Suppporting Information

Isoindoline-based fluorogenic probes bearing a self-immolative linker for the sensitive and selective detection of O-GlcNAcase activity

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Content list

S1. Experimentsal section

General. All chemicals and reagents were purchased commercially at analytical grade unless otherwise noted. N-acetyl-β-D-hexosaminidase (from *Canavalia ensiformis*) was purchased from Shanghai Yuanye Bio-Technology Company (#S10237). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AM 400MHz spectrometer with tetramethylsilane as the internal reference. UV-vis absorption spectra were measured on a Varian Cary 500 UV-vis spectrophotometer. Fluorescence spectra were measured on a Cary Eclipse Fluorescence spectrophotometer.

OGA expression and purification. pT7-OGA (human)-6×His were transformed in *E. coli* BL21(DE3) cells and cells were grown in LB medium containing kanamycin (50 μg mL^{-1}) at 37 °C until OD_{600nm} reached 0.5–0.6. The temperature was then decreased to 16°C and cells were induced with 0.5 mM IPTG overnight. After induction, cells were collected by centrifugation (5000 rpm for 15 min at 4 °C) and washed by phosphate buffered saline (PBS) once (8000 rpm for 20 min at 4°C). Collected cells were then resuspended in lysis buffer (50 mM tris base pH 8.0, 200 mM NaCl, 1% Triton-100(v/v) and 4 mM β-mercaptoethanol) and lysed using a sonic dismembrator for 30 min (3 sec on and then 6 sec off) until clear. The lysate was clarified by centrifugation at 8000 RPM at 4 °C for 20 min twice. The supernatant was mixed and incubated with Ni-NTA Agarose Resin for 3 hrs at 4 °C and then loaded to a column. A lower concentration of imidazole elution buffer (50 mM tris base pH 7.4, 200 mM NaCl, 5% glycerin (v/v) and 10/50 mM imidazole) was prepared to elute undesired protein and a higher concentration of imidazole elution buffer (50 mM tris base pH 7.4, 200 mM NaCl, 5% glycerin (v/v) and 300 mM imidazole) was used to elute OGA. Fractions containing OGA were dialyzed at 4 °C overnight and the end product was collected and stored at -80 ℃ for further measurements.

Fluorescence measurements. Fluorescence experiments were performed using a SpectraMax M5 Microplate Reader with the excitation/emission wavelength being 405/515 nm. Working buffer was prepared with 100 mM NaCl, 0.1% BSA, 50 mM NaH_2PO_4 in deionized water (pH 7.0). 100 μL DMSO was added into 900 μL deinoized water to afford a 10% DMSO solution. Solutions of **GlcNAc-BHID (**10 mM**)**, **GlcNAc-Bn-BHID (**10 mM**)**, **GlcNPr-Bn-BHID (**10 mM**)**, **4-MUF-NAG (**20 mM**)** and **Thiamet-G** (10 mM) were prepared in DMSO and solution of OGA was prepared in the buffer mentioned above. Data

were plotted and fitted using Prism 9.5.0. Enzyme assays were performed in Perkin-Elmer 384 well black plate.

Solvent effect of BHID. A DMSO solution of **BHID** (5 mM) was diluted into different solvents (MeCN, deionized water and phosphate buffered saline (PBS, 0.01 M, pH 7.4) to afford a final concentration of 10 μM, and the fluorescence spectra of corresponding solutions were recorded on a Cary Eclipse Fluorescence spectrophotometer with excitation at 405 nm.

Optimization of substrate concentration. A DMSO solution of **GlcNAc-Bn-BHID** and **GlcNAc-BHID** (10 mM) was half-diluted from 50 μM to 1.6 μM with working buffer and a control group without substrate was used. OGA concentration was set as 1 μg mL^{-1} and microplates were placed at room temperature for 90 min before fluorescence detection. The K_m (Michalies constant) value was fitted by GraphPad Prism 9.5.0. All experiments were repeated three times.

Optimization of OGA concentration. OGA was half diluted from 150 μg mL^{-1} to 0.146 μg mL^{-1} with working buffer and a control group without OGA was used. **GlcNAc-Bn-BHID** and **GlcNAc-BHID** were set as 50 μM (diluted with working buffer) and mixed with OGA. Plates were placed at room temperature for 90 minutes before fluorescence detection. All experiments were repeated three times.

Selectivity assay. Different enzymes including β-galactosidase (3 U mL^{-1}), α-glucosidase (3 U mL^{-1}), α-galactosidase (3 U mL^{-1}), lysozyme (3 U mL^{-1}), pepsin (3 U mL^{-1}), glucose oxidase (3 U mL^{-1}) were diluted in PB. 50 μM **GlcNAc-Bn-BHID** (diluted with PBS) was incubated with the enzymes for 90 minutes with OGA as a positive control.

Determination of IC_{50}. Half maximal inhibitory concentration (IC_{50}) for a commercially available OGA inhibitor **Thiamet-G** was determined by measuring the changes in fluorescence intensity of **GlcNAc-Bn-BHID** and a commercial OGA probe **4-MUF-NAG**. Inhibition assays were determined in the presence or absence of various concentrations of the inhibitor and at a fixed substrate concentration of 50 μM. **Thiamet-G** was serially diluted to the desired range of concentrations in a deinoized water containing 10% DMSO, and then

was mixed in 384 well black plate with substrate and OGA for 90 min. Then, fluorescence intensities were measured and IC_{50} was fitted by GraphPad Prism 9.5.0.

Specificity between OGA and commercial hexosaminidase. 50 μM **GlcNPr-Bn-BHID** and **GlcNAc-Bn-BHID** was incubated with 1 ng μL^{-1} OGA or 0.03 U mg^{-1} commercial hexosaminidase (diluted with PBS) at room temperature. Measurements were performed every 15 min for six times in total.

