Supporting Information

Site-Specific Glycoconjugation of Protein via Bioorthogonal Tetrazine Cycloaddition with a Genetically Encoded trans-Cyclooctene or Bicyclononyne

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1. General
All reagents and solvents for the organic synthesis were purchased from commercial sources and were used without further purification. Anhydrous solvents were obtained by passing them through commercially available alumina column (Innovative Technology, Inc., VA). Acidic resin DOWEX® 50Wx2 was generated by shaking the resin with 2M HCl and washing with deionized waster before use. Reverse phase purification was performed using Isolera Biotage with SNAP Cartridge KP-C18-HS of 60g or 12g. HPLC purification was performed with an Agilent 1260 infinity using ZORBAX 300SB-C18 column (9.4 x 250 mm). NMR spectra were measured using the following instruments Bruker 300 UltraShield (300 MHz), Bruker AVANCE 3 HD (400 MHz), Bruker 500 UltraShield (500 MHz) using deuterated solvents (CDCl$_3$, CD$_3$OD, or D$_2$O) referenced to undeuterated peak (CDCl$_3$ ($\delta$=7.26) CD$_3$OD ($\delta$=4.79)). LC-MS spectra were recorded using a DIONEX Ultimate 3000 UHPLC with a PINNACLE DB C18 column (1.9 µm, 50 x 2.1 mm) and with a Thermo LCQ Fleet Mass Spectrometer System operated in positive ion mode in electrospray ionization (ESI). MALDI-TOF Mass spectra were recorded using a Bruker Daltonics Autoflex spectrometer operated in positive ion mode. Fluorescence intensities were measured using a Molecular Devices Spectra Max M5. Electrophoresis was performed using Novex NuPAGE 4-12% Bis-Tris Gel. In gel fluorescence analysis were performed using a GE Healthcare Ettan DIGE Imager.

2. Synthesis of alkyne-substituted tetrazine 2

\[
\begin{align*}
\text{S1} & \xrightarrow{\text{formamidine acetate, Ni(OTf)$_2$, sulfur}} \text{S2} \\
\text{propargyl amine, HATU, lutidine} & \xrightarrow{\text{DMF, 82%}} \text{2} \\

\end{align*}
\]

2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetic acid (S2)

Tetrazine phenyl acetic acid (S2, CAS: 1380500-92-4) is a commercial product. Conversely, it can be prepared according to the following procedure: To the mixture of 2-(4 cyanophenyl)acetic acid S1 (687 mg, 4.27 mmol), nickel triflate (269 mg, 0.75 mmol), sulfur (136 mg, 4.27 mmol), formamidine acetate (4.44g, 42.7 mmol) was added anhydrous hydrazine (6.70 ml, 214 mmol, prepared by drying hydrazine monohydrate using flamed dried 3Å molecular sieves overnight) and stirred at 30°C for 24 h under nitrogen atmosphere. The reaction mixture was transferred to 500ml glass beaker and sodium nitrite solution (15.3 g, 222 mmol) in water (45 ml) was added to the reaction mixture followed by
1M HCl until gas evolution ceased and the pH reached 3. The mixture was extracted with CH₂Cl₂ and the organic phase was washed with brine, dried over sodium sulfate, filtrated and concentrated in vacuo. The residue was purified by silica gel column chromatography (CH₂Cl₂:MeOH=50:1) to obtain S₂ as a red solid (429 mg, 47%). NMR data for this compound matched literature report.[1]

H¹ NMR (300 MHz, CDCl₃): δ=10.22 (s, 1H), 8.62 (d, J = 8.4 Hz, 2H), 7.55 (d, J = 8.4 Hz, 2H), 3.80 (s, 2H).

2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-N-(prop-2-yn-1-yl)acetamide (2)

To a solution of S₂ (100 mg, 0.46 mmol), 2,6-lutidine (107 µl, 0.92 mmol) in dry DMF (3 ml) was added HATU (350 mg, 0.92 mmol) and the reaction mixture was stirred for 5 min at 0 ºC under nitrogen atmosphere. Propargyl amine (68 µl, 1.06 mmol) was then added and the reaction was stirred for 2 h at 0 ºC. Saturated sodium bicarbonate solution was added to the reaction mixture and the product was extracted with CH₂Cl₂. The organic phase was washed with brine, dried over sodium sulfate, filtrated and concentrated in vacuo. The residue was purified by silica gel column chromatography (CH₂Cl₂:MeOH=10:1) furnishing 2 as a red solid (95 mg, 82%).

H¹ NMR (400 MHz, D₂O): δ=10.23 (s, 1H), 8.63 (d, J = 8.4 Hz, 2H), 7.54 (d, J = 8.4 Hz, 2H), 4.07 (dd, J = 2.4 Hz, 5.2 Hz, 2H), 3.71 (s, 2H), 2.22 (t, J = 2.4 Hz, 1H); C¹³ NMR (100 MHz, D₂O): δ=169.4, 166.2, 157.9, 139.8, 130.8, 130.4, 128.9, 79.1, 71.9, 43.4, 29.5; LRMS (ESI): calcd. for C₁₃H₁₂N₃O [M+H]⁺, 254.11. found 254.13.

3. Preparation of azide-glycan 1

General procedures
Azido glycan 1 were prepared according to the procedure reported by Shoda et al.[2] Briefly, a mixture of reducing glycan 4, Et₃N, 2-chloro-1.3-dimethylimidazolium chloride (DMC), NaN₃, in 1,4-dioxane/ H₂O=1/1 was stirred for 1 h at – 10 ºC. The solvent was evaporated and the residue was purified directly by column chromatography. In some cases, it was found to be more practical to transiently acetylate the product for purification purposes. For convenience, the reactions were also performed in D₂O which facilitates reaction monitoring by NMR. In a
2.0ml Eppendorf tube, glycan 4, NaN₃, 2-chloro-1.3-dimethylimidazolium chloride (DMC), and Et₃N or 2,6-lutidine were added to D₂O. The mixture was shaken vigorously until the mixture became homogeneous. The mixture was then transferred to an NMR tube and the progress was monitored by H¹-NMR with intermittent shaking. Upon completion, the reaction was transferred back to the Eppendorf and lyophilized.

To the residue was added Ac₂O (300 μl) and pyridine (1 ml) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated in vacuo and the product was isolated by reverse phase chromatography (12g of C18 column, 50 ml of H₂O, then 100 ml of acetonitrile). The acetonitrile fraction was concentrated in vacuo then lyophilized. To the residue was added sodium methoxide (6 mg, 0.1 mmol) and dry methanol. The reaction mixture was stirred over night under nitrogen atmosphere. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered and lyophilized. After filtration, the mixture was concentrated in vacuo and the residue was purified by reverse phase column (12g of C18 column, flowed 200 ml of H₂O). Lyophilizing solvent gave desired azide-sugar.

The mixture was then tra 1 M NaOH (100 μl) and stirred 3 h.

