Electronic Supplementary Material (ESI) for ChemComm.


*Supporting Information*

# A mechanism-based GlcNAc-inspired cyclophellitol inactivator of the peptidoglycan recycling enzyme NagZ reverses resistance to β-lactams in *Pseudomonas aeruginosa*

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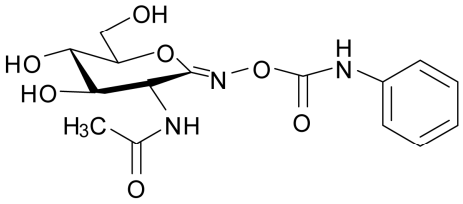
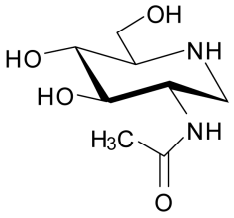
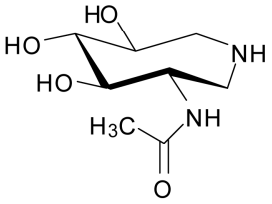
## *Models*




**PUGNAc**

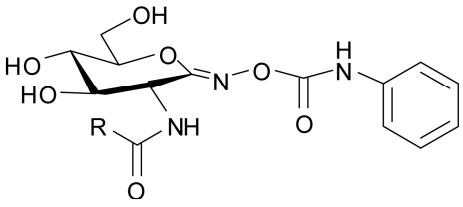



**NHAc-DNJ**

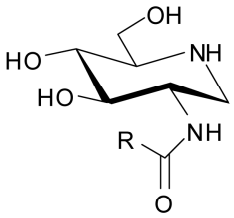



**GlcNAc-azepane**

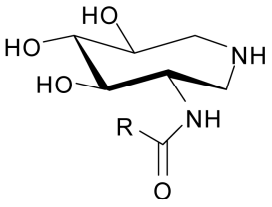
## *Derivatives*




R = $CH(CH_2CH_3)_2$




R = $CH_2CH_2CH_3$




R = $CH_2CH_2CH_3$
R = $CF_3$

**Fig. S1.** Examples of model compounds as inhibitors of NagZ centring on taking advantage of the oxocarbenium ion-like transition states that are thought to flank the covalent glycosyl-enzyme intermediate and derivatives to drive selectivity for NagZ over important human enzymes.

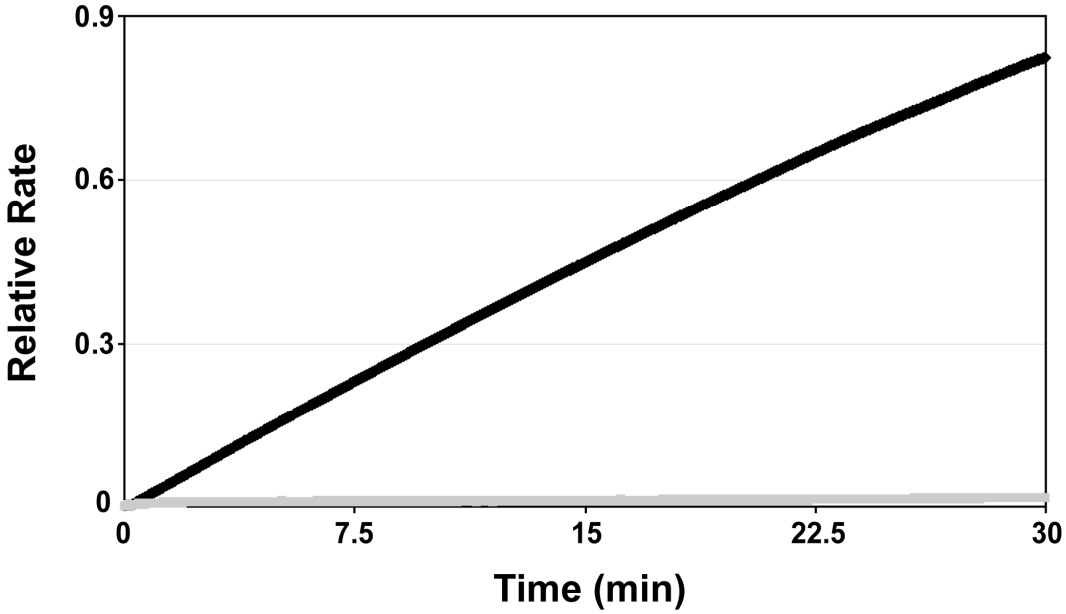



**Fig S2.** Demonstration of the irreversible inactivation of *Bc*NagZ by **2**. After inactivation of *Bc*NagZ by **2** the sample is diluted to allow for determination of reversibility of enzyme inactivation. Black line - Enzyme plus substrate, Grey line - Enzyme incubated with inhibitor plus substrate. Linear regression of the data of enzyme incubated with inhibitor plus substrate gives slope and $R^2$ values respectively of 0.0004 and 0.982 (2-6 minutes), 0.0003 and 0.976 (24-30 minutes) and 0.0003 and 0.979 (overall), strongly supporting that there is no upward curvature of the line and thus no reactivation of enzyme activity, confirming the irreversible nature of inactivator binding.

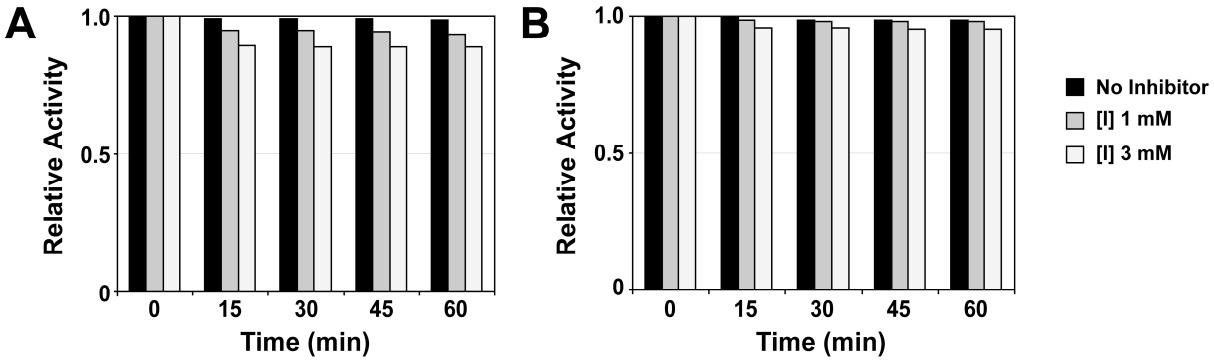
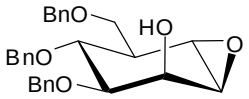



**Fig S3.** Residual activity of relevant human enzymes after incubation with **2**, compared to a control of enzyme with no inhibitor added. A 60 minute time course was conducted using exemplar enzymes (A) HexB and (B) NAGLU that possess the different catalytic mechanism exhibited by human enzymes. In addition *O*-GlcNAcase was also analysed against **2** at a concentration of 1 mM and at the 60 minute time point the residual activity was 85%. All reactions were conducted in triplicate with the average shown. These data support **2** not being an inactivator of HexB, NAGLU, and *O*-GlcNAcase, which all use different catalytic mechanisms that differ from NagZ. Errors were within ±2%.