Table S1. K_m value of **GlcNAc-BHID** and **GlcNAc-Bn-BHID**

Probe	*K*m (μM)
GlcNAc-BHID	17.88
GlcNAc-Bn-BHID	6.716

Table S2. IC_{50} of **4-MUF-NAG** and **GlcNAc-Bn-BHID**

Probe	IC_{50} (nM)
4-MUF-NAG	1.673
GlcNAc-Bn-BHID	0.8055

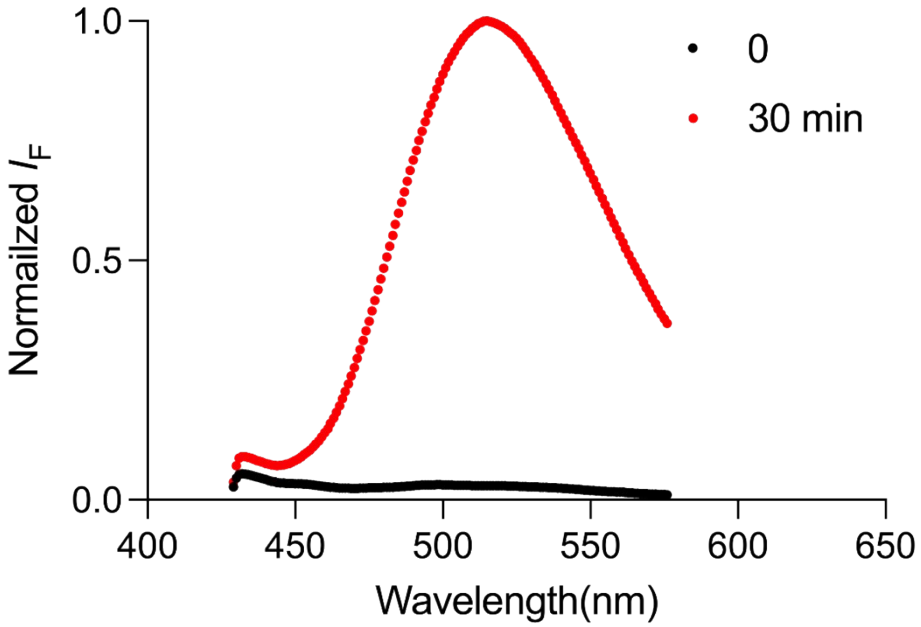
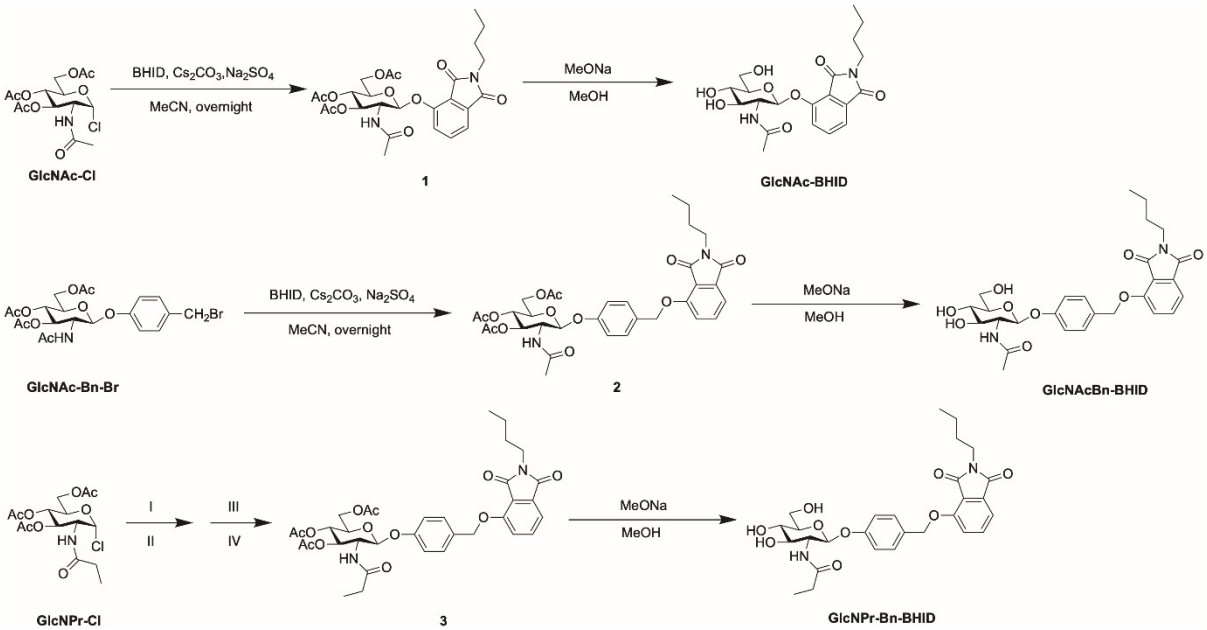


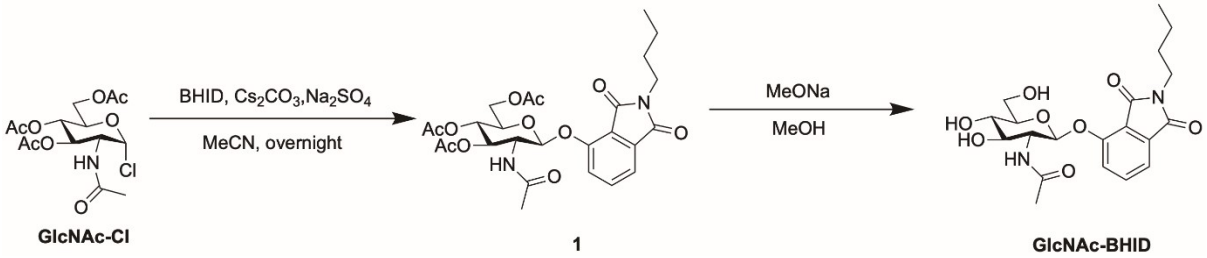
Figure S1. Fluorescence emission spectra of **GlcNPr-Bn-BHID** (50 μM) before and after incubated with OGA (1 μg mL^{-1}) for 30 min in working buffer with an excitation wavelength of 405 nm.

S2. Synthesis of the OGA probes and original spectra of new componds



Scheme S1. Reagents and conditions: (I) 4-Hydroxybenzaldehyde, Ag_2O and KI in MeCN at r.t. (II) $NaBH_4$ in $CHCl_3$/*i*-PrOH (3:1, v/v). (III) PBr_3 in CH_2Cl_2. (IV) **BHID**, Cs_2CO_3, and Na_2SO_4 in MeCN at r.t.

Synthesis of GlcNAc-BHID.



Compound **1.** A solution of **GlcNAc-Cl** (200 mg, 0.55 mmol),[1] **BHID** (60 mg, 0.27 mmol), Cs_2CO_3 (445 mg, 1.37 mmol) and Na_2SO_4 (155 mg, 1.07 mmol) in MeCN (10 ml) was vigorously stirred overnight. The reaction was monitored by TLC until the color turned to pale yellow. The solvent was removed under reduced pressure and the residue was dissolved by DCM (30 mL), washed with water (30 mL), brine (30 mL), dried (Na_2SO_4) and concentrated. The crude product was purified by column chromatography (PE/EtOAc = 50/1 to 1/3, v/v) to provide Compound **1** as a white solid (100 mg, 66.7 %). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.69 – 7.60 (m, 2H), 7.46 – 7.37 (m, 1H), 6.71 (d, J = 8.3 Hz, 1H), 5.40 – 5.30 (m, 2H), 5.18 (t, J = 9.6 Hz, 1H), 4.30 – 4.19 (m, 2H), 4.13 – 4.04 (m, 1H), 3.74 – 3.68 (m, 1H), 3.65 (t, J = 7.3 Hz, 2H), 2.11 – 2.00 (m, 9H), 1.99 (s, 3H), 1.70 – 1.58 (m, 2H), 1.46 – 1.30 (m, 2H), 0.96 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$-*d*) δ 171.1, 170.7, 170.6,

169.4, 167.5, 167.3, 153.51, 135.8, 134.0, 128.2, 120.5, 119.7, 101.0, 72.6, 72.2, 68.4, 61.8, 54.4, 37.9, 30.6, 23.3, 20.7, 20.7, 20.6, 20.1, 13.6. HRMS (ESI, m/z): $[M+Na]^+$: caled for $C_{26}H_{32}N_2O_{11}Na^+$ 571.1900, found 571.1904.