**β-D-Glucopyranosyl azide (1a)**

\[
\begin{align*}
\text{D-Glucose monohydrate} & \quad (198 \text{ mg, } 1.0 \text{ mmol}), \\
\text{DMC} & \quad (508 \text{ mg, } 3.0 \text{ mmol}), \\
\text{NaN}_3 & \quad (584 \text{ mg, } 9.0 \text{ mmol}), \\
\text{triethylamine} & \quad (1.25 \text{ ml, } 9.0 \text{ mmol}) \text{ in H}_2\text{O (2 ml) and 1,4-dioxane (2 ml) were used.} \\
\text{Silica gel column chromatography (CH}_2\text{Cl}_2:\text{MeOH=5:1) gave β-D-glucopyranosyl azide (200 mg, 98%) as colorless solid. Spectral characteristics of this compound matched the reported data.}\ [\text{2}] \\
\text{H¹ NMR} (400 MHz, D₂O): δ=4.74 (d, J = 8.4 Hz, 1H), 3.92 (dd, J = 2.0 Hz, 12.4 Hz, 1H), 3.75 (dd, J = 5.6 Hz, 12.4 Hz, 1H), 3.49-3.56 (m, 2H), 3.42 (t, J = 10.0 Hz, 1H), 3.26 (t, J = 8.4 Hz, 1H)
\end{align*}
\]

**α-D-Mannopyranosyl azide (1b)**

\[
\begin{align*}
\text{D-Mannose} & \quad (45 \text{ mg, } 0.25 \text{ mmol}), \\
\text{DMC} & \quad (127 \text{ mg, } 0.75 \text{ mmol}), \\
\text{NaN}_3 & \quad (146 \text{ mg, } 2.25 \text{ mmol}), \\
\text{triethylamine} & \quad (313 \mu l, 2.25 \text{ mmol}) \text{ in H}_2\text{O (500 μl) and 1,4-dioxane (500 μl) was used. Silica gel column chromatography (CH}_2\text{Cl}_2:\text{MeOH=5:1) afforded α-D-mannopyranosyl azide (41}
\end{align*}
\]
mg, 80%) as colorless solid. Spectral characteristics of this compound matched the reported data.\[3\]

\[\text{H}^1 \text{NMR} \ (400 \text{ MHz, D}_2\text{O}): \delta=5.38 \text{ (d, } J=2.0 \text{ Hz, 1H), 3.79-3.85 \text{ (m, 2H), 3.64-3.73 \text{ (m, 3H), 3.57 \text{ (t, } J=9.6 \text{ Hz, 1H)}}}\]

**D-Galactopyranosyl azide (1c)**

\[
\text{D-Galactose (54 mg, 0.3 mmol), DMC (153 mg, 0.9 mmol), NaN}_3 \ (291 mg, 4.5 mmol), triethylamine (375 µl, 2.7 mmol) in deutrium oxide (1.2 ml) were shaken for 1 h at room temperature to afford a mixture of } \alpha \text{ and } \beta \text{ anomers. The solvent was evaporated in vacuo and to the residues were treated with } \text{Ac}_2\text{O (900 µl) and pyridine (3 ml). After stirring overnight at room temperature, the solvent was removed in vacuo and the residues were purified by silica gel column chromatography (Pentane:EtOAc=10:1) to separate } \alpha \text{ and } \beta \text{ anomers. Each anomer was dissolved in dry methanol (1 mL) and treated with sodium methoxide (6 mg, 0.1 mmol). The reaction mixture was stirred overnight under nitrogen atmosphere. The mixture was neutralized with acidic resin (DOWEX® 50WX2), diluted with water (5 mL) and lyophilized to obtain } \alpha-\text{D-Galactopyranosyl azide (9 mg, 15%)) as colorless solid and } \beta-\text{D-Galactopyranosyl azide (33 mg, 53%)) as colorless solid. Spectral characteristics of this compound matched the reported data.}\[3\]

\[\begin{align*}
\text{α anomer: } & \text{H}^1 \text{NMR (400 MHz, D}_2\text{O): } \delta=5.56 \text{ (d, } J=4.4 \text{ Hz, 1H), 4.07 \text{ (m, 1H), 3.98 \text{ (d, } J=2.8 \text{ Hz, 1H), 3.93 \text{ (dd, } J=4.4 \text{ Hz, 10.4 Hz, 1H), 3.73-3.80 \text{ (m, 3H), C}^{13} \text{NMR (100 MHz, D}_2\text{O): } \delta=89.4, 73.0, 69.2, 69.0, 67.6, 61.2}}
\end{align*}\]

\[\begin{align*}
\text{β anomer: } & \text{H}^1 \text{NMR (300 MHz, D}_2\text{O): } \delta=4.65 \text{ (d, } J=8.7 \text{ Hz, 1H), 3.94 \text{ (d, } J=3.3 \text{ Hz, 1H), 3.72-3.80 \text{ (m, 3H), 3.66 \text{ (dd, } J=3.3 \text{ Hz, 9.6 Hz, 1H), 3.49 \text{ (dd, } J=8.7 \text{ Hz, 9.6 Hz, 1H)}}.}
\end{align*}\]

**L-Fucopyranosyl azide (1d)**

\[
\text{L-Fucose (48 mg, 0.3 mmol), DMC (153 mg, 0.9 mmol), NaN}_3 \ (291 mg, 4.5 mmol), triethylamine (375 µl, 2.7 mmol) in deutrium oxide (1.2 ml) were shaken for 1 h at room temperature. The solvent was evaporated in vacuo and to the residues were treated with } \text{Ac}_2\text{O (900 µl) and pyridine (3 ml). After stirring overnight at room temperature, the solvent was removed in vacuo and the residues were purified by silica gel column chromatography (Pentane:EtOAc=10:1) to separate } \alpha \text{ and } \beta \text{ anomers. Each anomer was dissolved in dry}
\]
methanol (1 mL) and treated with sodium methoxide (6 mg, 0.1 mmol). The reaction mixture was stirred overnight under nitrogen atmosphere. The mixture was neutralized with acidic resin (DOWEX® 50WX2), diluted with water (5 mL) and lyophilized to obtain α-L-Fucopyranosyl azide (7 mg, 12%) as colorless solid and β-L-Fucopyranosyl azide (45 mg, 80%) as colorless solid.

α anomer: H¹ NMR (400 MHz, D₂O): δ=5.50 (d, J = 4.4 Hz, 1H), 4.19 (q, J = 6.4 Hz, 1H), 3.88 (dd, J = 4.4 Hz, 10.0 Hz, 1H), 3.81 (d, J = 3.2 Hz, 1H), 3.75 (dd, J = 3.2 Hz, 10.0 Hz, 1H), 1.24 (d, J = 6.4 Hz, 1H); C¹³ NMR (100 MHz, D₂O): δ=89.6, 71.5, 69.3, 68.9, 67.3, 15.4.