| **Table S1: Crystallographic and refinement statistics for *Bc*NagZ bound to 2** | |
|---|---|
| **Crystallographic data** | |
| X-ray source | Rigaku MicroMax 007HF |
| Space group | $P2_1$ |
| Unit cell dimensions | $a$ = 48.91Å, $b$ = 88.14 Å, $c$ = 67.31 Å<br>$\alpha = \gamma = 90°$, $\beta = 92.03°$ |
| Wavelength (Å) | 1.54 |
| Resolution (Å) | 67.27 – 1.93 (1.97 – 1.93) |
| $R_{merge}$ | 0.082 (0.309) |
| Total observations | 154081(9411) |
| Unique observations | 42981 (2882) |
| Multiplicity | 3.6 (3.3) |
| CC(1/2) | 0.993 (0.833) |
| I / σI | 9.3 (3.3) |
| Completeness (%) | 99.9 (98.9) |
| Wilson B-factor | 14.87 |
| **Refinement** | |
| Reflections used for $R_{free}$ | 2010 (204) |
| Non-hydrogen atoms | |
| Protein | 5149 |
| Ligands | 36 |
| Solvent | 843 |
| $R_{work}$ / $R_{free}$ | 0.15/ 0.20 |
| Average B-factors (Å$^2$) | 15.45 |
| Protein | 14.73 |
| Ligands | 11.83 |
| Solvent | 20.01 |
| RMSDs | |
| Bond lengths (Å)/angles (°) | 0.003/0.65 |
| Ramachandran favoured/allowed (%) | 96.00/4.00 |

*General Experimental*

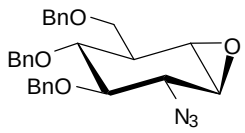
$^{1}H$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance IIIHD 500 (500 MHz for $^{1}H$ and 125.8 MHz for $^{13}C$) or Bruker Avance IIIHD 600 spectrometer (600 MHz for $^{1}H$ and 150.9 MHz for $^{13}C$). The solvent used for NMR was deuteriochloroform ($CDCl_3$) with $CHCl_3$ ($^{1}H$, δ 7.26 ppm) or $CDCl_3$ ($^{13}C$, δ 77.16 ppm) or deuteriomethanol ($CD_3OD$) with $CH_3OH$ ($^{1}H$. 3.31) or $CD_3OD$ ($^{13}C$, 49.00) used as an internal standard. High resolution mass spectra (HR-MS) were obtained on a Waters LCT Premier XE spectrometer, run in W-mode, using the electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) method with $CH_3CN$:0.1% HCOOH (9:1) as a matrix. Infrared spectra were obtained on a PerkinElmer spectrum one FTIR spectrometer fitted with a PerkinElmer Universal ATR sampling accessory. Samples were analysed as neat samples and recorded in wave numbers ($cm^{-1}$). Flash chromatography was performed on Merck silica gel using the specified solvents. Thin layer chromatography (TLC) was effected on Merck silica gel 60 F254 aluminium-backed plates that were visualised using a UV lamp.




(1S,2S,3R,4R,5R,6R)-3,4-bis(benzyloxy)-5-((benzyloxy)methyl)-7-oxabicyclo[4.1.0]heptan-2-ol **4**

*meta*-Chloroperoxybenzoic acid (501 mg, 2.90 mmol) was added to a solution of allylic alcohol **3**[1] (614 mg, 1.42 mmol) and dry $CH_2Cl_2$ (20 mL). The resultant solution was left to stir overnight. After this time, the solution was diluted with $CH_2Cl_2$ (50 mL), then washed with saturated aqueous $Na_2CO_3$ (3×30 mL) and brine (30 mL). The aqueous layers were extracted further with EtOAc (2×50 mL). The combined organic layers were then filtered through a plug of silica gel to dry, and concentrated. The resultant residue was purified *via*

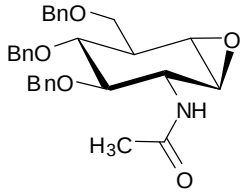
flash chromatography (EtOAc:hexanes 1:4 → 2:3) to yield the title compound **4** as a white solid (489 mg, 77%). $R_f$ 0.56 EtOAc:hexanes (2:3). The $^1$H and $^{13}$C NMR spectra of the isolated compound were consistent with known data in the literature.[1] $^1$H NMR (600 MHz, $CDCl_3$): δ = 7.38-7.20 (m, 15H), 4.73 (d, *J* = 11.7 Hz, 1H), 4.65 (d, *J* = 12.0 Hz, 1H), 4.61 (d, *J* = 12.0 Hz, 1H), 4.56 (d, *J* = 12.0 Hz, 1H), 4.50 (d, *J* = 12.0 Hz, 1H), 4.46 (d, *J* = 11.7 Hz, 1H), 4.28 (t, *J* = 4.5 Hz, 1H), 3.75-3.64 (m, 2H), 3.60-3.48 (m, 3H), 3.38 (t, *J* = 4.0 Hz, 1H), 2.38-2.30 (m, 1H) ppm. $^{13}$C NMR (150.9 MHz, $CDCl_3$): δ =138.31, 138.18, 137.74, 128.62, 128.50, 128.48, 128.09, 128.07, 127.87, 127.75, 127.71, 79.88, 74.08, 73.33, 73.29, 73.10, 69.09, 65.48, 55.48, 53.53, 41.34 ppm. HR-MS (ESI): calc. for $C_{28}H_{31}O_5$ $[M+H]^+$ 447.2172, found 447.2160.




(1S,2R,3R,4R,5R,6R)-2-azido-3,4-bis(benzyloxy)-5-((benzyloxy)methyl)-7-oxabicyclo[4.1.0]heptane **5**

The alcohol **4** (489 mg, 1.09 mmol) and triphenylphosphine (372 mg, 1.41 mmol) were dissolved in dry toluene and cooled to -10ºC in a dry ice/acetone bath. At this same temperature, DPPA (285 μL, 1.43 mmol) and DEAD (220 μL, 1.40 mmol) were then added drop-wise, successively. The resultant solution was warmed to 0ºC and left to stir for 2 hours. After this time, the reaction mixture was filtered through a plug of silica gel and concentrated. The residue was purified *via* flash chromatography ($CH_2Cl_2$:hexanes 1:3 → EtOAc:hexanes 1:9) to yield the title compound **5** as a white solid (441 mg, 86%) $R_f$ 0.45 EtOAc:hexanes (1:4). $^1$H NMR (600 MHz, $CDCl_3$): δ =7.38-7.27 (m, 13H), 7.19-7.16 (m, 2H), 4.89 (d, *J* = 10.8 Hz, 1H), 4.83 (d, *J* = 10.8 Hz, 1H), 4.80 (d, *J* = 10.8 Hz, 1H), 4.54 (d, *J* = 12.6 Hz, 1H), 4.51 (d, *J* = 10.8 Hz, 1H), 4.43 (d, *J* = 10.8 Hz, 1H), 3.87 (d, *J* = 9.0 Hz, 1H),

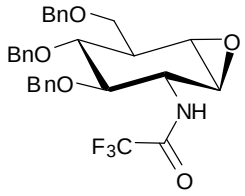
3.74 (dd, $J$ = 3.6, 9.0 Hz, 1H), 3.57 (t, $J$ = 9.0 Hz, 1H), 3.49-3.42 (m, 2H), 3.33 (t, $J$ = 9.6 Hz, 1H), 3.11 (d, $J$ = 3.6 Hz, 1H), 2.32-2.26 (m, 1H) ppm. δ = $^{13}$C NMR (151 MHz, $CDCl_3$): 138.25, 138.01, 137.86, 128.59, 128.57, 128.23, 128.09, 128.04, 127.99, 127.81, 127.78, 84.43, 76.02, 75.57, 73.40, 68.51, 63.20, 55.745, 54.29, 42.78 ppm. FTIR (neat, $cm^{-1}$): 2106, 1750, 1725, 1367. HR-MS (ESI): calc. for $C_{28}H_{30}N_3O_4$ $[M+H]^+$ 472.2236, found 472.2236.