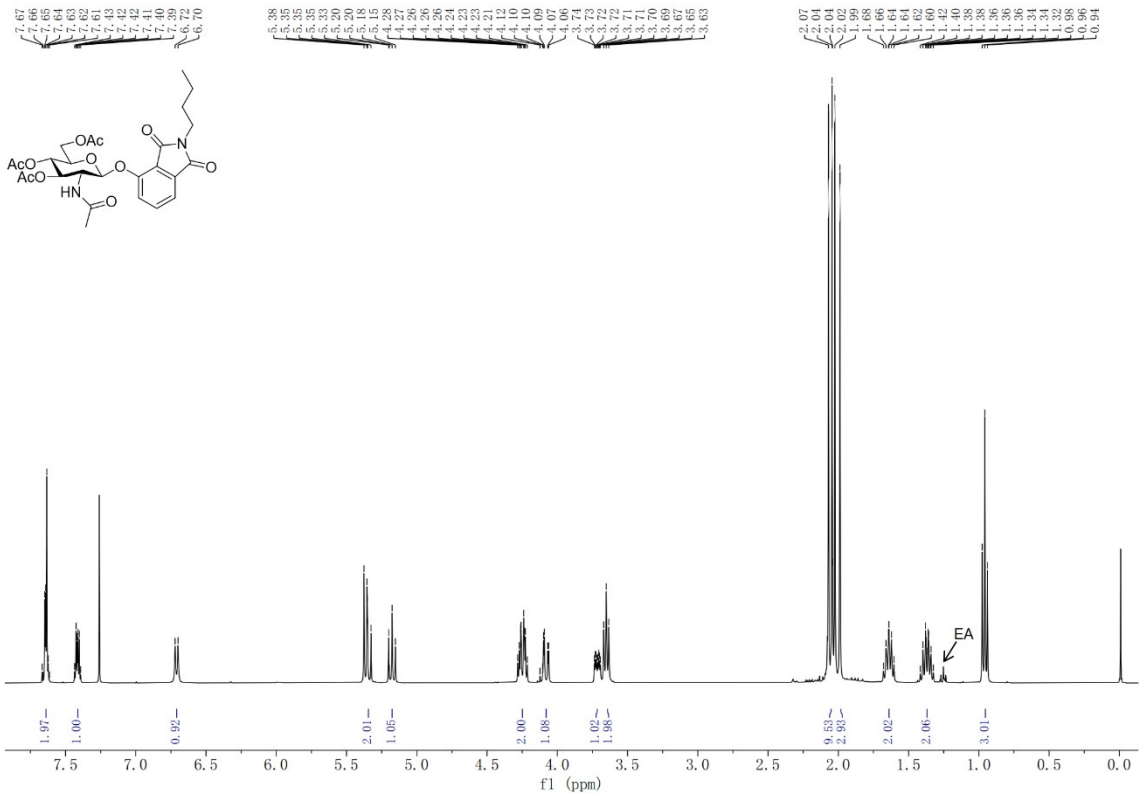


Figure S2. ^1H NMR of **1** in $CDCl_3$.

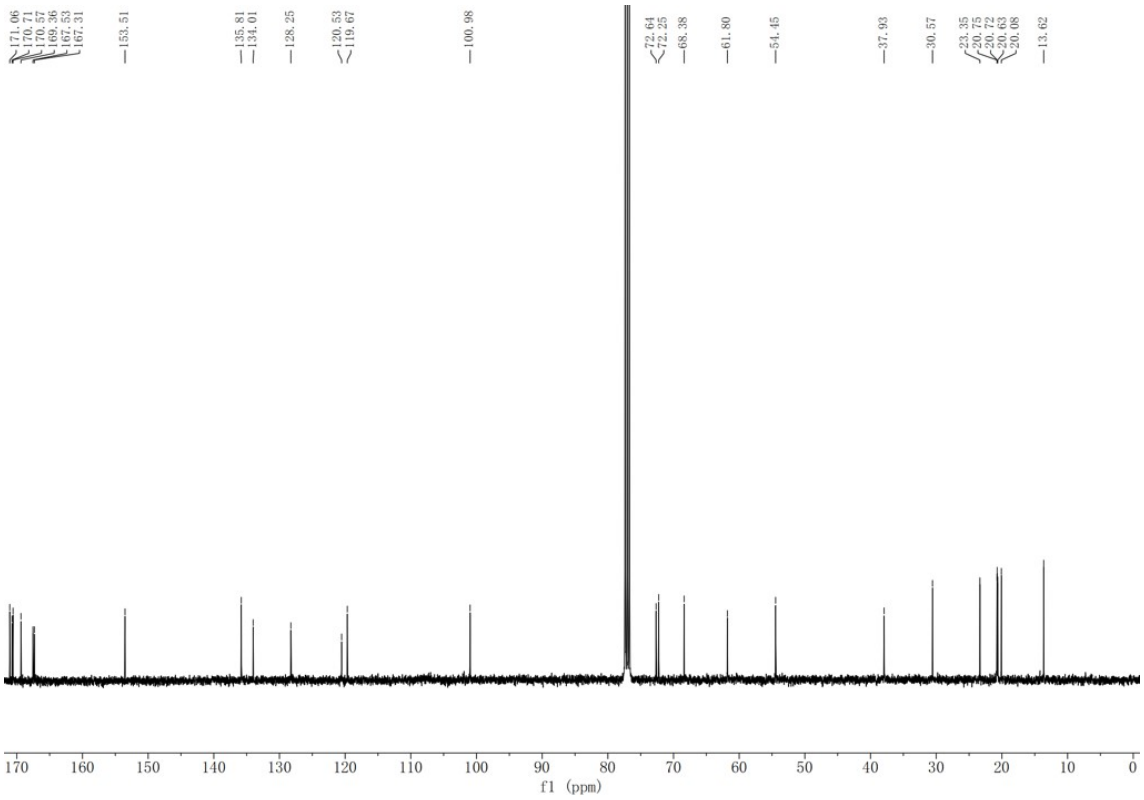


Figure S3. ^{13}C NMR of **1** in $CDCl_3$.

GlcNAc-BHID. To a stirred solution of Compound **1** (100 mg, 0.18 mol) in EtOH (15 mL) was added MeONa (30 mg, 0.55 mmol) at room temperature. The reaction mixture was stirred for 3 h at room temperature. The solvent was removed under the reduced pressure and The residue was purified by recrystallization using MeOH to afford as a white solid (36.2 mg, 47.8%): ^1H NMR (400 MHz, DMSO-d_6) δ 7.83 – 7.70 (m, 2H), 7.52 (d, J = 8.5 Hz, 1H), 7.46 (d, J = 7.4 Hz, 1H), 5.33 (d, J = 8.3 Hz, 1H), 5.12 (dd, J = 14.9, 5.4 Hz, 2H), 4.62 (s, 1H), 3.76 – 3.60 (m, 2H), 3.51 – 3.40 (m, 5H), 3.23 – 3.17 (m, 1H), 1.76 (d, J = 2.1 Hz, 1H), 1.55 – 1.48 (m, 2H), 1.29 – 1.20 (m, 2H), 0.87 (t, J = 7.1 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 169.8, 167.8, 166.0, 154.3, 136.7, 133.9, 122.8, 118.1, 116.9, 99.12, 78.1, 74.6, 70.6, 61.1, 56.0, 37.3, 30.4, 23.5, 19.9, 13.9. HRMS (ESI, m/z): $[M+Na]^+$: caled for $C_{20}H_{26}N_2O_8Na^+$ 445.1588, found 445.1587.