β anomer: H¹ NMR (400 MHz, D₂O): δ=4.63 (d, J = 8.8 Hz, 1H), 3.88 (q, J = 6.8 Hz, 1H), 3.78 (d, J = 3.6 Hz, 1H), 3.67 (dd, J = 3.6 Hz, 10.0 Hz, 1H), 3.47 (dd, J = 8.8 Hz, 10.0 Hz, 1H), 1.27 (d, J = 6.8 Hz, 1H); C¹³ NMR (100 MHz, D₂O): δ=90.5, 73.1, 72.8, 71.1, 70.0, 15.3

2-Acetamido-2-deoxy-β-D-glucopyranosyl azide (1e)

N-acetyl-D-glucosamine (22 mg, 0.1 mmol), DMC (51 mg, 0.3 mmol), NaN₃ (97 mg, 1.5 mmol), 2,6-lutidine (70 µl, 0.6 mmol) in deuterium oxide (500 µl) were shaken for 36 h. The reaction was lyophilized and purified by silica gel column chromatography (CH₂Cl₂:MeOH=5:1) to afford 2-acetamido-2-deoxy-β-D-glucopyranosyl azide 1e (24 mg, 97%), as colorless solid. Spectral characteristics of this compound matched the reported data.[⁴]

H¹ NMR (400 MHz, D₂O): δ=4.76 (d, J = 9.2 Hz, 1H), 3.93 (d, J = 12.4 Hz, 1H), 3.69-3.79 (m, 2H), 3.48-3.60 (m, 3H), 2.06 (s, 3H).

β-D-Glucopyranuronic azide (1f)

D-glucuronic acid (19 mg, 0.1 mmol), DMC (85 mg, 0.5 mmol), NaN₃ (58 mg, 0.9 mmol), triethylamine (125 µl, 0.9 mmol) in deuterium oxide (500 µl) were shaken for 1 h, lyophilized and purified by reversed phase chromatography to afford β-D-glucopyranuronic azide 1f (11 mg, 50%) as colorless solid.

H¹ NMR (400 MHz, D₂O): δ=4.82 (d, J = 8.8 Hz, 1H), 3.99 (d, J = 10.0 Hz, 1H), 3.54-3.60 (m, 2H), 3.32 (dd, J = 8.8 Hz, 9.2 Hz, 1H); C¹³ NMR (100 MHz, D₂O): δ=173.3, 90.0, 76.7, 75.4, 72.4, 71.1.
**β-D-Maltosyl azide (1g)**

\[
\begin{align*}
\text{D-Maltose monohydrate (36 mg, 0.1 mmol), DMC (85 mg, 0.5 mmol), NaN}_3 (97 mg, 1.5 \\
\text{mmol), triethylamine (208 µl, 1.5 mmol) in deutrium oxide (500 µl) were shaken for 1 h and lyophilized. To the residue was added Ac}_2\text{O (300 µl) and pyridine (1 ml) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated in vacuo and the product was isolated by reverse phase chromatography (12g of C18 column, 50 ml of H}_2\text{O, then 100 ml of acetonitrile). The acetonitrile fraction was concentrated in vacuo then lyophilized. To the residues was added sodium methoxide (6 mg, 0.1 mmol) and dry methanol. The reaction mixture was stirred overnight under nitrogen atmosphere. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered and lyophilized to afford β-D-maltosyl azide 1g (27 mg, 74%) as colorless solid. Spectral characteristics of this compound matched the reported data.}^{[2]} \\
\text{H}^1\text{NMR (400 MHz, D}_2\text{O): δ=5.41 (d, J = 4.0 Hz, 1H), 4.75 (d, J = 8.8 Hz, 1H), 3.94 (d, J = 12.4 Hz, 1H), 3.66-3.87 (m, 8H), 3.57 (dd, J = 4.0 Hz, 10.0 Hz, 1H), 3.41 (t, J = 9.2 Hz, 1H), 3.30 (t, J = 9.2 Hz, 1H)}
\end{align*}
\]

**β-D-Cellobiosyl azide (1h)**

\[
\begin{align*}
\text{D-Cellobiose (34 mg, 0.1 mmol), DMC (85 mg, 0.5 mmol), NaN}_3 (97 mg, 1.5 mmol), triethylamine (208 µl, 1.5 mmol) in deutrium oxide (500 µl) were shaken for 1 h and lyophilized. To the residue was added Ac}_2\text{O (300 µl) and pyridine (1 ml) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated in vacuo and the product was isolated by reverse phase chromatography (12g of C18 column, 50 ml of H}_2\text{O, then 100 ml of acetonitrile). The acetonitrile fraction was concentrated in vacuo then lyophilized. To the residues was added sodium methoxide (6 mg, 0.1 mmol) and dry methanol. The reaction mixture was stirred overnight under nitrogen atmosphere. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered and lyophilized to afford β-D-cellobiosyl azide 1h (30 mg, 82%) as colorless solid. Spectral characteristics of this compound matched the reported data.}^{[2]} \\
\text{H}^1\text{NMR (400 MHz, D}_2\text{O): δ=4.77 (d, J = 8.8 Hz, 1H), 4.51 (d, J = 8.0 Hz, 1H), 3.65-4.00 (m, 3H), 3.65-3.76 (m, 4H), 3.46-3.53 (m, 2H), 3.41 (t, J = 9.2 Hz, 1H), 3.29-3.35 (m, 2H).}
\end{align*}
\]
**β-D-Lactosyl azide (1e)**

D-Lactose monohydrade (36 mg, 0.1 mmol), DMC (85 mg, 0.5 mmol), NaN₃ (97 mg, 1.5 mmol), triethylamine (208 µl, 1.5 mmol) in deutrium oxide (500 µl) were shaken for 1 h and lyophilized. To the residue was added Ac₂O (300 µl) and pyridine (1 ml) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated *in vacuo* and the product was isolated by reverse phase chromatography (12g of C18 column, 50 ml of H₂O, then 100 ml of acetonitrile). The acetonitrile fraction was concentrated *in vacuo* then lyophilized. To the residues was added sodium methoxide (6 mg, 0.1 mmol) and dry methanol. The reaction mixture was stirred overnight under nitrogen atmosphere. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered and lyophilized to afford β-D-Lactosyl azide (36 mg, 99%) as colorless solid. Spectral characteristics of this compound matched the reported data.²

H¹ NMR (400 MHz, D₂O): δ=4.77 (d, J = 8.8 Hz, 1H), 4.44 (d, J = 10.4 Hz, 1H), 3.91-4.00 (m, 2H), 3.65-3.84 (m, 8H), 3.54 (m, 1H), 3.31 (m, 1H).

**Fuca₁-2Galβ₁-3[Fuca₁-4]GlcNAcβ₁-3Galβ₁-4Glc-1N₃ (1j)**

Lacto-N-difucohexaose (Fuca₁-2Galβ₁-3[Fuca₁-4]GlcNAcβ₁-3Galβ₁-4Glc, 5.0 mg, 0.005 mmol), DMC (8.5 mg, 0.05 mmol), NaN₃ (8.0 mg, 0.125 mmol), triethylamine (10.4 µl, 0.075 mmol) in deutrium oxide (100 µl) were shaken for 8 h and lyophilized. To the residue was added Ac₂O (50 µl) and pyridine (150 µl) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated *in vacuo* and the product was isolated by reverse phase chromatography (1g of C18 column, 5 ml of H₂O, then 10 ml of acetonitrile). The acetonitrile fraction was concentrated *in vacuo* then lyophilized. To the residues was added sodium methoxide (2 mg) and dry methanol (100 µl). The reaction mixture was stirred overnight under nitrogen atmosphere. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered, washed with water and lyophilized to afford 1j (4.0 mg, 78%) as colorless solid.
HRMS (MALDI-TOF): calcd. for C_{41}H_{70}N_{4}O_{25}Na [M+Na]^+, 1047.3605. found 1047.3641.