*N*-((1S,2R,3R,4R,5R,6R)-3,4-bis(benzyloxy)-5-((benzyloxy)methyl)-7-oxabicyclo[4.1.0]heptan-2-yl)acetamide **6**

The azide **5** (100 mg, 0.21 mmol) was dissolved in a mixture of THF:$H_2O$ (20:1, 3 mL) and cooled to 0ºC in an ice bath. At this same temperature, trimethylphosphine (1 M in toluene, 250 µL, 0.25 mmol) was added drop-wise, and the resultant solution was then left to stir at 0ºC, for 2 hours. After this time, the reaction solvent was removed at room temperature under reduced pressure. The crude residue was then co-evaporated with toluene (5x), and dried for 30 minutes under reduced pressure. The resulting white solid was dissolved in dry $CH_2Cl_2$ (2 mL) and cooled to 0ºC in an ice bath. At this same temperature, $Ac_2O$ (47 µL, 0.5 mmol) and pyridine (200 µL, 2.5 mmol) were then added drop-wise, successively. The resultant solution was left to stir at 0ºC for 1 hour. After this time, the reaction mixture was quenched with methanol and stirred at 0ºC for 15 minutes. The reaction mixture was then concentrated under reduced pressure, at room temperature, and then co-evaporated with toluene (3x). The crude solid was then washed with (EtOAc:hexanes 3:7) to yield the title compound **6** as a white solid (65 mg, 63%) $R_f$ 0.34 EtOAc:hexanes (7:3). $^1$H NMR (500 MHz, $CDCl_3$): δ = 7.38-7.27 (m, 13H), 7.24-7.21 (m, 2H), 5.53 (d, $J$ = 7.5 Hz, 1H), 4.79-4.72 (m, 2H), 4.60-4.48 (m, 4H),

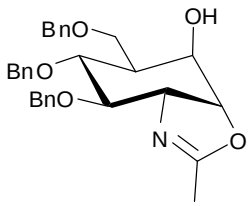
3.99 (t, *J* = 7.5 Hz, 1H), 3.76-3.71 (dd, *J* = 4.6, 9.0 Hz, 1H), 3.66 (t, *J* = 8.3 Hz, 1H), 3.54 (t, *J* = 8.3 Hz, 1H), 3.45 (d, *J* = 8.4 Hz, 1H), 3.43-3.40 (m, 1H), 3.13 (d, *J* = 3.7 Hz, 1H), 2.45-2.35 (m, 1H), 1.74 (s, 3H) ppm. $^{13}$C NMR (126 MHz, $CDCl_3$): δ = 169.92, 138.31, 138.26, 137.92, 128.61, 128.49, 128.38, 128.32, 127.99, 127.96, 127.88, 127.65, 127.58, 80.07, 76.14, 74.51, 74.01, 73.26, 68.78, 54.89, 54.51, 51.25, 41.25, 41.55, 23.16 ppm. FTIR (neat, $cm^{-1}$): 3310, 1636 (C=O), 1152, 695. HR-MS (APCI): calc. for $C_{30}H_{34}NO_5$ $[M+H]^+$ 488.2437, found 488.2438.




*N*-((1S,2R,3R,4R,5R,6R)-3,4-bis(benzyloxy)-5-((benzyloxy)methyl)-7-oxabicyclo[4.1.0]heptan-2-yl)-2,2,2-trifluoroacetamide **7**

The azide **5** (265 mg, 0.56 mmol) was dissolved in a mixture of THF:$H_2O$ (20:1, 8 mL) and cooled to 0ºC in an ice bath. At this same temperature, trimethylphosphine (1 M in toluene, 700 µL, 0.67 mmol) was added drop-wise, and the resultant solution was then left to stir at 0ºC, for 2 hours. After this time, the reaction solvent was removed at room temperature under reduced pressure. The crude residue was then co-evaporated with toluene (5x) and dried for 30 minutes under reduced pressure. The resulting white solid was dissolved in dry $CH_2Cl_2$, and cooled to 0ºC in an ice bath. At this same temperature, pyridine (140 µL, 1.69 mmol) and $(CF_3CO)_2O$ (120 µL, 0.84 mmol) were then added drop-wise, successively. The resultant solution was left to stir at 0ºC for 30 minutes. After this time, the reaction mixture was quenched with methanol (0ºC). The reaction mixture was then concentrated under reduced pressure, at room temperature, and then co-evaporated with toluene (3x). The residue obtained was then filtered through a plug of silica gel (eluted with EtOAc) and concentrated.

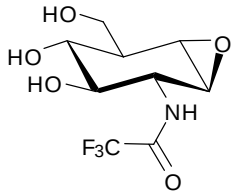
Trituration (10:5:85 EtOAc:$CH_2Cl_2$:hexanes) of the crude reside gave the title compound **7** as a white solid (176 mg, 61%). $R_f$ 0.48 EtOAc:hexanes (3:7). $^1$H NMR (500 MHz, $CDCl_3$): δ = 7.40-7.28 (m, 11H), 7.27-7.19 (m, 4H), 6.76 (d, *J* = 7.8 Hz, 1H), 4.71-4.64 (m, 2H), 4.61-4.48 (m, 4H), 4.29-4.25 (m, 1H), 3.74-3.67 (m, 2H), 3.59-3.52 (m, 2H), 3.42 (t, *J* = 3.2 Hz, 1H), 3.15 (d, *J* = 3.5 Hz, 1H), 2.54-2.47 (m, 1H) ppm. $^{13}$C NMR (125.8 MHz, $CDCl_3$): δ = 157.07 (q, *J* = 37 Hz), 138.29, 137.57, 137.48, 128.83, 128.71, 128.56, 128.28, 128.24, 128.15, 128.13, 127.83, 115.66 (q, *J* = 288 Hz), 78.60, 75.20, 74.12, 73.92, 73.47, 68.76, 53.57, 53.53, 50.12, 40.64 ppm. FTIR (neat, cm$^{-1}$): 3295, 3033, 2864, 1699, 1558. HR-MS (APCI): calc. for $C_{30}H_{31}F_3NO_5$ $[M+H]^+$ 542.2154, found 542.2164.