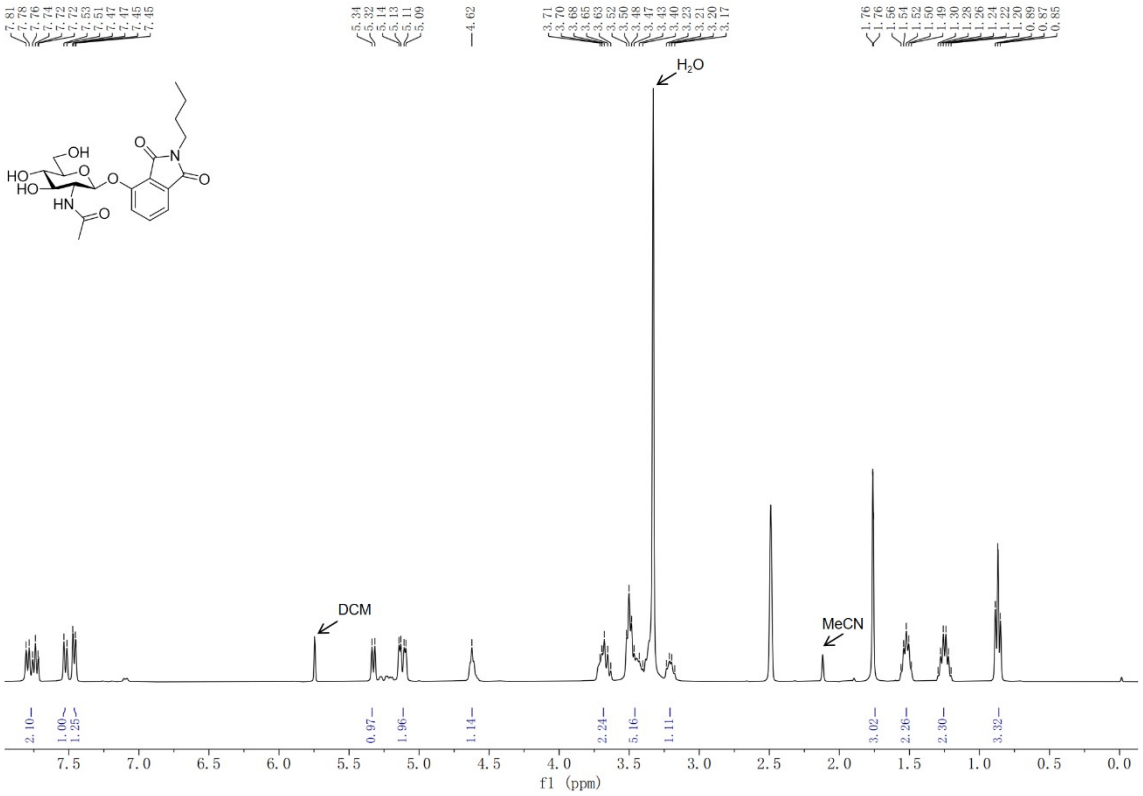


Figure S4. ^{1}H NMR of **GlcNAc-BHID** in DMSO-d_6.

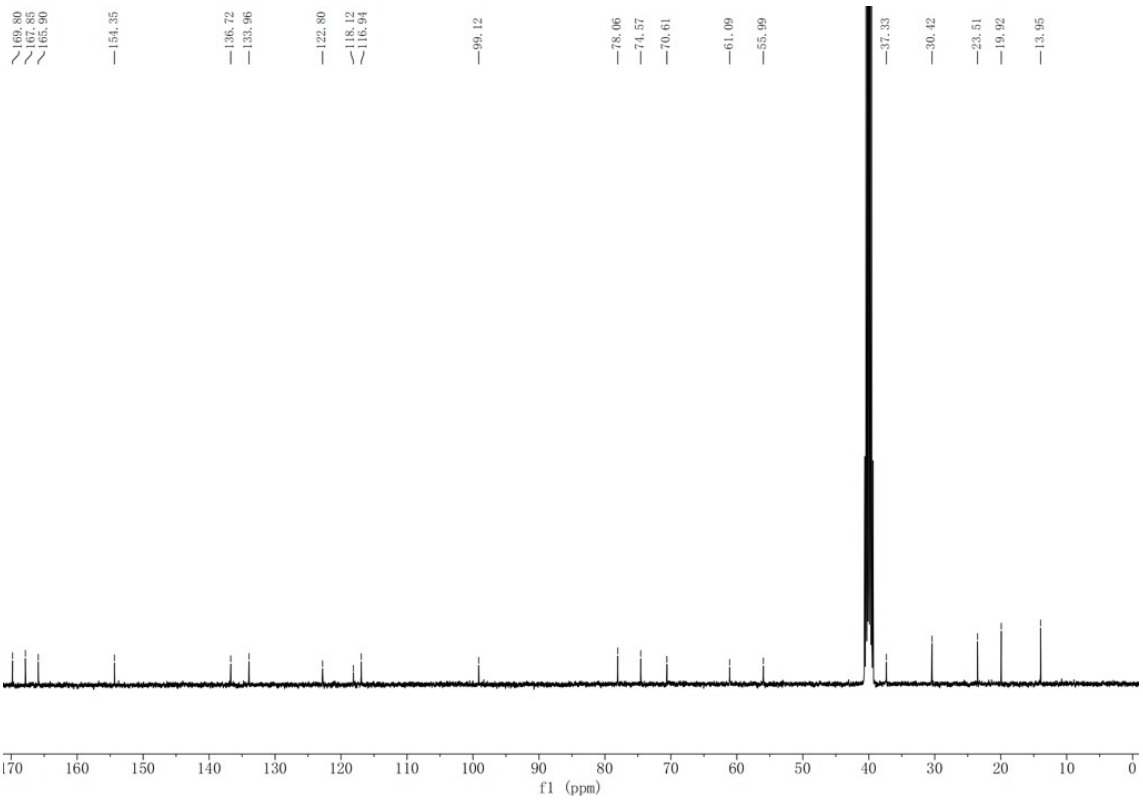
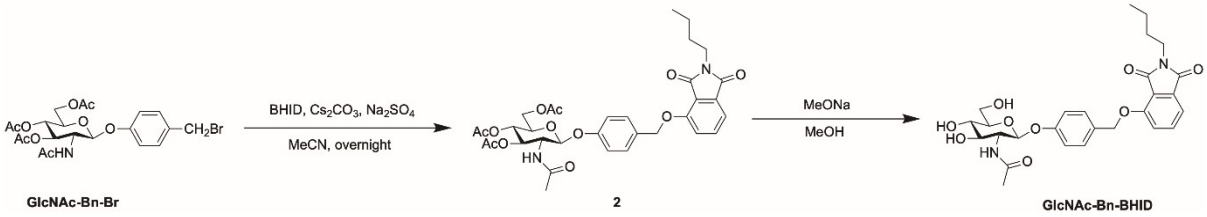


Figure S5. ^{13}C NMR of **GlcNAc-BHID** in DMSO-d_6.

Synthesis of GlcNAc-Bn-BHID.



Compound **2.** A solution of **GlcNAc-Bn-Br**[1] (200 mg, 0.39 mmol), **BHID** (42.5 mg, 0.19 mmol), Cs_2CO_3 (315 mg, 0.97 mmol) and Na_2SO_4(1.1 g, 0.775 mmol) in MeCN (15 ml) was vigorously stirred overnight. The reaction was monitored by TLC until the color turned to pale yellow. The solvent was removed under reduced pressure and the residue was dissolved with DCM (30 mL), washed with water (30 mL), brine (30 mL), dried (Na_2SO_4) and concentrated. The crude product was purified by column chromatography (PE/EtOAc = 50/1 to 1/3, v/v) to provide Compound **2** as a white solid (37 mg, 16.3 % yield). ^{1}H NMR (400 MHz, Chloroform-d) δ 7.59 (dd, J = 8.4, 7.3 Hz, 1H), 7.42 (dd, J = 7.9, 5.1 Hz, 3H), 7.18 (d, J = 8.4 Hz, 1H), 7.05 – 6.94 (m, 2H), 5.67 – 5.60 (m, 1H), 5.41 (dd, J = 10.5, 9.2 Hz, 1H), 5.30 – 5.23 (m, 3H), 5.15 (t, J = 9.6 Hz, 1H), 4.29 (dd, J = 12.2, 5.3 Hz, 1H), 4.20 – 4.06 (m, 2H), 3.88 – 3.84 (m, 1H), 3.65 (t, J = 7.2 Hz, 2H), 2.13 – 2.02 (m, 9H), 1.95 (s, 3H), 1.70 – 1.60 (m, 2H), 1.44 – 1.29 (m, 2H), 0.93 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$-*d*) δ 170.8, 170.7, 170.5, 169.4, 168.1, 167.0, 156.9, 155.5, 135.8, 134.4, 130.5, 128.5, 119.3, 118.1, 117.1, 115.8, 98.90, 72.0, 70.5, 68.5, 62.1, 54.8, 37.7, 30.6, 23.3, 20.7, 20.7, 20.6, 20.1, 13.7. HRMS (ESI, m/z): $[M+K]^+$: caled for $C_{33}H_{38}N_2O_{12}K^+$, 693.2063 found 693.2062.

