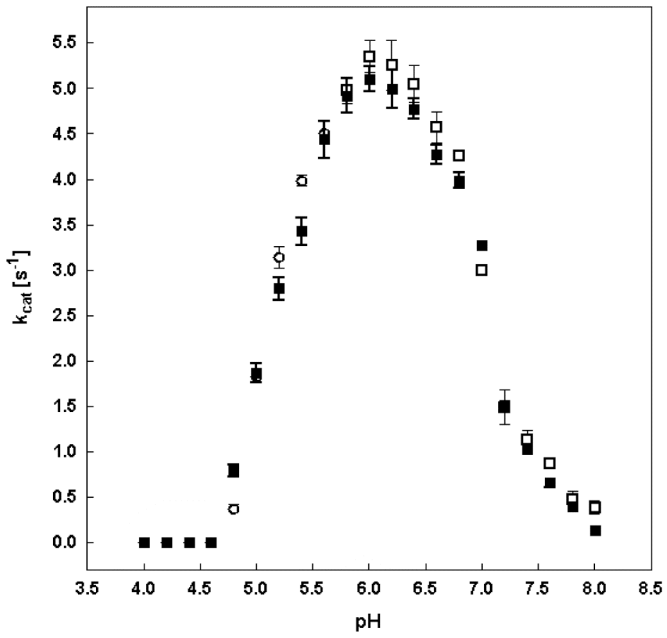



**Figure S2. pH activity profile of NagZ.** The $k_{cat}$ versus pH plot revealed a bell-shaped curve with a pH-optimum in the range of 5.8 to 6.2 with 4-methylumbelliferyl-β-*N*-acetyl-D-glucosaminide (4-Mu-β-GlcNAc) as the substrate. The buffers were: 0.1 M citric acid/0.2 M disodium phosphate buffer (McIlvaine) ranging from pH 4.0 to 8.0 (■); 0.2M sodium acetate-acetic acid buffer ranging from 4.0 to 5.6 (○) and Clark and Lubs solution (0.1 M $KH_2PO_4$/0.1 M NaOH) in the range of pH 5.8 to 8.0 (□).

## References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