The Bioorthogonal Isonitrile–Chlorooxime Ligation

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1. General Aspects and Materials

Reactions requiring anhydrous conditions were performed under dry nitrogen or argon atmospheres in flame-dried glassware. Reaction mixtures were stirred magnetically. Air- and moisture-sensitive liquids and solutions were transferred via syringe or cannula into the reaction vessels through rubber septa. All reagents and solvents were purchased at the highest commercial quality available and used as received. Anhydrous solvents were obtained from a purification column composed of activated alumina (A₂). Yields refer to spectroscopically pure compounds unless otherwise stated. Flash chromatography was performed on silica gel (Merck Kieselgel 60 F254 230–400 mesh). TLC was performed on aluminium-backed silica plates (0.2 mm, 60 F254) or glass-backed silica plates (Merck, Silica Gel 60 F254) which were developed using standard visualizing agents: UV fluorescence (254 and 366 nm), iodine, ninhydrine / Δ, phosphomolybdic acid / Δ, anisaldehyde / Δ, potassium permanganate / Δ, Magic Seebach Stain/ Δ (Helv. Chim. Acta 1987, 70, 448). Rₚ values given in the experimental procedure correspond to the Rₚ value of the obtained product.

¹H-NMR: Spectra were recorded on 300, 400, 500 or 600 MHz instruments. Chemical shifts (δH) are quoted in parts per million (ppm) and referenced to the appropriate NMR solvent peak(s). ¹³C-NMR: Spectra were recorded on a 101 MHz, 125 MHz and 150 MHz instruments. Chemical shifts (δC) are quoted in ppm, referenced to the appropriate solvent peak(s) and are assigned C, CH, CH₂ and CH₃. COSY, HSQC, ¹⁵N-HSQC, and HMBC spectra were used where necessary in assigning NMR spectra (proton (H-x) and carbon (C-x) numbers are listed and correspond to assigned numbers in the chemical structure). ¹⁹F-NMR: Chemical shifts (δF) are quoted in parts per million (ppm) and are not referenced. HRMS: High resolution mass spectra were recorded on an ESI-Q-TOF, MALDI-TOF-MS or an EI-Sector-MS. HPLC: Analytical reversed-phase (RP) high-performance liquid chromatography (HPLC) was performed on a Dionex UHPLC, Ultimate 3000 (ThermoFisher Scientific, Waltham/USA). All literature known compounds were characterized by comparison of at least two pieces of spectral data with those reported in the literature.

Cell culture: Chinese hamster ovary cells (CHO-K1) were obtained from the Health Protection Agency (www.HPA.org.uk). The cells were grown in a humidified 5% CO₂ atmosphere at 37 °C using Kaighn’s Modification of Ham’s F-12 medium (F-12K™) supplemented with L-glutamine (4 mM), penicillin (100 U/mL penicillin), streptomycin (100 μg/mL), and 10% fetal calf serum (FCS) superior (standardized). Culture medium DMEM high glucose, F-12KTM, L-glutamine (200 mM), penicillin (10.000 U/mL), streptomycin (10 mg/mL), and trypsin blue solution were purchased from Sigma, Invitrogen, ATCC or BioConcept. Trypsin-EDTA (0.05%/0.02%) in Ca²⁺- and Mg²⁺-deficient phosphate buffered saline (PBS) (1:250) was purchased from Amimed. PBS (pH 7.4) was purchased from Invitrogen. FCS superior was bought from Oxoid AG and Biochrom AG. Cell culture flasks as well as serological pipettes were purchased from BD Biosciences and Sarstedt. Ethylenediaminetetraacetic acid (EDTA) and propidium iodide (PI) were purchased from Sigma-Aldrich. Hoechst 33342 was purchased from Invitrogen. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Merck. Live cell imaging solution was purchased from ThermoFisher. FACS: The samples were run in triplicate, and each experiment was repeated at least three times. Alexa Fluor 488 and PI were excited at 488 nm and monitored with 530/30 bandpass and 650 longpass filters, respectively, on a BDLSFRortessa flow cytometer. Events corresponding to cellular debris were removed by gating on forward and side scatter. Mean fluorescence values were determined from the histograms using FlowJo 10.0.6 software while dead cells were removed according to PI.
staining. **Confocal Microscopy:** Fluorescence images of cells were collected using a Nikon Eclipse T1 microscope equipped with a Yokogawa spinning-disk confocal scanner unit CSU-W1-T2, two sCMOS cameras (Orca Flash 4.0 V2) and a LUDL BioPrecision2 stage with piezo focus. Emission in the blue channel was filtered with a 450/50 bandpass filter, emission in the green channel was filtered with a 525/50 bandpass filter and emission in the far-red channel with a 700/75 bandpass filter. Fluorescence images were obtained using an oil-immersion objective with a magnification of 100 × 1.49 CFI Apo TIRF. The microscope was operated using VisiVIEW (Metamorph). **MTT:** For the cell viability read out a SPARK multimode microplate reader from TECAN was used and the data was analyzed with the software SPARKCONTROL.