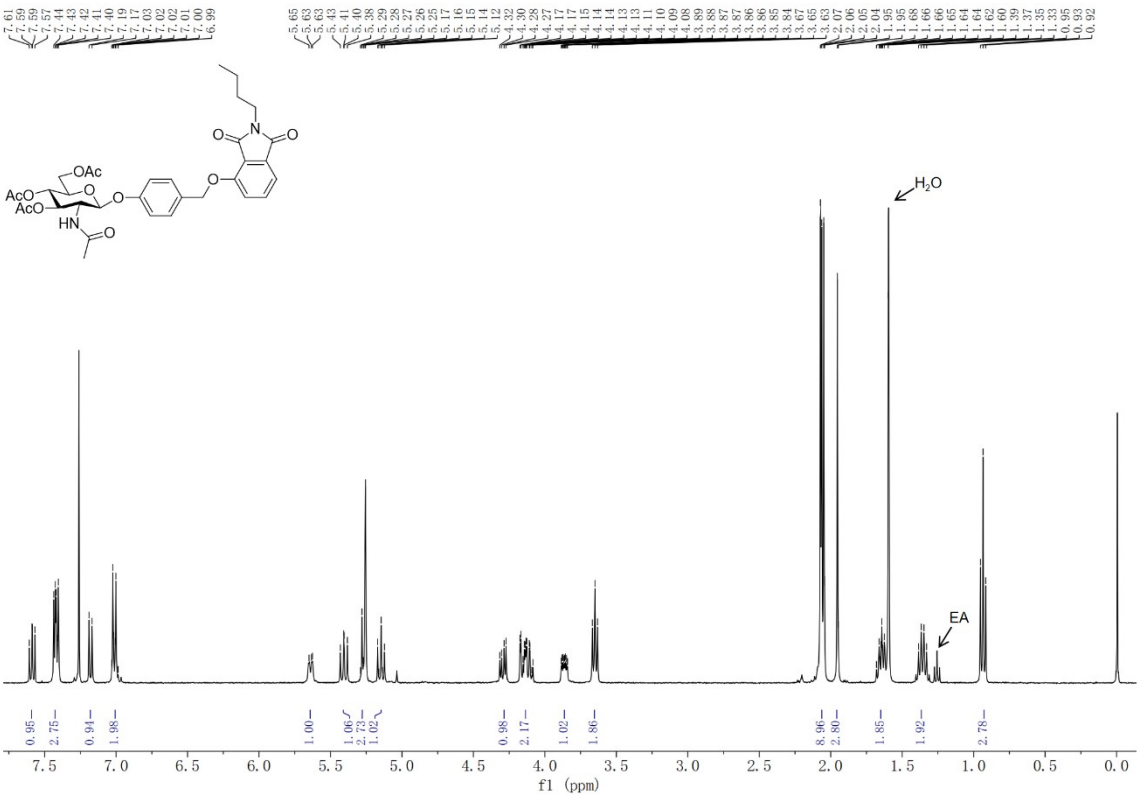


Figure S6. ^{1}H NMR of **2** in $CDCl_3$.

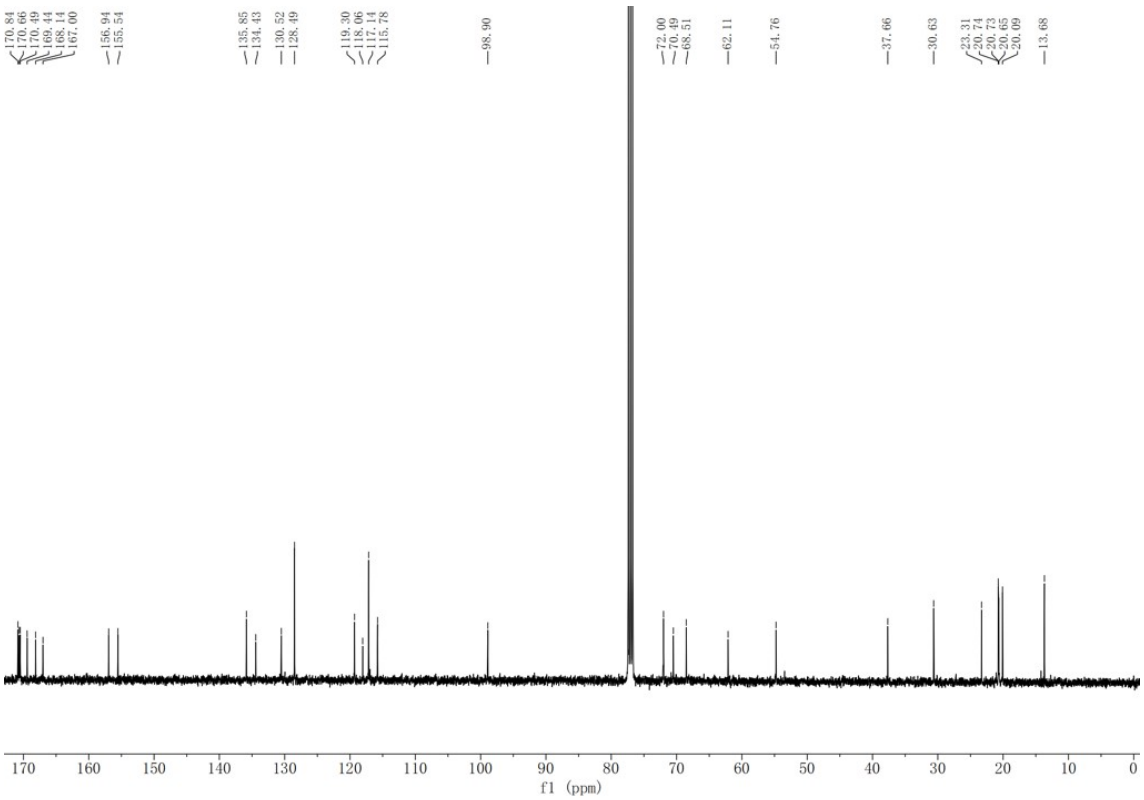


Figure S7. ^{13}C NMR of **2** in $CDCl_3$.

GlcNAc-Bn-BHID. To a stirred solution of Compound **2** (37 mg, 0.06 mol) in MeOH (10 mL) was added MeONa (9 mg, 0.17 mmol) at room temperature. The reaction mixture was stirred for 3 h at room temperature. The solvent was removed under the reduced pressure and The residue was purified by recrystallization using MeOH to afford GlcNPr-Bn-BHID as a white solid (21 mg, 66.7%): ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.83 – 7.70 (m, 2H), 7.52 (d, J = 8.5 Hz, 1H), 7.45 – 7.36 (m, 3H), 7.02 – 6.95 (m, 2H), 5.26 (s, 2H), 5.08 (dd, J = 16.4, 5.4 Hz, 2H), 4.97 (d, J = 8.5 Hz, 1H), 4.61 (t, J = 5.9 Hz, 1H), 3.75 – 3.60 (m, 2H), 3.54 – 3.43 (m, 3H), 3.42-3.36(m, 1H), 3.31-3.25 (m, 1H), 3.20-3.12 (m, 1H), 1.80 (s, 3H), 1.58 – 1.46 (m, 2H), 1.32 – 1.18 (m, 2H), 0.87 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.7, 168.0, 166.7, 157.8, 155.7, 136.9, 134.2, 130.2, 129.5, 120.4, 117.4, 116.8, 115.6, 99.6, 77.7, 74.6, 70.7, 70.2, 61.2, 55.9, 37.3, 30.4, 23.6, 19.9, 13.9. HRMS (ESI, m/z): $[M+Na]^+$: caled for $C_{27}H_{32}N_2O_9Na^+$ 551.2005, found 551.2006.