(3aR,4R,5R,6S,7R)-4,5-bis(benzyloxy)-6-((benzyloxy)methyl)-2-methyl-3a,4,5,6,7,7a-hexahydrobenzo[d]oxazol-7-ol **8**

The epoxide **6** (47 mg, 0.1 mmol) was dissolved in MeOH and stirred for at room temperature for 1 hour. The solution was concentrated and flash chromatography of the residue (EtOAc:hexanes 3:2) gave the title compound **8** as a white solid (44 mg, 94%). $R_f$ 0.45 EtOAc: hexanes 7:3. $^1$H NMR (600 MHz, $CDCl_3$): δ = 7.38-7.25 (m, 3H), 7.23-7.21 (m, 2H), 4.88 (d, *J* = 11.3Hz, 1H), 4.75 (d, *J* = 11.3 Hz, 1H), 4.66 (d, *J* = 11.3 Hz, 1H), 4.54 (dd, *J* = 3.5, 9.2 Hz, 1H), 4.48 (s, 2H), 4.44 (d, *J* = 11.3 Hz, 1H), 4.33-4.29 (m, 1H), 4.29-4.26 (m, 1H), 3.87 (dd, *J* = 5.3, 9.0 Hz, 1H), 3.77 (dd, *J* = 6.1, 9.0 Hz, 1H), 3.69-3.61 (m, 3H), 1.97 (d, *J* = 1.1 Hz, 3H), 1.97-1.92 (m, 1H) ppm. $^{13}$C NMR (151 MHz, $CDCl_3$): δ = 165.03, 138.42, 138.06, 137.61, 128.52, 128.42, 128.33, 128.00, 127.90, 127.75, 127.64, 83.33, 80.98, 75.39, 73.57, 73.54, 72.91, 69.61, 69.43, 68.56, 41.11, 14.09 ppm. FTIR (neat, cm$^{-1}$): 3030, 2861,

1661, 1496, 1454, 1388, 1362, 1222. HR-MS (APCI): calc. for $C_{30}H_{34}NO_5$ $[M+H]^+$ 488.2437 found 488.2440.




N-((1S,2R,3R,4R,5R,6R)-3,4-dihydroxy-5-(hydroxymethyl)-7-oxabicyclo[4.1.0]heptan-2-yl)-2,2,2-trifluoroacetamide **2**

Palladium hydroxide-on-carbon (20%, 2.8 mg) was added to a solution of compound **7** (22 mg, 0.04 mmol) dissolved in MeOH (1 mL). The reaction vessel was sealed, then evacuated and backfilled three times with $H_2$ gas. The resultant mixture was left to stir under a $H_2$ gas atmosphere for two hours at room temperature. After this time, the reaction mixture was gravity filtered through a plug of cotton wool (2x), and then concentrated under reduced pressure. The crude white solid obtained was then washed with EtOAc (2x), and dried under reduced pressure at room temperature for two hours to give the title compound **2** as a white solid (10 mg, 91%) $R_f$ 0.04 EtOAc:hexanes (1:1). $^1$H NMR (600 MHz, $CD_3OD$): δ = 4.03 (dd, *J* = 4.8, 10.8 Hz, 1H), 3.94 (d, *J* = 9.6 Hz, 1H), 3.72 (dd, *J* = 9.0, 9.0 Hz, 1H), 3.48-3.42 (m, 2H), 3.16 (t, *J* = 9.6, Hz, 1H), 3.02 (d, *J* = 3.6 Hz, 1H), 2.05-1.96 (m, 1H) ppm. $^{13}$C NMR (151 MHz, $CD_3OD$): δ = 159.15 (q, *J* = 37 Hz), 117.55 (q, *J* = 286 Hz), 74.49, 69.61, 62.40, 56.07, 56.03, 54.36, 45.64 ppm. FTIR (neat, $cm^{-1}$): 3540, 3304, 1698, 1561, 1342. HR-MS (ESI): calc. for $C_9H_{13}F_3NO_5$ $[M+H]^+$ 272.0745, found 272.0740.

**Kinetic Analysis of 2:** The time-dependent inactivation of *Bc*NagZ by **2** was monitored by measuring the residual enzyme activity over time. This was accomplished by incubation of the enzyme (0.016 mg $mL^{-1}$ for *Bc*NagZ) in 200 μL of buffer solution (25 mM Hepes, 0.5 M

NaCl, 5% glycerol (v/v), pH = 7.0, adjusted with NaOH) containing the inactivator **2**. Five inactivation concentrations (0, 1, 5, 10, 25, and 40 μM) were used with the control mixture containing the same concentration of the appropriate enzyme but no inactivator. Both inactivation and control samples were incubated at 28°C, and at various time intervals an aliquot (20 μL) of each inactivation mixture was added to a solution of the substrate, 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in buffer solution (25 mM Hepes, 0.5 M NaCl, 5% glycerol (v/v), pH = 7.0, adjusted with NaOH) so that the final assay contained *Bc*NagZ (active + inactive) at a concentration of 1.6 μg $mL^{-1}$ and 0.5 mM substrate in buffer; the total volume was 200 μL. The initial rates were then measured under steady-state conditions by spectrophotometric monitoring of the release of 4-nitrophenolate ion at 354 nm. Pseudo-first-order rate constants, $k_{obs} = k_i[I]/(K_i+[I])$, for the decay of activity at each inactivator concentration were determined from fitting the decay curve to a single exponential decay equation using the nonlinear regression analysis package of the program Grafit.

*Reversibility study* – Investigation into the time-dependent reactivation of *Bc*NagZ was monitored by measuring the residual enzyme activity over time. This was accomplished by first incubating the enzyme (0.16 mg $mL^{-1}$ for *Bc*NagZ) in 200 μL of buffer solution (25 mM Hepes, 0.5 M NaCl, 5% glycerol (v/v), pH = 7.0, adjusted with NaOH) containing the inactivator **2** at a concentration of either zero or 50 μM for 60 minutes. An aliquot (20 μL) of each mixture was then added to a solution of the substrate, 4-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in buffer solution (25 mM Hepes, 0.5 M NaCl, 5% glycerol (v/v), pH = 7.0, adjusted with NaOH) so that the final assay contained *Bc*NagZ at a concentration of 1.6 μg $mL^{-1}$ (1 in 100 dilution) and 0.5 mM substrate in buffer;. The rate of these solutions was then measured under steady-state conditions by spectrophotometric monitoring of the release of 4-nitrophenolate ion at 354 nm.

*Other enzymes* - Assays against human *O*-GlcNAcase (OGA), β-hexosamindase B and NAGLU were carried out in a similar fashion with preincubation of the enzyme with the inhibitor in a buffer solution (200 μL) for various times before an aliquot of sample (20 μL) was added to a solution of the substrate in buffer solution (total volume 200 μL) and activity obtained. Activity assays for β-hexosaminidase B and NAGLU were carried out in triplicate at 37 °C for 30 minutes using a stopped assay procedure in which the enzymatic reactions were quenched by the addition of a 4-fold excess of quenching buffer (200 mM glycine, pH 10.75). Assays against OGA were carried out in triplicate at 37°C for 20 minutes using a continuous assay procedure. Assays against β-hexosaminidase B were conducted in buffer (50 mM citrate, 100 mM NaCl, pH 4.25) and OGA (PBS, pH 7.4 buffer, 0.03% BSA (w/v)) using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as substrate. For NAGLU, assays were performed in acetate buffer (100 mM, pH 4.3), containing BSA (0.5 mg $ml^{-1}$) using 4-methylumbelliferyl *N*-acetyl-α-D-glucosaminide as substrate. For β-hexosaminidase B and NAGLU assays, release of 4-methylumbelliferone was measured using a Varian CARY Eclipse Fluorescence Spectrophotometer 96-well plate system with readings taken at excitation and emission wavelengths of 368 nm and 450 nm respectively, with 5 mm slit openings. For OGA the extent of 4-methylumbelliferone release was determined using a BioTek Synergy Plate Reader at excitation and emission wavelengths of 350 and 445 nm respectively. Assays for β-hexosaminidase B and NAGLU contained a substrate concentration of 0.5 mM and OGA 0.2 mM, and the enzyme was at a concentration of 20 nM for β-hexosaminidase B, 100 nM for NAGLU and 20 nM for OGA.