β-3'-Sialyllactosyl azide (entry 1k)

3'-Sialyllactose (Neu5Acβ2-3Galβ1-4Glc, 10.0 mg, 0.015 mmol), DMC (25.5 mg, 0.15 mmol), NaN₃ (24 mg, 0.375 mmol), triethylamine (31.2 µl, 0.225 mmol) in deuterium oxide (250 µl) were shaken for 5 h and lyophilized. To the residue was added Ac₂O (150 µl) and pyridine (450 µl) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated in vacuo and the product was isolated by reverse phase chromatography (2g of C18 column, 10 ml of H₂O, then 20 ml of acetonitrile). The acetonitrile fraction was concentrated in vacuo then lyophilized. To the residues was added LiOH (2 mg) and methanol:water (1:1, 100 µl) and stirred overnight. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered, washed with water and lyophilized to afford β-3'-Sialyllactosyl azide 1k (8.0 mg, 80%) as colorless solid.

HRMS (MALDI-TOF): calcd. for C_{31}H_{51}N_{5}O_{22}Na[M+Na]^+, 681.2079. found 681.2071.

β-6'-Sialyllactosyl azide (entry 1l)

6'-Sialyllactose (Neu5Acβ2-6Galβ1-4Glc, 10.0 mg, 0.015 mmol), DMC (25.5 mg, 0.15 mmol), NaN₃ (24 mg, 0.375 mmol), triethylamine (31.2 µl, 0.225 mmol) in deuterium oxide (250 µl) were shaken for 5 h and lyophilized. To the residue was added Ac₂O (150 µl) and pyridine (450 µl) and the reaction mixture was stirred overnight at room temperature. The reaction was concentrated in vacuo and the product was isolated by reverse phase chromatography (2g of C18 column, 10 ml of H₂O, then 20 ml of acetonitrile). The acetonitrile fraction was concentrated in vacuo then lyophilized. To the residues was added LiOH (2 mg) and methanol:water (1:1, 100µl) and stirred overnight. The reaction mixture was neutralized with acidic resin (DOWEX® 50WX2), filtered, washed with water and lyophilized to afford β-6'-Sialyllactosyl azide 1l (9.1 mg, 91%) as colorless solid.

HRMS (MALDI-TOF): calcd. for C_{31}H_{51}N_{5}O_{22}Na[M+Na]^+, 681.2079. found 681.2071.
4. Synthesis of glycan-tetrazine probe 3

General procedures

Procedure 1 (entry 1-9, Table 1)
Azido glycan 1 (1.0 eq.) and CuSO₄ (0.2 eq.) were dissolved in water (40 mM). To this solution at 0 °C was added a solution of sodium ascorbate (2.0 eq.), TBTA (Tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine, 0.2 eq.) in DMF:t-BuOH (20mM) followed by the alkyne 2 (1.0 eq.) was added to the reaction mixture and stirred at 0°C for 5 h. The reaction mixture was directly loaded on a column for reverse phase purification (column: SNAP Cartridge KP-C18-HS 60g, flow rate: 35ml/min with collection based on UV detection) to afford the triazole product 3.

Procedure 2 (used for smaller scale reaction, entry 10-12, Table 1)
Azido glycan 1 (1.0 eq.) and CuSO₄ (2 mg, 12 mM) were dissolved in water (200 µl). To the mixture were added sodium ascorbate (14 mg, 120 mM), TBTA (Tris[(1-benzyl-1,2,3-triazol-4-yl)methyl]amine, 4 mg, 12 mM), DMF (200 µl), t-BuOH (200 µl) and the mixture was kept at 0 °C. Alkyne 3 (9 mg, 60 mM) was added to the reaction mixture and stirred at 0°C for 5 h and the reaction was lyophilized. The residues were dissolved in water (200 µl) and the precipitates were removed by centrifugation. Purification by HPLC (column: ZORBAX 300SB-C18 column (9.4 x 250 mm), flow rate : 3 ml/min) afforded the triazole product.

Tetrazine-glucose conjugate 3a

β-D-Glucopyranosyl azide 1a (10 mg, 0.1 mmol) was used following procedure 1. Reverse phase purification (eluent: acetonitrile/water=1/4) afforded conjugate 3a (18 mg, 80%) as red solid.

H¹ NMR (400 MHz, MeOD): δ=10.32 (s, 1H), 8.53 (d, J = 8.4 Hz, 2H), 8.04 (s, 1H), 7.57 (d, J = 8.4 Hz, 2H), 5.59 (d, J = 8.8 Hz, 1H), 4.50 (s, 2H), 3.3-3.9 (m, 8H); C¹³ NMR (100 MHz, MeOD): δ=173.2, 167.6, 159.2, 142.2, 133.4, 132.1, 131.4, 131.3, 129.3, 89.5, 81.1, 78.4,
74.0, 70.9, 62.4, 43.5, 35.9. HRMS (MALDI-TOF): calcd. for C_{19}H_{22}N_{8}O_{6}Na [M+Na]^+, 481.1560. found 481.1553.

**Tetrazine-mannose conjugate 3b**

α-D-Mannopyranosyl azide 1b (20 mg, 0.1 mmol) was used following procedure 1. Reverse phase purification (eluent: acetonitrile/water=1/4) afforded conjugate 3b (35 mg, 76%) as red solid.

H\textsuperscript{1}NMR (400 MHz, D\textsubscript{2}O): δ=10.20 (s, 1H), 8.24 (d, J = 7.6 Hz, 2H), 7.83 (s, 1H), 7.39 (d, J = 7.6 Hz, 2H), 5.89 (d, J = 2.4 Hz, 1H), 4.37 (s, 2H), 3.93 (dd, J = 3.6 Hz, 8.8 Hz, 1H), 3.58-3.65 (m, 6H), 3.05-3.09 (m, 1H); C\textsubscript{13}NMR (100 MHz, D\textsubscript{2}O): δ=173.8, 166.3, 157.3, 140.3, 130.5, 130.2, 129.7, 128.6, 126.0, 70.5, 68.2, 66.4, 60.4, 42.2, 34.6; HRMS (MALDI-TOF): calcd. for C_{19}H_{22}N_{8}O_{6}Na [M+Na]^+, 481.1560. found 481.1553.

**Tetrazine-galactose conjugate 3c**

β-D-Galactopyranosyl azide 1c (10 mg, 0.05 mmol) was used following procedure 1. Reverse phase purification (eluent: acetonitrile/water=1/4) afforded conjugate 3c (11 mg, 50%) as red solid.