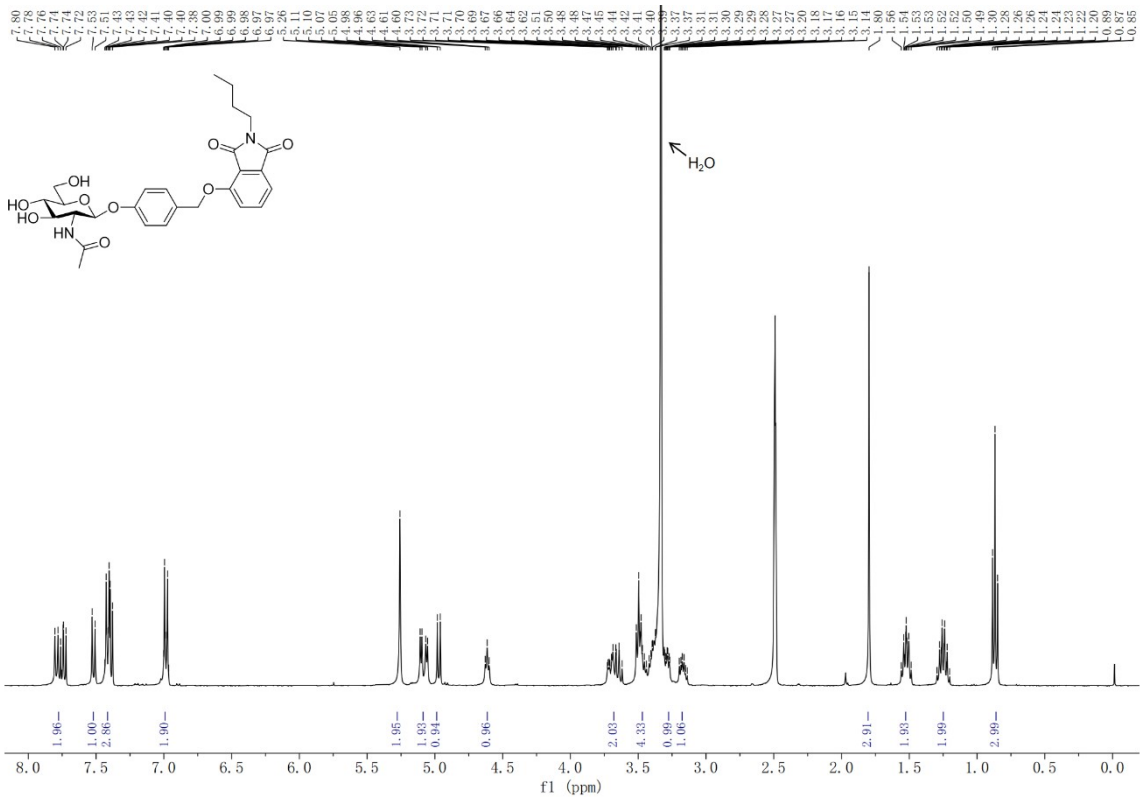


Figure S8. ^{1}H NMR of **GlcNAc-Bn-BHID** in DMSO-d_6.

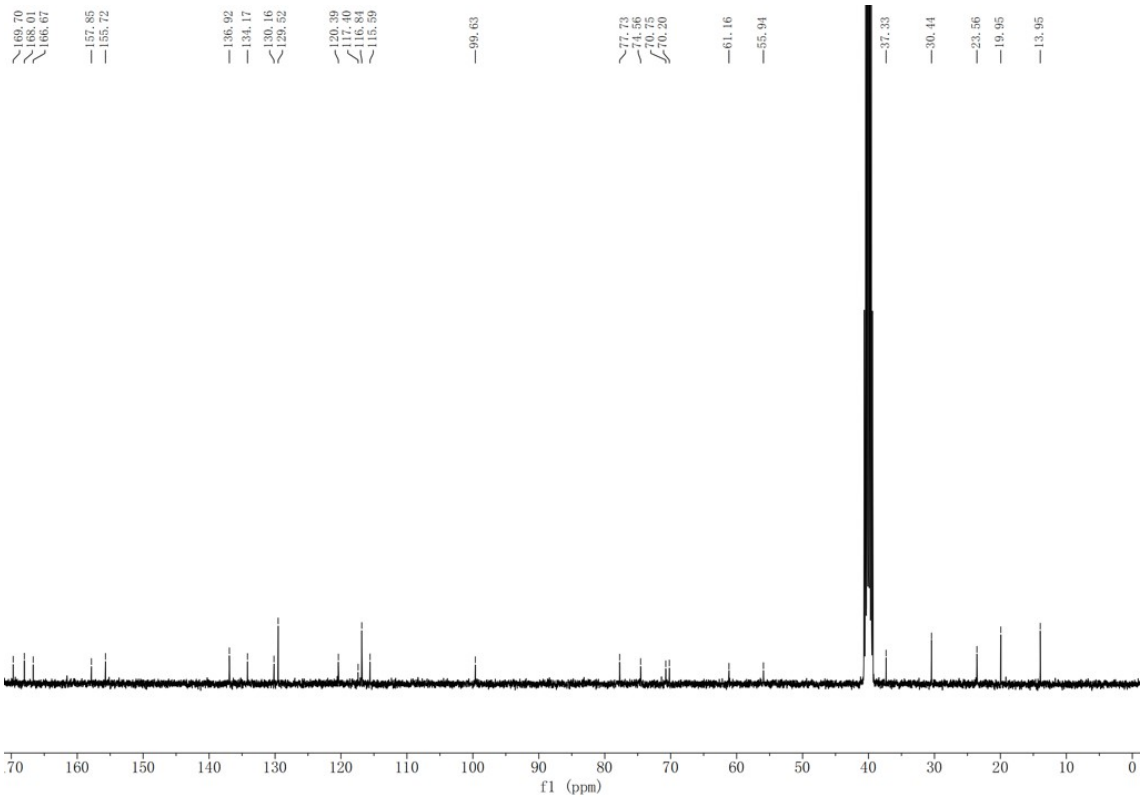
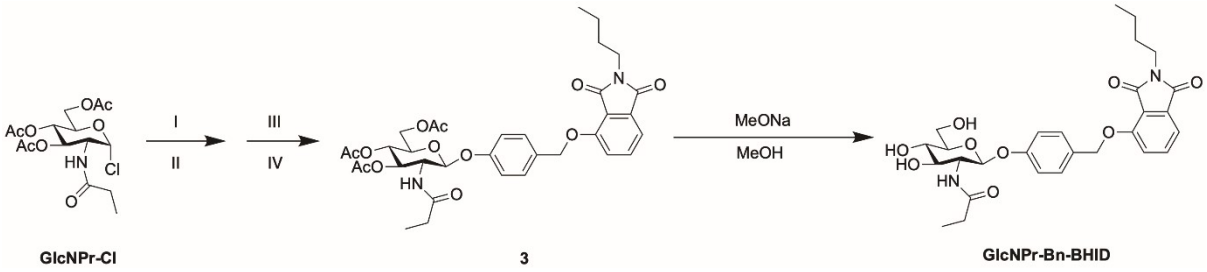


Figure S9. ^{13}C NMR of **GlcNAc-Bn-BHID** in DMSO-d_6 .

Synthesis of GcNPr-Bn-BHID.



Scheme S2. Reagents and conditions: (I) 4-hydroxybenzaldehyde, Ag_2O, KI in MeCN at r.t. (II) $NaBH_4$, $CHCl_3$/*i*-PrOH (3:1, v/v). (III) PBr_3, CH_2Cl_2. (IV) **BHID**, Cs_2CO_3, Na_2SO_4 in MeCN at r.t.