**_Bc_NagZ Crystallization, Structure Determination and Refinement:**

*Expression and purification of BcNagZ*

Expression of recombinant *Bc*NagZ was induced in E. coli BL21(DE3)Gold cells using IPTG and the soluble, hexahistidine-tagged protein subsequently purified as described.[2]

*Crystallization and structure determination of BcNagZ bound to **2***

*Bc*NagZ crystals were grown at room temperature using the hanging drop vapour-diffusion method by mixing equal volumes of reservoir buffer (30-32% PEG8000 (w/v), 0.1M MES pH 6.6-6.8) with protein solution (12-15 mg/ml). Compound **2** was added to a droplet containing several *Bc*NagZ crystals in reservoir buffer at a final concentration of 2 mM, followed by a 24-hour incubation room temperature. Crystals incubated with **2** were cryo-protected by sweeping through a solution of 30% PEG3500 (w/v), 15% PEG8000 (w/v) and 0.1M MES pH 6.6 and then cooled to 100K in $N_2$(g) by mounting directly onto the goniometer of the X-ray diffractometer (Rigaku MicroMax007HF, Raxis $4^{++}$, X-stream 2000 cryostat). X-ray data were indexed using Mosflm[3], then scaled and averaged using SCALA (CCP4 package)[4]. The *Bc*NagZ:inhibitor complex structures were determined by molecular replacement using PHASER (from within the PHENIX package)[5] and a solvent-free structure of *Bc*NagZ (PDB ID: 4G6C) as the search model. Restraint and linkage files for **2** were generated using PHENIX eLBOW[5] and a model of the inhibitor was manually fitted into its electron density. Subsequent refinement of the complex and addition of solvent was carried out using PHENIX.REFINE[5] and COOT[6]. Crystallographic and refinement statistics are presented in Table S1.

**Determination of the Minimal Inhibitory Concentration of β-lactams:**

Cultures were prepared by inoculating 5 ml of Mueller-Hinton (MH) Broth with a small amount of a glycerol stock of *P. aeruginosa* PA01 Δ*dacB*[7] and then were grown at 37 ºC to

an $OD_{600}$ of ≈ 0.5. 96-well plates containing a range of concentrations of β-lactams varying by factors of 2 were prepared. Each well contained 90 μl of the antibiotic in MH Broth and the volume was made up to 100 μl by addition of either 10 μl of **2** (1 mM in MH Broth) or 10 μl MH Broth. These broths were inoculated with 100 μl of the culture and allowed to incubate at 37 ºC for 18 hours. The optical density at 595 nm was measured for all cultures and the MIC determined from the concentration of antibiotic at which no growth was observed. All MIC determinations were performed in triplicate.

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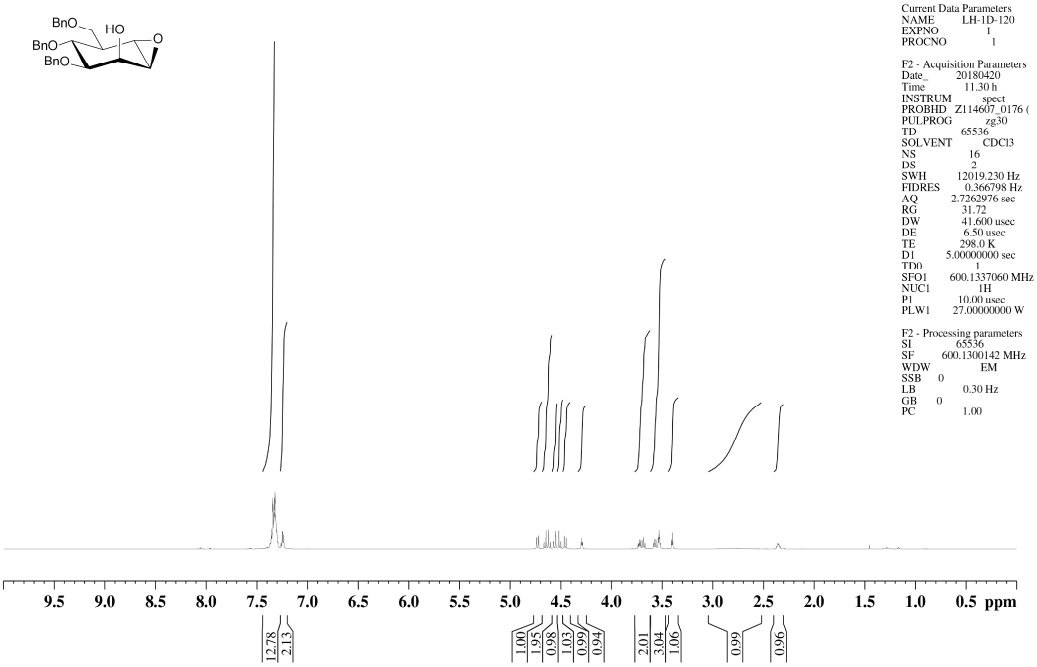
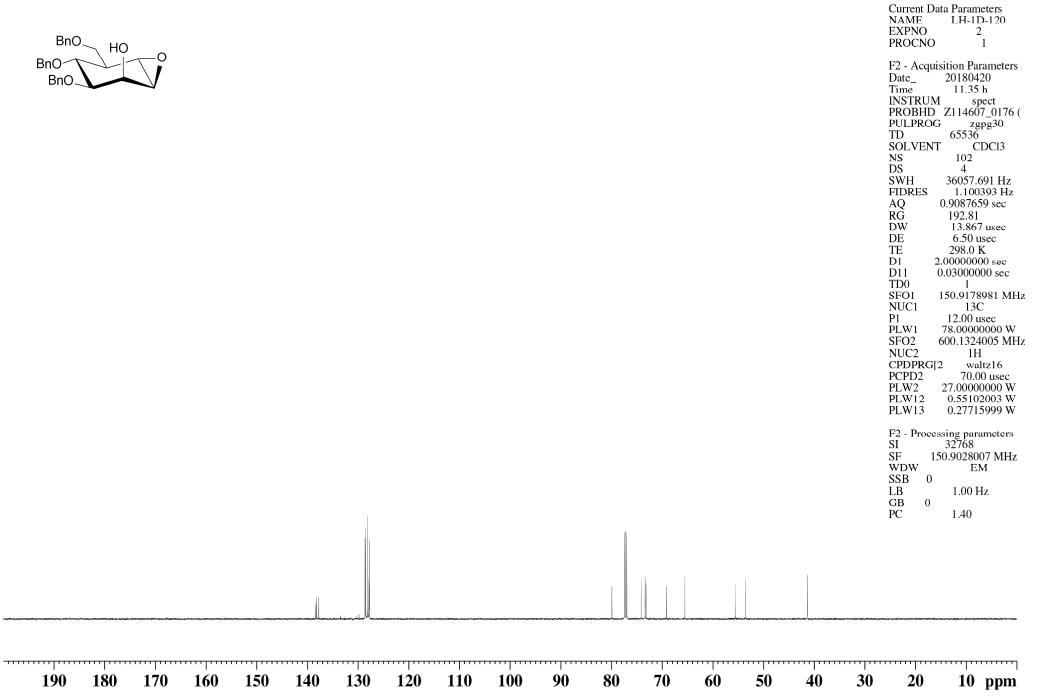
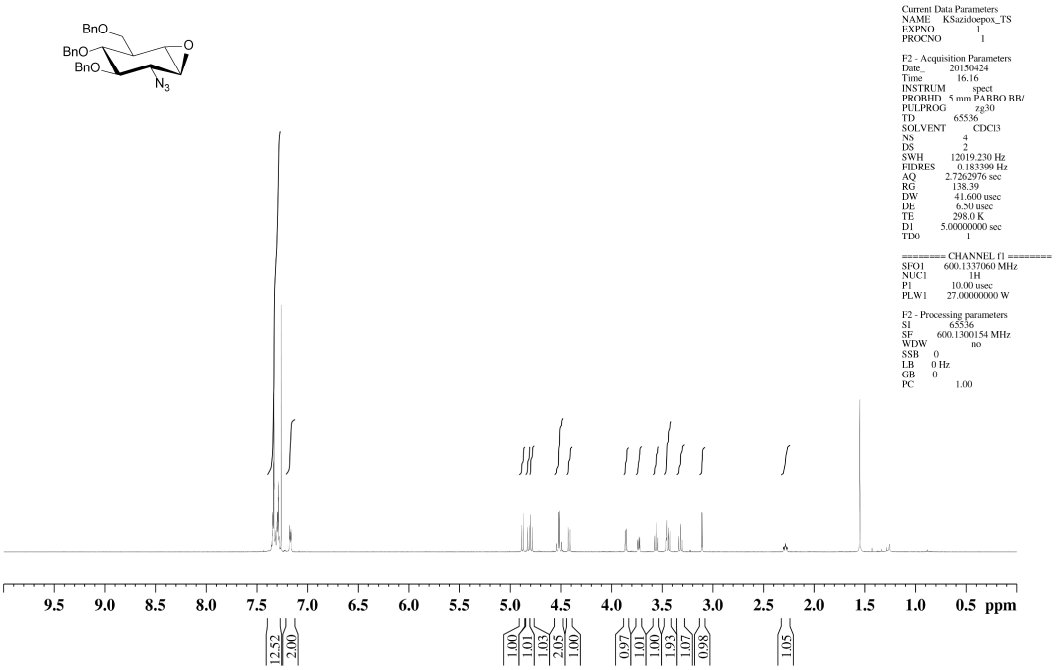
$^{1}$H NMR spectrum of **4**