H\textsuperscript{1}NMR (500 MHz, D\textsubscript{2}O): δ=10.31 (s, 1H), 8.35 (broad s, 2H), 8.08 (s, 1H), 7.42 (broad s, 2H), 5.61 (d, J = 9.0 Hz, 1H), 4.49 (s, 2H), 4.13 (dd, J = 9.0 Hz, 9.0 Hz, 1H), 4.04 (d, J = 3 Hz, 1H), 3.94 (dd, J = 5.5 Hz, 6.0 Hz, 1H), 3.82 (dd, J = 3.0 Hz, 9.0 Hz, 1H), 3.72-3.73 (m, 4H); C\textsuperscript{13}NMR (125 MHz, D\textsubscript{2}O): δ=173.9, 166.3, 157.3, 140.3, 132.7, 130.2, 129.8, 129.6, 128.5, 88.0, 78.2, 72.9, 69.9, 68.5, 60.8, 42.1, 34.7; HRMS (MALDI-TOF): calcd. for C_{19}H_{22}N_{8}O_{6}Na [M+Na]^+, 481.1560. found 481.1578.

**Tetrazine-fucose conjugate 3d**

β-L-Fucopyranosyl azide 1d (9 mg, 0.05 mmol) was used following procedure 1. Reverse phase purification (eluent: acetonitrile/water=1/4) afforded conjugate 3d (15 mg, 72%) as red solid.
**Tetrazine-**N-**actyl glucosamine conjugate 3e**

![Structure of conjugate 3e]

2-Acetamido-2-deoxy-β-D-glucopyranosyl azide 1e (13 mg, 0.05 mmol) was used following procedure 1. Reverse phase purification (eluent: acetonitrile/water=1/4) afforded conjugate 3e (18 mg, 70%) as red solid.

H\textsuperscript{1} NMR (500 MHz, D\textsubscript{2}O): δ=10.36 (s, 1H), 8.45 (d, J = 8.5 Hz, 2H), 7.96 (s, 1H), 7.58 (d, J = 8.5 Hz, 2H), 5.78 (d, J = 9.5 Hz, 1H), 4.50 (s, 2H), 4.17 (dd, J = 10.0 Hz, 10.0 Hz, 1H), 3.90 (dd, J = 2.0 Hz, 12.5 Hz, 1H), 3.70-3.81 (m, 5H), 3.64 (dd, J = 9.0 Hz, 9.5 Hz, 1H), 1.76 (s, 3H); C\textsuperscript{13} NMR (100 MHz, D\textsubscript{2}O): δ=174.4, 173.8, 166.4, 157.3, 140.4, 130.3, 130.2, 128.6, 86.3, 78.8, 73.4, 69.2, 60.3, 55.3, 42.3, 34.5, 21.5; HRMS (MALDI-TOF): calcd. for C\textsubscript{21}H\textsubscript{25}N\textsubscript{9}O\textsubscript{7}Na [M+Na]\textsuperscript{+}, 522.1863. found 522.1863.

**Tetrazine-glucoronic acid conjugate 3f.**

![Structure of conjugate 3f]

β-D-Glucopyranuronic azide 1f (11 mg, 0.05 mmol) was used following procedure 1. Reverse phase purification (eluent: acetonitrile/water=1/4) conjugate 3f (14 mg, 57%) as red solid.

H\textsuperscript{1} NMR (400 MHz, D\textsubscript{2}O): δ=10.35 (s, 1H), 8.42 (broad s, 2H), 7.99 (s, 1H), 7.56 (broad s, 2H), 5.74 (d, J = 9.2 Hz, 1H), 4.49 (s, 2H), 4.18 (m, 1H), 3.95 (dd, J = 9.2 Hz, 9.2 Hz, 1H), 3.77 (s, 2H), 3.70 (m, 2H); C\textsuperscript{13} NMR (100 MHz, D\textsubscript{2}O): δ=173.9, 166.4, 157.3, 140.3, 132.7, 130.3, 130.2, 129.9, 128.6, 87.0, 75.5, 75.4, 71.8, 70.9, 42.3, 34.7; HRMS (MALDI-TOF): calcd. for C\textsubscript{19}H\textsubscript{20}N\textsubscript{8}O\textsubscript{7}Na [M+Na]\textsuperscript{+}, 495.1353. found 495.1394.

**Tetrazine-maltose conjugate 3g**

![Structure of conjugate 3g]
**β-D-Maltosyl azide 1g** (13 mg, 0.04 mmol) was used following procedure 1. Reverse phase purification (eluente: acetonitrile/water=1/4) afforded conjugate 3g (17 mg, 76%) as red solid. 

H<sup>1</sup>NMR (500 MHz, D<sub>2</sub>O): δ=10.37 (s, 1H), 8.46 (d, J = 8.5, 2H), 7.98 (s, 1H), 7.59 (d, J = 8.5, 2H), 5.71 (d, J = 9 Hz, 1H), 5.44 (d, J = 3.5 Hz, 1 H), 4.53 (s, 2H), 3.68-3.95 (m, 12H), 3.60 (dd, J = 3.5 Hz, 10.0 Hz, 1H), 3.43 (dd, J = 9.5 Hz, 9.5 Hz, 1H), 5.74 (d, J = 9.2 Hz, 1H), 4.49 (s, 2H), 4.18 (m, 1H), 3.95 (dd, J = 9.2 Hz, 9.2 Hz, 1H), 3.77 (s, 2H), 3.70 (m, 2H); C<sup>13</sup>NMR (125 MHz, D<sub>2</sub>O): δ=174.0, 166.4, 157.3, 140.4, 130.3, 1302, 128.6, 99.6, 87.2, 77.4, 76.2, 75.8, 72.8, 72.7, 72.1, 71.6, 69.3, 60.4, 60.3, 42.2, 34.7; HRMS (MALDI-TOF): calcd. for C<sub>25</sub>H<sub>32</sub>N<sub>8</sub>O<sub>11</sub>Na [M+Na]<sup>+</sup>, 643.2089. found 643.2122.

**Tetrazine-celllobiose conjugate 3h**

**β-D-Cellobiosyl azide 1h** (20 mg, 0.05 mmol) was used following procedure 1. Reverse phase purification (eluente: acetonitrile/water=1/4) afforded conjugate 3h (24 mg, 72%) as red solid.

H<sup>1</sup>NMR (500 MHz, D<sub>2</sub>O): δ=10.36 (s, 1H), 8.42 (d, J = 8.0, 2H), 8.03 (s, 1H), 7.56 (d, J = 8.0, 2H), 5.73 (d, J = 9.5 Hz, 1H), 4.44 (d, J = 7.5 Hz, 1 H), 4.53 (s, 2H), 3.72-3.99 (m, 12H), 3.53 (dd, J = 9.0 Hz, 9.0 Hz, 1H), 3.43 (dd, J = 9.0 Hz, 10.0 Hz, 1H); C<sup>13</sup>NMR (125 MHz, D<sub>2</sub>O): δ=173.9, 166.3, 157.3, 140.3, 130.3, 1302, 128.6, 102.5, 87.2, 77.6, 77.6, 76.0, 75.4, 74.4, 73.1, 72.0, 69.4, 60.5, 59.6, 42.2, 34.6; HRMS (MALDI-TOF): calcd. for C<sub>25</sub>H<sub>32</sub>N<sub>8</sub>O<sub>11</sub>Na [M+Na]<sup>+</sup>, 643.2089. found 643.2072.