GlcNPr-Cl. GlcNPr-Cl was synthesized using a previously reported method.[1]

Compound **3.** Compound **3** was synthesized using the same method as Compound **2.** ^1H NMR (400 MHz, Chloroform-d) δ 7.58 (dd, J = 8.4, 7.3 Hz, 1H), 7.45 – 7.37 (m, 3H), 7.17 (d, J = 8.4 Hz, 1H), 7.03 – 6.98 (m, 2H), 5.78 – 5.72 (m, 1H), 5.46 – 5.38 (m, 1H), 5.31 – 5.27 (m, 2H), 5.24 (s, 2H), 5.18 – 5.10 (m, 1H), 4.33 – 4.24 (m, 1H), 4.21 – 4.06 (m, 2H), 3.89 –

3.85 (m, 1H), 3.64 (t, J = 7.2 Hz, 2H), 2.17 – 2.10 (m, 2H), 2.09 – 2.02 (m, 9H), 1.66 – 1.59 (m, 2H), 1.41 – 1.28 (m, 2H), 1.09 (t, J = 7.6 Hz, 3H), 0.93 (t, J = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$-*d*) δ 174.2, 170.8, 170.6, 169.4, 157.0, 155.5, 135.8, 134.4, 130.5, 129.9, 128.4, 119.3, 118.1, 117.2, 116.9, 115.8, 99.0, 72.0, 70.5, 68.5, 62.1, 54.7, 37.7, 30.6, 29.8, 20.7, 20.7, 20.6, 20.1, 13.7, 9.8. HRMS (ESI, m/z): $[M+Na]^+$: caled for $C_{34}H_{40}N_2O_{12}Na^+$ 691.2480, found 691.2479.

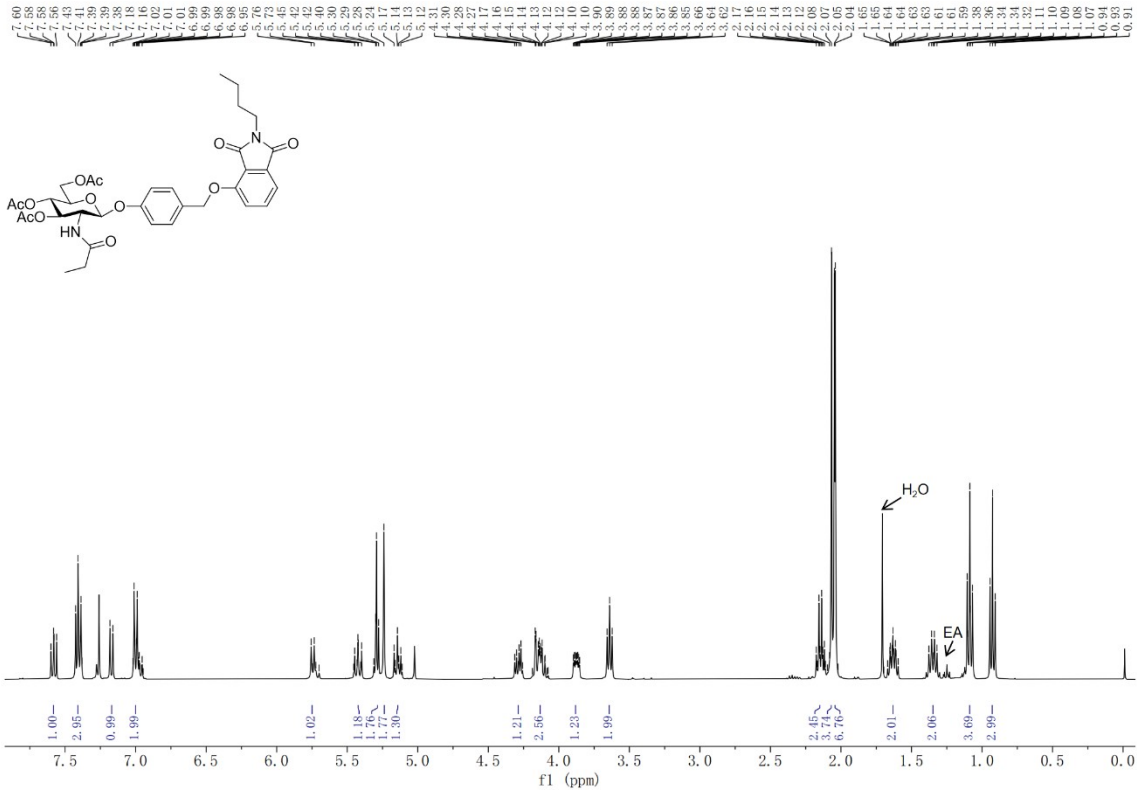


Figure S10. ^{1}H NMR of **3** in $CDCl_3$.

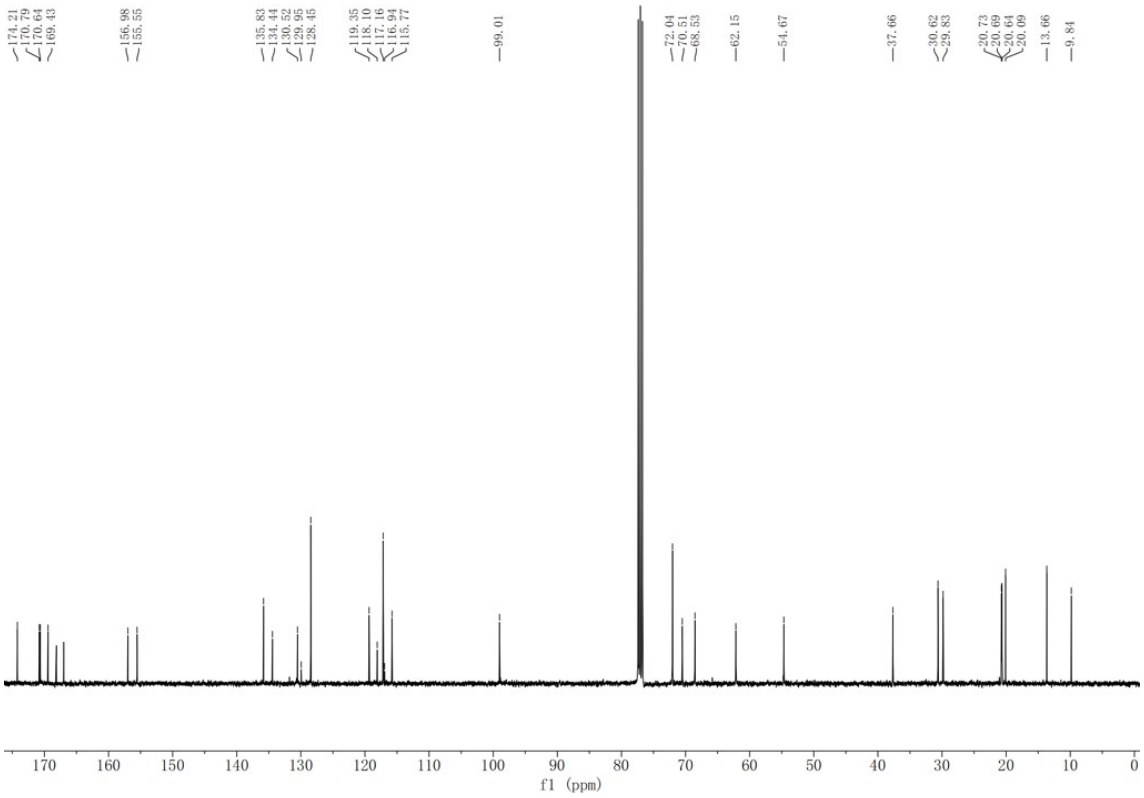


Figure S11. ^{13}C NMR of **3** in $CDCl_3$.