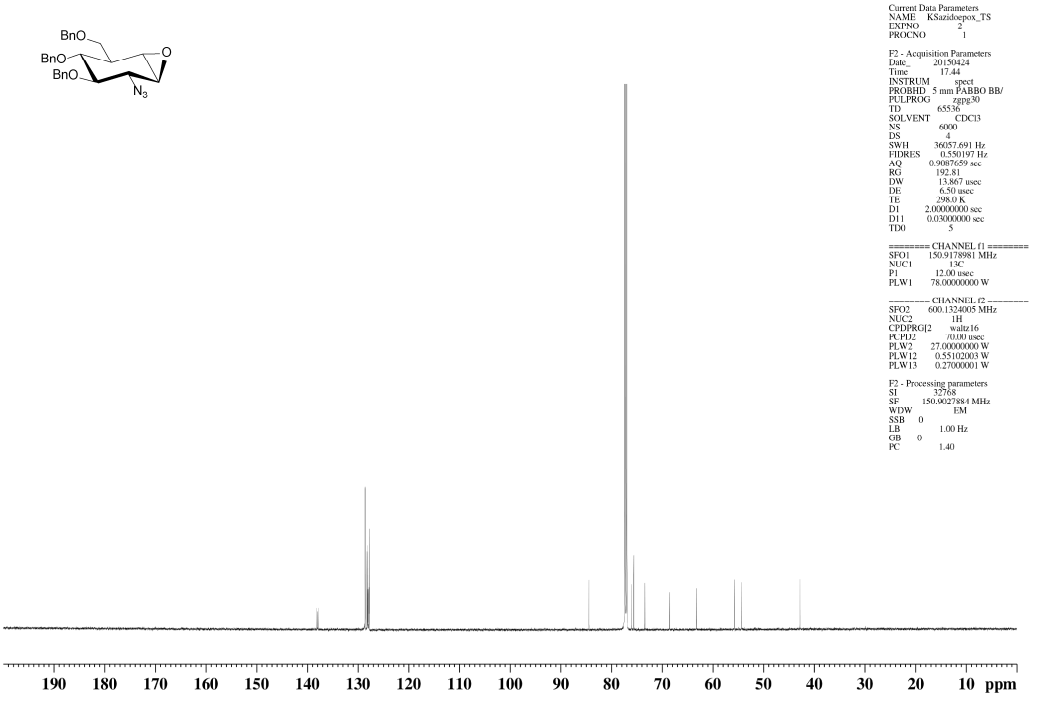

$^{13}$C NMR spectrum of **4**



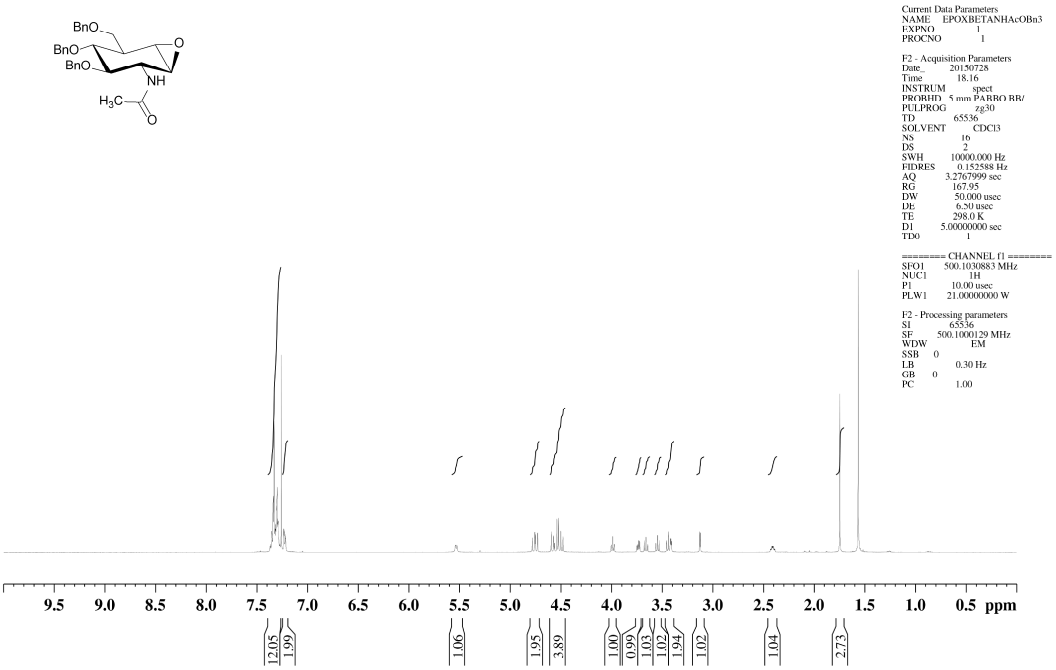
$^{1}$H NMR spectrum of **5**



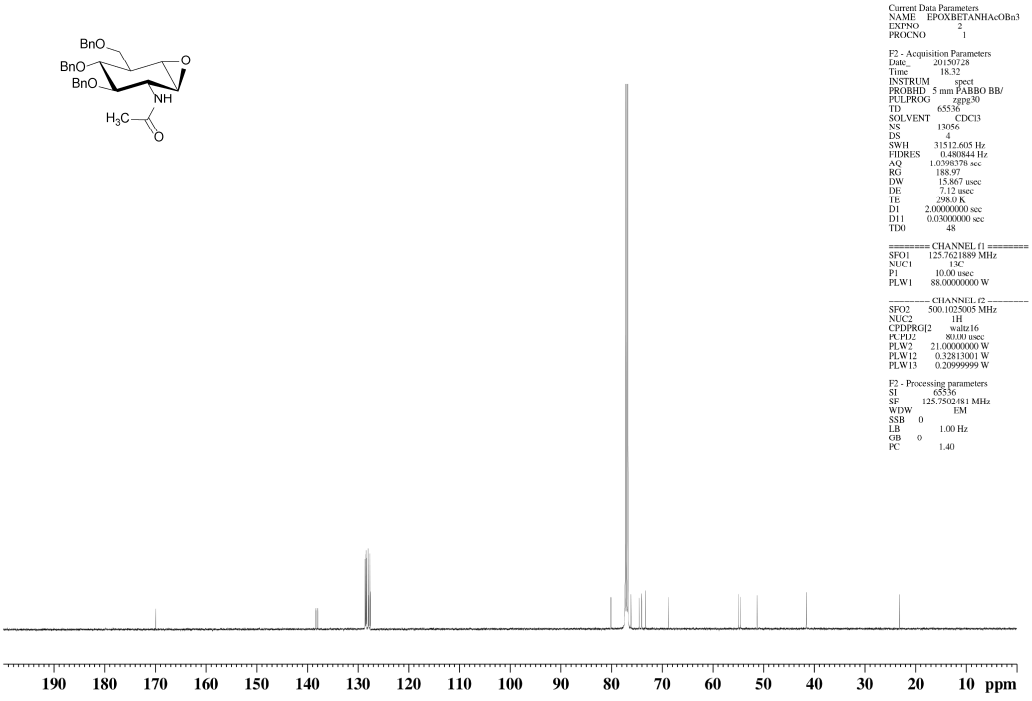

$^{13}$C NMR spectrum of **5**