**Tetrazine-lactose conjugate 3i**

**β-D-Lactosyl azide** (22 mg, 0.06 mmol) was used following procedure 1. Reverse phase purification (eluente: acetonitrile/water=1/4) afforded conjugate 3i (24 mg, 64%) as red solid.

H<sup>1</sup>NMR (400 MHz, D<sub>2</sub>O): δ=10.30 (s, 1H), 8.34 (d, J = 7.2 Hz, 2H), 8.05 (s, 1H), 7.49 (d, J = 7.2 Hz, 2H), 5.70 (d, J = 6.4 Hz, 1H), 4.45-4.48 (m, 3H), 3.51-3.97 (m, 14H); C<sup>13</sup>NMR (100 MHz, D<sub>2</sub>O): δ=173.8, 166.2, 157.3, 140.3, 132.7, 130.2, 129.9, 129.7, 128.5, 102.9, 87.2,
77.6, 77.3, 75.4, 74.5, 72.5, 71.9, 71.0, 68.5, 61.0, 59.7, 42.2, 34.6; HRMS (MALDI-TOF): calcd. for C_{25}H_{32}N_{8}O_{11}Na [M+Na]^+, 643.2089. found 643.2037.

**Tetrazine Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc conjugate 3j**

β-lacto-N-difucohexaosy azide 1j (4.0 mg, 0.0039 mmol) was used following procedure 2. HPLC purification (eluent: acetonitrile/water=1/4) afforded conjugate 3j (1.0 mg, 20%) as red solid.

HRMS (MALDI-TOF): calcd. for C_{51}H_{76}N_{9}O_{29} [M+H]^+, 1278.4750. found 1278.4632.

**Tetrazine-β-3′-sialyllactose conjugate 3k**

β-3′-sialyllactosyl azide 1k (3.4 mg, 0.0052 mmol) was used as starting sugar-azide in **General procedure2**. HPLC purification (eluent: acetonitrile/water=1/4) gave 2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)-N-(β-3′-sialyllactosyl-1,2,3-triazol-4-yl)methyl)acetamide (1.4 mg, 30%) as red solid.

HRMS (MALDI-TOF): calcd. for C_{36}H_{49}N_{9}O_{19}Na [M+Na]^+, 934.3043. found 934.3011.

**Tetrazine-β-6′-sialyllactose conjugate 3l**
β-6′-sialyllactosyl azide 1I (9.1 mg, 0.014 mmol) was used according to procedure 2. HPLC purification (eluent: acetonitrile/water=1/4) afforded conjugate 3I (3.5 mg, 17%) as red solid. HRMS (MALDI-TOF): calcd. for C_{36}H_{49}N_{9}O_{19}Na [M+Na]^+, 934.3043. found 934.3045.

5. Synthesis of 3-GluNAc-Cy3

To the mixture C33-COOH S3 (41 mg, 0.072 mmol) and 2.6-lutidine (10 µl, 0.087 mmol) in dry DMF (0.5 ml) was added HATU (33 mg, 0.087 mmol) at 0 ºC and reaction mixture was stirred for 5 min. Glucosamine hydrochloride (23 mg, 0.107 mmol) was added and the reaction was stirred for 1 h at 0 ºC. The mixture was directly loaded on a reverse phase column for purification (eluent: acetonitrile/water=3/7) to afford S4 (42 mg, 80%) as red solid.
**N-CY3-Glucosamine-azide (S5)**

![Diagram of S5](image)

Compound **S4** (30 mg, 0.041 mmol), NaN₃ (24 mg, 0.37 mmol) and triethylamine (51 µl, 0.37 mmol) in water (125 µl) and 1.4-dioxane (125 µl) were treated with DMC (35 mg, 0.21 mmol) and the reaction mixture was shaken for 1 h. The crude mixture was directly loaded on a reverse phase column (eluent: acetonitrile/water=3/7) to afford **S5** (24 mg, 78%) as red solid.

**H¹ NMR (400 MHz, MeOD):**  δ=8.56 (t, J = 13.6 Hz, 1H), 7.55 (d, J = 7.2 Hz, 2H), 7.46 (m, 2H), 7.34 (m, 4H), 6.47 (d, J = 13.6 Hz, 2H), 4.45 (d, J = 9.6 Hz, 1 H), 4.19 (m, 2H), 3.88 (d, J = 12.0 Hz, 1H), 3.71 (m, 5H), 3.46 (t, J = 8.0 Hz, 1H), 3.33 (m, 2H), 2.38 (m, 2H), 1.85 (m, 16H); C¹³ NMR (100 MHz, MeOD): δ=176.7, 176.0, 175.9, 152.1, 144.1, 143.3, 142.2, 142.1, 130.0, 129.9, 126.8, 126.7, 123.5, 123.3, 112.4, 112.2, 103.8, 103.7, 90.2, 80.4, 75.7, 71.8, 62.5, 56.5, 50.6, 50.6, 44.9, 36.5, 31.8, 28.3, 28.3, 28.1, 27.6, 24.1. HRMS (MALDI-TOF): calcd. for C₃₅H₄₆N₆O₆ [M-I]⁺, 629.3446. found 629.3441.

**3-GluNAc-Cy3**

![Diagram of 3-GluNAc-Cy3](image)
Compound S5 (24 mg, 0.03 mmol), Cul (12 mg, 0.06 mmol), TBTA (2 mg, 0.003 mmol) were taken up in DMF (1 ml) at 0 °C and alkyne 3 (11 mg, 0.04 mmol) was added. The reaction was stirred for 5 h at 0 °C and the crude reaction mixture was loaded directly on a reverse phase column (eluent: acetonitrile/water=2/3) to afford 3-GluNAc-Cy3 (4 mg, 11%) as red solid.

H1 NMR (400 MHz, MeOD): δ=10.29 (s, 1H), 8.52 (m, 3H), 8.07 (s, 1H), 7.57 (d, J = 8.0 Hz, 2H), 7.52 (m, 2H), 7.42 (m, 2H), 7.29 (m, 4H), 6.45 (m, 2H), 5.77 (d, J = 10.0 Hz, 1H), 4.44 (m, 2H), 4.20 (t, J = 10.0 Hz, 1H), 4.00 (m, 2H), 3.90 (m, 1H), 3.75 (m, 1H), 3.69 (m, 4H), 3.62 (s, 2H), 3.52 (m, 3H), 2.15 (m, 2H), 1.77 (m, 12H), 1.58 (m, 4H); C13 NMR (125 MHz, MeOD): δ=176.5, 175.8, 175.5, 172.8, 167.4, 159.2, 152.0, 146.3, 144.0, 143.3, 142.3, 142.1, 142.0, 132.0, 131.3, 129.9, 129.9, 129.2, 126.7, 126.6, 123.4, 123.3, 122.8, 112.4, 112.2, 104.0, 103.9, 88.2, 81.3, 75.5, 71.4, 62.2, 56.8, 50.6, 50.5, 44.8, 43.5, 37.7, 36.3, 35.9, 31.7, 28.3, 28.3, 28.1, 27.5, 23.9; HRMS (MALDI-TOF): calcd. for C48H56N11O6 [M-I]+, 882.4410. found 882.4410.