GlcNPr-Bn-BHID. To a stirred solution of Compound **3** (55 mg, 0.08 mmol) in MeOH (10 mL) was added MeONa (13mg, 0.24 mmol) at room temperature. The reaction mixture was stirred for 3 h at room temperature. The solvent was removed under the reduced pressure and The residue was purified by recrystallization using MeOH to afford **GlcNPr-Bn-BHID** as a white solid (11 mg, 25 %): ^1H NMR (400 MHz, DMSO-d_6) δ 7.78 – 7.66 (m, 2H), 7.52 (d, J = 8.5 Hz, 1H), 7.40 (dd, J = 9.7, 7.8 Hz, 2H), 6.97 (d, J = 8.7 Hz, 2H), 5.26 (s, 2H), 5.12 (d, J = 5.4 Hz, 1H), 5.06 (d, J = 5.5 Hz, 1H), 4.95 (d, J = 8.5 Hz, 1H), 4.63 (t, J = 5.8 Hz, 1H), 3.75 – 3.62 (m, 2H), 3.51-3.44 (m, 3H), 3.44 – 3.35 (m, 1H), 3.28 (t, J = 5.0 Hz, 1H), 3.20 – 3.12 (m, 1H), 2.07 – 2.02 (m, 2H), 1.55 – 1.48 (m, 2H), 1.29 – 1.20 (m, 2H), 0.97 (t, J = 7.6 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 173.5, 168.0, 166.7, 158.0, 155.7, 136.9, 134.2, 130.2, 129.5, 120.4, 117.4, 116.8, 115.6, 99.9, 77.8, 74.5, 70.8, 70.2, 61.2, 55.8, 37.3, 30.4, 29.4, 19.9, 13.9, 13.9, 10.5. HRMS (ESI, m/z): $[M+Na]^+$: caled for $C_{28}H_{34}N_2O_9Na^+$ 565.2161, found 565.2162.

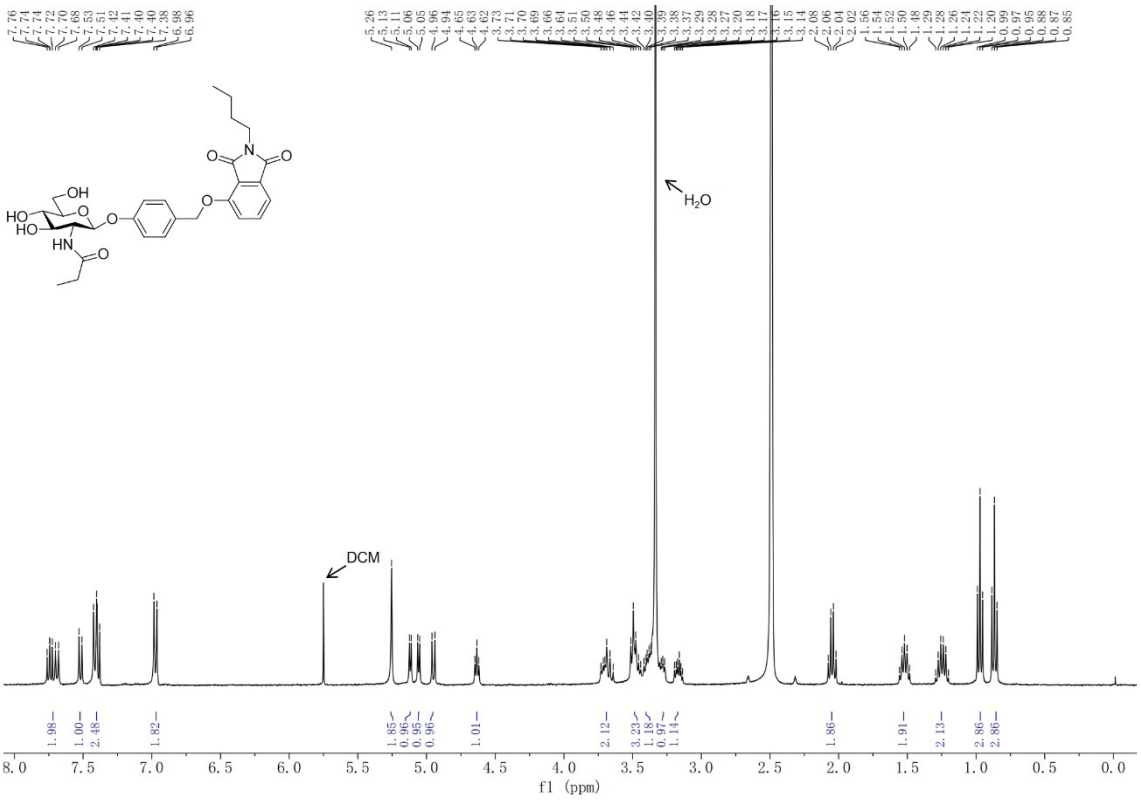


Figure S12. ^{1}H NMR of **GlcNPr-Bn-BHID** in DMSO-d_6.

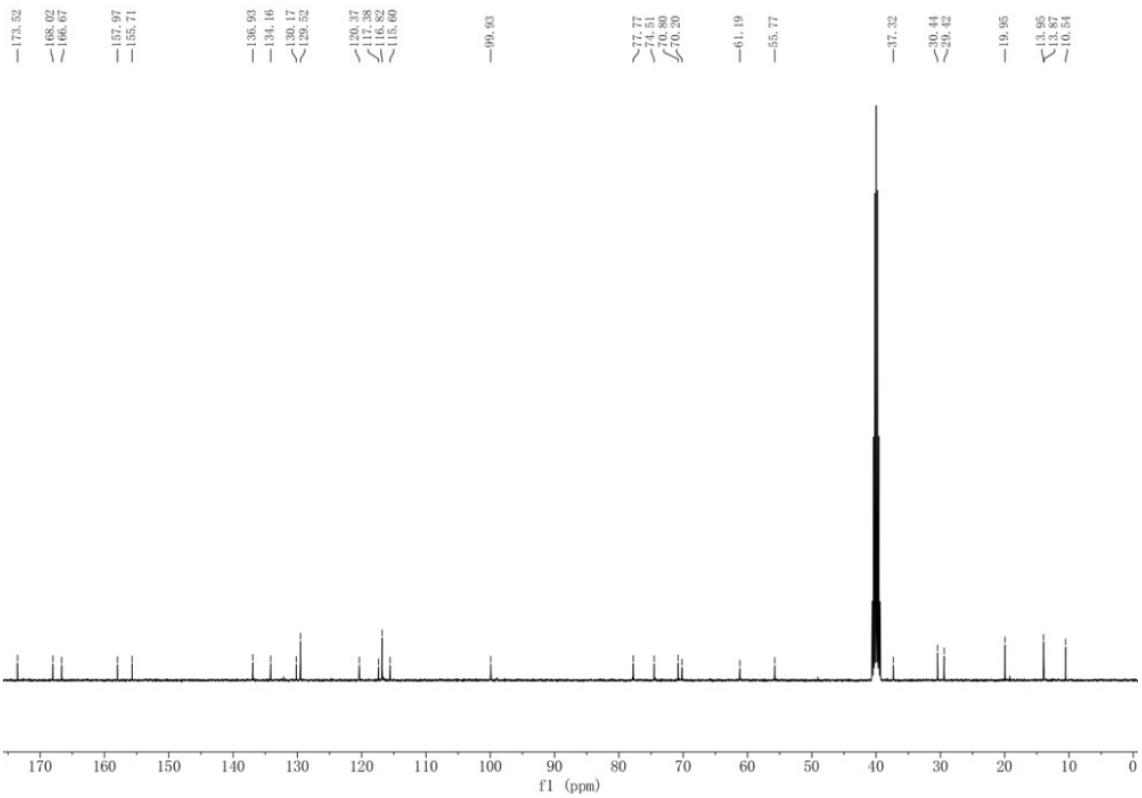


Figure S13. ^{13}C NMR of **GlcNPr-Bn-BHID** in DMSO-d_6.

S3. Additional references

[1] H. Jung, S. H. Park, W. H. Yang, J. W. Cho and I. Shin, *Sens. Actuat., B*, 2022, **367**, 132093.