$^{1}$H NMR spectrum of **6**




$^{13}$C NMR spectrum of **6**



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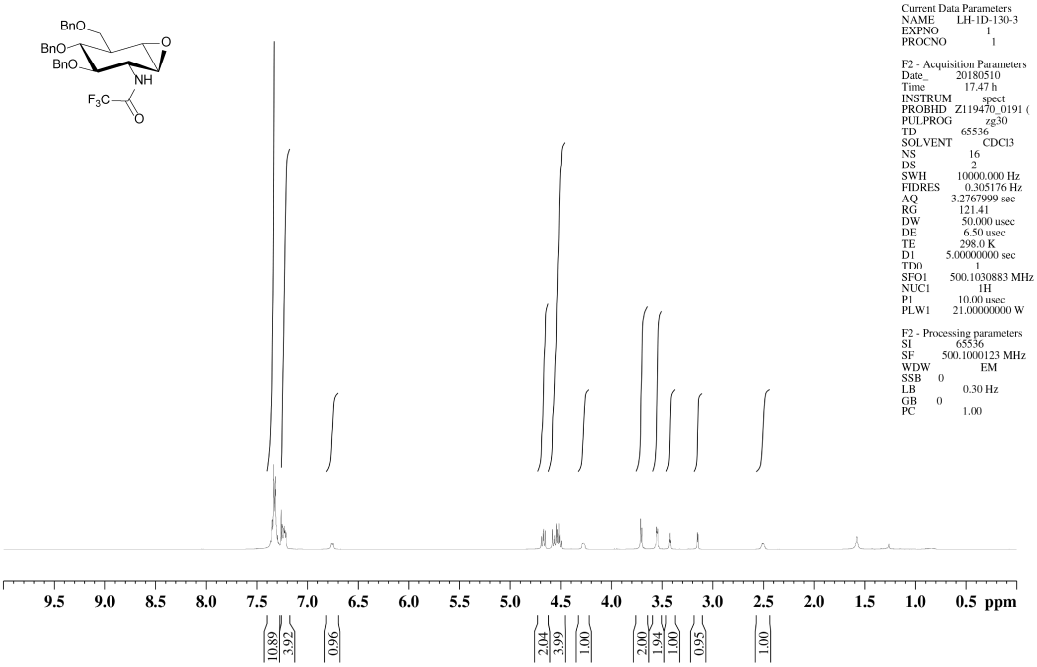
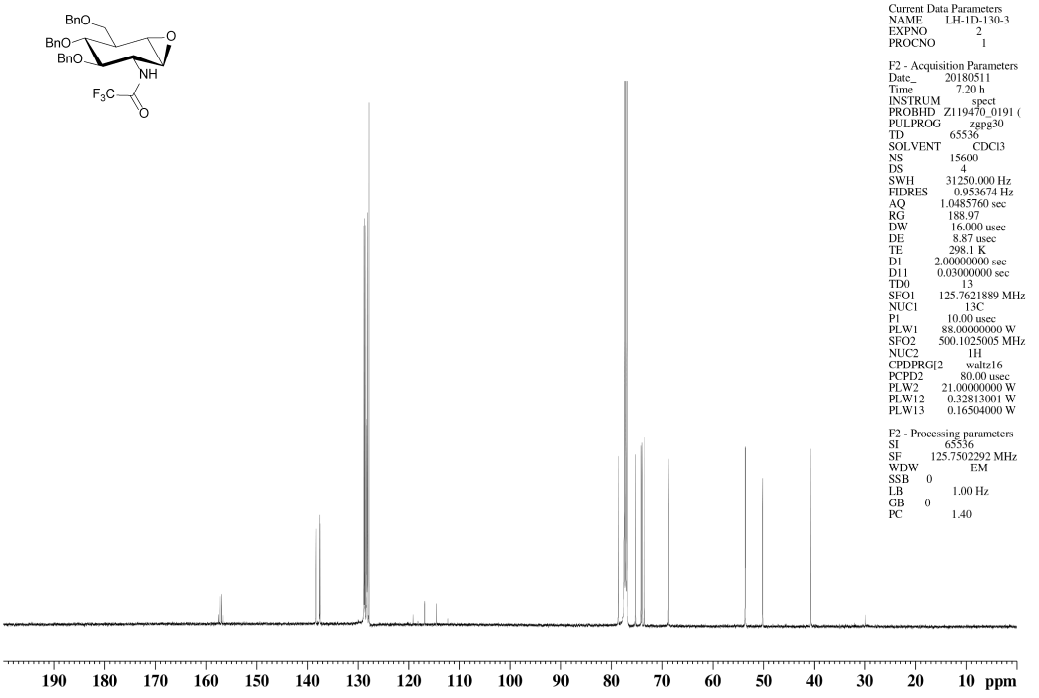