6. Synthesis of trans-cyclooctene derivative S7 for kinetic study

(Z)-cyclooct-4-en-1-ylmethanol (S7)

Compound S6 [5] (1.1 g, 8.15 mmol) in CH2Cl2 (33 ml) was treated dropwise with disobutylaluminium hydride (1.0 M in toluene, 12.2 ml, 12.2 mmol) at -78 °C and stirred for 1 h under nitrogen atmosphere. The reaction mixture was warmed up to 0 °C and 2 M HCl (20 ml) was added dropwise to the reaction mixture. The mixture was stirred for another 30 min at room temperature then the organic compounds were extracted with ethyl acetate, washed with brine, dried over Na2SO4, filtrated and concentrated in vacuo. The residue were taken up
in MeOH (33 ml) at room temperature and treated with sodium borohydride (620 mg, 16.3 mmol). After 1 h, the reaction was quenched with saturated NH₄Cl and the organic compounds were extracted with ethyl acetate, washed with brine, dried over Na₂SO₄, filtrated and concentrated in vacuo. Purification by silica gel column chromatography (pentane : ethyl acetate = 10 : 1) afforded S7 (690 mg, 61%) as colorless oil.

H¹ NMR (400 MHz, CDCl₃): δ=5.63-5.67 (m, 2H), 3.35-3.44 (m, 2H), 2.07-2.35 (m, 4H), 1.51-1.72 (m, 4H), 1.30-1.39 (m, 2H), 1.09-1.18 (m, 1H); C¹³ NMR (100 MHz, CDCl₃): δ=130.2, 130.1, 69.2, 40.5, 31.7, 29.6, 28.1, 25.9, 24.6.

Epoxide S8 (S8)

Compound S7 (358 mg, 2.56 mmol) dissolved in CH₂Cl₂ (5 ml) at room temperature was treated with triethylamine (534 µl, 3.84 mmol) and chlorotriethylsilane (645 µl, 3.84 mmol). The reaction mixture was stirred for 1 h then quenched with saturated NaHCO₃ and the organic compounds were extracted with ethyl acetate, washed with brine, dried over Na₂SO₄, filtrated and concentrated in vacuo. The residue were taken back up in CH₂Cl₂ (5 ml) at 0 ºC and treated with m-chloroperbenzoic acid (<70%, 1.28 g, 5.12 mmol). The reaction mixture was stirred for 2 h at 0 ºC, quenched with saturated Na₂S₂O₃ and NaHCO₃ and the organic compounds were extracted with ethyl acetate, washed with brine, dried over Na₂SO₄, filtrated and concentrated in vacuo. Purification by silica gel column chromatography (pentane : ethyl acetate = 20 : 1) gave S8 (471 mg, 68%, mixture of diasteromer) as colorless oil.

H¹ NMR (400 MHz, CDCl₃): δ=3.22-3.31 (m, 2H), 2.77-2.91 (m, 2H), 2.12-2.26 (m, 1H), 1.96-2.06 (m, 1H), 1.46-1.85 (m, 4H), 1.06-1.37 (m, 4H), 0.89 (t, J = 8.0 Hz, 9H), 0.52 (q, J = 8.0 Hz, 6H); C¹³ NMR (100 MHz, CDCl₃): δ=68.9, 55.9, 55.3, 41.1, 30.3, 29.4, 28.0, 25.8, 23.4, 6.82, 4.39.

(E)-(cyclooct-4-en-1-ylmethoxy)triethylsilane (S9)

According to the previously reported procedure⁷ to chlorodiphenylphosphine (723 µl, 4.0 mmol) in dry THF (5 ml) was slowly added lithium wire (62 mg, 8.8 mmol) washed in dry THF (5 ml). The mixture was stirred vigorously at room temperature overnight to give dark red lithium diphenylphosphide solution. This solution was added to S8 (471 mg, 1.74 mmol) dissolved in a few drops of THF and the reaction mixture was stirred for 2 d at room
temperature (the color changed to pale yellow). The reaction mixture was cooled to 0 °C and AcOH (251 µl, 4.4 mmol) followed by 35% H₂O₂ (466 µl, 4.8 mmol) were slowly added to the stirring for 1 h at room temperature. The organic compounds were extracted with CH₂Cl₂, washed with brine, dried over Na₂SO₄, filtrated and concentrated in vacuo. To the residue dissolved in DMF (10 ml) were added to NaH (<60%, 209 mg, 5.22 mmol, washed with dry hexane before using) and stirred for 1 h at room temperature. The reaction was quenched with saturated NH₄Cl and the organic compounds were extracted with ethyl acetate, washed with brine, dried over Na₂SO₄, filtrated and concentrated in vacuo. Purification by silica gel column chromatography (pentane : ethyl acetate = 10 : 1) gave S9 (113 mg, 26%, mixture of atropisomers) as colorless oil.

H¹ NMR (400 MHz, CDCl₃): δ=5.55-5.74 (m, 2H), 3.77-3.85 (m, 1H), 1.45-2.36 (m, 12H)
C¹³ NMR (100 MHz, CDCl₃): δ=134.9, 133.3, 68.4, 45.0, 38.2, 35.5, 35.0, 33.9, 32.8, 6.78, 4.05.

(E)-cyclooct-4-en-1-ylmethanol (S10)

To the mixture of S9 (20 mg, 0.08 mmol) in THF (1 ml) was added tetrabuthylammonium fluoride trihydrate (37 mg, 0.12 mmol) at 0 °C and reaction mixture was stirred for 1 h at 0 °C. Water was added to the reaction mixture and the organic compounds were extracted with ethyl acetate, washed with brine, dried over Na₂SO₄, filtrated and concentrated in vacuo. Purification by silica gel column chromatography (pentane : ethyl acetate = 10 : 1) gave S10 (5 mg, 50%, mixture of atropisomers) as colorless oil.

H¹ NMR (400 MHz, CDCl₃): δ=5.49-5.69 (m, 2H), 3.33-3.46 (m, 1H), 1.18-2.44 (m, 12H)
C¹³ NMR (125 MHz, CDCl₃): δ=134.6, 133.5, 69.3, 45.0, 38.2, 35.4, 35.0, 33.9, 32.9.

7. Kinetic study of tetrazine-TCO cycloaddition

Kinetic studies of tetrazine-trans-cyclooctene (TCO) cycloaddition were performed by monitoring the change in CY3 fluorescence resulting from the cycloaddition[S] (Figure SI1). Stock solutions of 3-GluNAc-Cy3 and TCO in DMSO (1 mM) were diluted in Tris buffer pH 7.4 to final concentration of 1.0 µM. Reactions were performed with 1.0 and 10 eq of TCO
S7 while monitoring Cy-3 fluorescence (blue and red curve respectively line, curve are the average of triplicates). As a control, the same reaction was performed in the absence of TCO S7. From the observed fluorescent intensity, 1/[sub] values were plotted (Figure SI2). Second order constant (k₂, M⁻¹s⁻¹) was calculated from the slope of the line from 1/[sub] value (k₂=8649 M⁻¹s⁻¹).