$^{13}$C NMR spectrum of **7**



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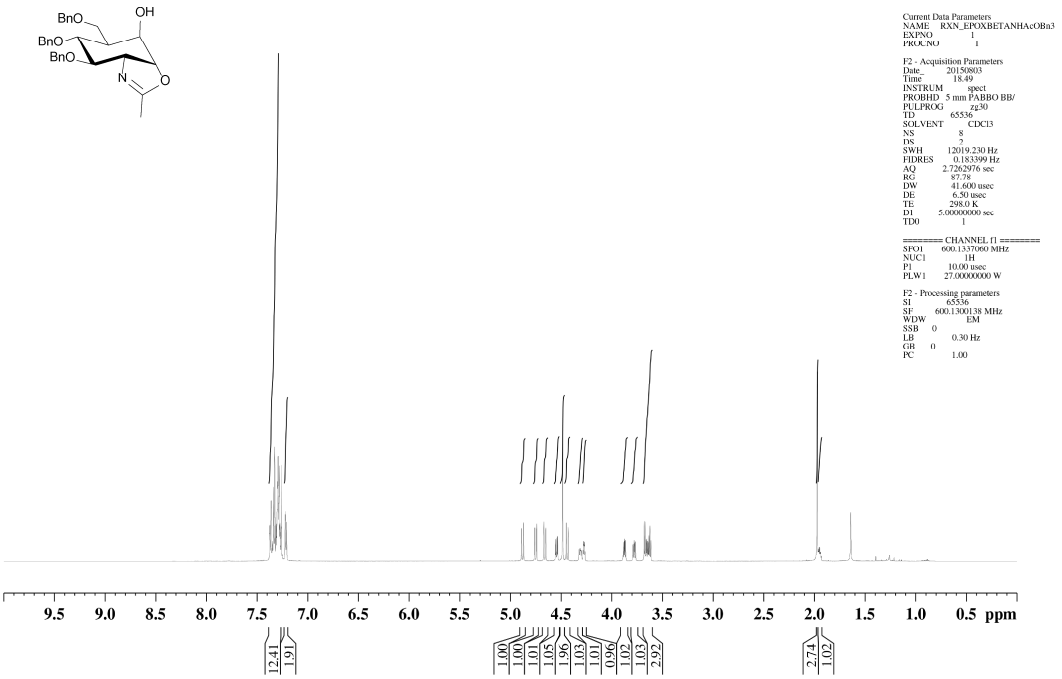
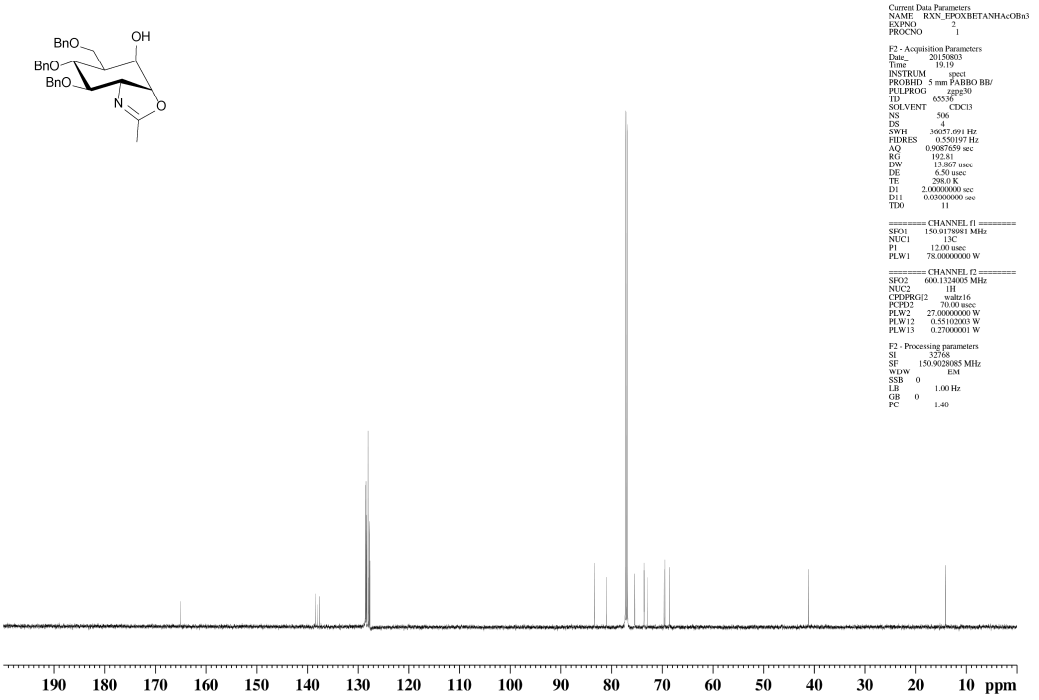
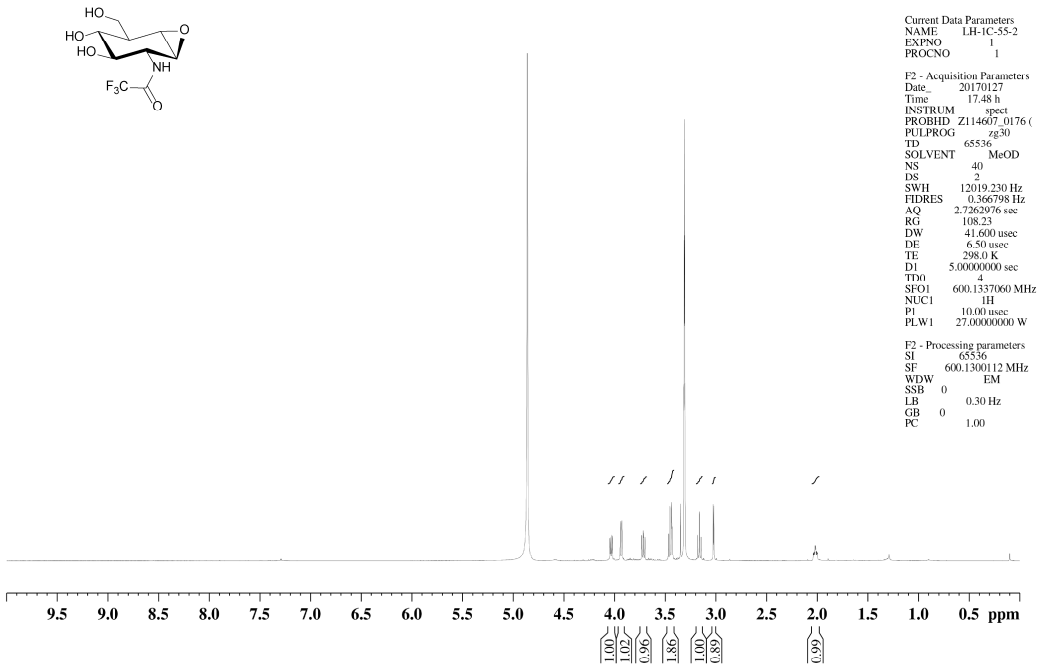



$^{13}$C NMR spectrum of **8**



$^{1}$H NMR spectrum of **2**




$^{13}$C NMR spectrum of **2**