**Figure SI1**: Observation value of fluorescent intensity of tetrazine-CY3 at 570 nm.

**Supplementary figure2**: Slope of 1/[sub] value from the fluorescence intensity.
8. Protein conjugation with purified sfGFP-TCOK

Purified sfGFP-TCOK and sfGFP-BocK were obtained according to published protocols.[8] The mixture of sfGFP-TCOK (13.5 μM in Tris buffer pH 7.4, 5 μl) and 3-GluNAC-Cy3 (1 mM in DMSO, 0.68 μl) was incubated for 12 h at 37 °C. The mixture of sfGFP-BocK (13.5 μM in Tris buffer pH 7.4, 10 μl) and 3-GluNAC-Cy3 (1 mM in DMSO, 1.36 μl) were also incubated as control experiment. Each sample was mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer and analyzed by 4-12 % SDS-PAGE. The gel was scanned with Ettan DIGE Imager to visualize in-gel fluorescence and stained by silver-staining. The completion of conjugation was assayed by MALDI-TOF mass spectroscopy.

9. Protein conjugation with purified sfGFP-TCOK

Purified sfGFP-BCNK were prepared according to published protocols.[8] To the solution of sfGFP-BCNK in Tris buffer pH 7.4 (13.5, 1, 0.1 μM, respectively) was added tetrazine-glycan at 10 equivalents (entry 1) or 5 equivalents (entry 2-4) and the solution was incubated for 10 min at room temperature with gentle agitation on an orbital shaker. The reaction was quenched by addition of 100 eq. of TCO-OH S10 and the product was analyzed by MALDI-TOF. The same procedure was performed for azide-glycan (entry 5) for 10 min and 3 hours and the reaction was quenched by addition of 100 eq. of tetrazine 2 instead of TCO-OH S10.

10. Protein modification of E. coli expressing sfGFP-TCOK

E. coli pellet (50 μl) expressing sfGFP-TCOK or sfGFP-BocK were obtained as previously described from 3 mL of culture.[8] The pellets were washed 3x with PBS to remove excess TCOK or BOCK. An aliquot of each E. coli pellet (15 μl) were treated with 100 μl of a 25 μM solution of 3-GluNAC-Cy3 in PBS buffer and incubated for 8h at room temperature with gentle agitation on an orbital shaker. The E. coli were re-pelleted by centrifugation and washed twice with PBS (centrifuge and discard supernatant). Then, 30 μl of LDS buffer was added to the pellet and the mixture was incubated for 10 min at room temperature. The mixture was analyzed by 4-12 % SDS-PAGE. The gel was characterized by in-gel fluorescence imaging and silver-staining.
10. MALDI analysis of Glycoconjugation

GFP-TCOK glycoconjugation (figure 3)

before reaction
MW: 27884.6 (left)
    28015.4 (middle)
    28165.1 (right)

after reaction
(glycoconjugated product)
MW: 28739.1 [left, 27884.6 + 854.8] 28869.9 [middle, 28015.4 + 854.8(tetrazine adduct)]; 29019.9 [right, 28165.1 + 854.8(tetrazine adduct)]
Table 2

Entry 1

before reaction
MW: 28015.4

after reaction
MW: 28869.8
(glycoconjugation)

Entry 2

before reaction
MW: 28015.4

after reaction
MW: 28607.6
(glycoconjugation)
Entry 3

before reaction
MW: 28015.4

after reaction
MW: 28898.7
(glycoconjugation)

Entry 4

before reaction
MW: 28015.4

before reaction
MW: 28015.4

after reaction
MW: 28898.7
(glycoconjugation)
Entry 5 (10 min)

before reaction
MW: 28015.4

after reaction
Quenched tetrazine
adduct: MW = 28240.5 (1)

Entry 5 (3 h)

before reaction
MW: 28015.4

after reaction
Quenched tetrazine
adduct: MW = 28240.5 (1)
Glycoconjugate: MW = 28644.7 (2)
12. Spectra

Tetrazine-glucose conjugate 3a

\[ \text{H}^1\text{-NMR} \]

\[ \text{C}^{13}\text{-NMR} \]
Tetrazine-mannose conjugate 3b

H\textsuperscript{1}-NMR

C\textsuperscript{13}-NMR
Tetrazine-galactose conjugate 3c

H\textsuperscript{1}-NMR

C\textsuperscript{13}-NMR
Tetrazine-fucose conjugate 3d

H$^1$-NMR

C$^{13}$-NMR
$^{1}H$-NMR

$^{13}C$-NMR
Tetrazine-\(N\)-actyl glucosamine conjugate 3e

\begin{align*}
\text{H}^1\text{-NMR} \\
\includegraphics[width=\textwidth]{H1_NMR}
\end{align*}

\begin{align*}
\text{C}^{13}\text{-NMR} \\
\includegraphics[width=\textwidth]{C13_NMR}
\end{align*}
Tetrazine-glucoronic acid conjugate 3f

H1-NMR

C13-NMR
Tetrazine-maltose conjugate 3g

H\textsuperscript{1}-NMR

C\textsuperscript{13}-NMR
Tetrazine-cellobiose conjugate 3h

H¹-NMR

C¹³-NMR
Tetrazine-lactose conjugate 3i

\[\text{H}^1\text{-NMR} \]

\[\text{C}^{13}\text{-NMR} \]
Tetrazine Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc conjugate 3j
H$^\text{1}$-NMR

MALDI-TOF mass

$[\text{M+H}]^+$

$[\text{M+Na+4H}]^+$

$[\text{M+Li+4H}]^+$
Tetrazine-β-3'-sialyllactose conjugate 3k

LC-MS

NL:
3.70E4
Total Scan
PDA
Avalon
TM9-67-
AGAIN-107
Tetrazine-\(\beta\)-6'-sialyllactose conjugate 3l

**LC-MS**

- RT: 1.22
- \([\text{M+H}]^+\) at 912.18
- Mass values: 224.26, 292.07, 621.09, 868.95, 928.02, 1350.81, 1494.56, 1823.96
**N-CY3-Glucosamine (S4)**

![Chemical Structure](image)

**LC-MS**

![LC-MS Graph](image)

- RT: 2.37
- 604.63 [M-I]^+
- NL: 6.24E4
- Total Scan
- PDA
- Genesis
- TM6-74-
- SPEC
**N-CY3-Glucosamine-azide (S5)**

![Chemical Structure](image)

**LC-MS**

![LC-MS Graph](image)

RT: 2.51

629.51 $[M-I]^+$

- NL:
  - 1.25E5
- Total Scan
- PDA
- Genesis
- TMS-76
- SPEC

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SI-45
H-NMR

C$^{13}$-NMR
3-GluNAc-Cy3

LC-MS

NL: 1.03E5
Total Scan
PDA
Avalon
TM9-73-
BIO-83
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