2. **Synthesis and Analytical Data of All Compounds**

2.1 **Synthesis of Lysine-derived Isonitrile (1)**

\[ \text{CbzHN} \text{COBn} \text{O} \text{NH}_{3} \text{OTs} \xrightarrow{\text{Et}_{3}\text{N}, \text{HCO}_{2}\text{Et}} \xrightarrow{\text{POCl}_{3}, \text{Et}_{3}\text{N}, \text{CH}_{2}\text{Cl}_{2}} \text{CbzHN} \text{COBn} \text{O} \text{NC} \]

\( S-1 \)\( \rightarrow \) \( S-2 \) \( \rightarrow \) \( 1 \)

\( S-2: \) In a flame-dried round bottom flask salt \( S-1 \) (3.0 g, 5.67 mmol, 1 equiv.) was dissolved in ethyl formate (9 ml). Triethylamine (0.869 ml, 6.24 mmol, 1.1 equiv.) was then added and the temperature was raised to 60°C. The stirred reaction was monitored by TLC (eluent: EtOAc/hexane 2:1, \( R_{f} = 0.3 \)). After 40 h the reaction was cooled to room temperature and the solvent was removed in vacuo using a rotatory evaporator. Purification by column chromatography on silica gel (eluent: mixtures of EtOAc/hexane from 2:1 to 5:1) provided 1.53 g (71%, 4.02 mmol) of formamide \( S-2 \) as a white solid.

\( ^1\text{H} \text{NMR} \) (400 MHz, CDCl\(_3\), 25°C): \( \delta/p pm = 8.07 \) (s, 1H; H-15), 7.29-7.39 (m, 10H; arom., H-5–H-9/H-19–H-23), 5.57 (bs, 1H; NH, H-14), 5.40 (d, \( J = 8.8 \) Hz, 1H; NH, H-16), 5.17 (dd, \( J = 15.9 \), 12.0 Hz, 2H; H-3), 5.10 (d, \( J = 1.58 \) Hz, 2H; H-17), 4.41 (td, \( J = 8.18, 8.14, 4.92 \) Hz, 1H; H-1), 3.22 (td, \( J = 6.54, 6.54, 6.55 \) Hz, 2H; H-13), 1.78-1.92 (m, 1H; H-10a), 1.62-1.74 (m, 2H; H-10b), 1.41-1.58 (m, 2H; H-12), 1.20-1.40 (m, 2H; H-11). \( ^{13}\text{C} \text{NMR} \) (75.4 MHz, CDCl\(_3\), 25°C): \( \delta/p pm = 172.2 \) (C\(_{q}\), C-2), 161.3 (C-H, C-15), 156.1 (C\(_{q}\), C-16'), 136.3 (C\(_{q}\), C-4), 135.3 (C\(_{q}\), C-18), 128.8 (CH\(_{ar}\), C-6/C-8), 128.7 (CH\(_{ar}\), C-20/C-22), 128.6 (CH\(_{ar}\), C-7/C-21), 128.4 (CH\(_{ar}\), C-5/C-9), 128.2 (CH\(_{ar}\), C-19/C-23), 67.4 (CH\(_{2}\), C-17), 67.2 (CH\(_{2}\), C-3), 41.5 (CH, C-1), 37.8 (CH\(_{2}\), C-13), 32.4 (CH\(_{2}\), C-10), 28.8 (CH\(_{2}\), C-12), 22.4 (CH\(_{2}\), C-11). **HRMS (ESI):** \( m/z \) : calcd. for \([C_{22}H_{26}N_{2}NaO_{5}]^{+}\): [M+Na] \(^{+}\) = 421.1734; found: 421.1737 [M+Na]\(^{+}\).
1: In a flame-dried round bottom flask, formamide S-2 (1.59 g, 4.18 mmol, 1.0 equiv.) was dissolved in anhydrous dichloromethane (8 ml) under nitrogen atmosphere and triethylamine (2.025 g, 20 mmol, 5.0 equiv.) was added. The stirred solution was cooled to 0°C using an ice-bath and POCl₃ (766 mg, 5 mmol, 1.25 equiv.) was added dropwise. After 1 h TLC analysis revealed full consumption of the starting material (eluent: hexane/EtOAC 1:1). The reaction mixture was quenched with a 1M NaHCO₃ aqueous solution, the organic phase was separated and washed twice with 1M NaHCO₃, with brine and then dried over sodium sulphate. Column-chromatography (eluent: hexane/EtOAC 1:1, Rₚ = 0.73) gave 1.42 g (85%, 3.56 mmol) of isonitrile 1 as a white solid.

**1H NMR** (400 MHz, D₂O: THF-d₈ (3:2), 25°C): δ/ppm = 7.41-7.47 (m, 10H; H-10–H-14/H-18–H-22, arom.), 5.13-5.30 (m, 4H; H-8/H-17, benzyl.), 4.37 (m, 1H; H-6), 3.54-3.58 (m, 2H; H-2), 1.81-1.87 (m, 1H; H-5), 1.73-1.76 (m, 2H; H-3), 1.62-1.65 (m, 2H; H-4).

**13C NMR** (75.4 MHz, D₂O: THF-d₈ (3:2), 25°C): δ/ppm = 173.3 (Cq, C-7), 157.5 (Cq, C-16), 155.1 (Cq, C-1), 137.4 (Cq, C-23), 136.4 (Cq, C-9), 129.1, 129.0, 128.88, 128.5, 128.21 (CH arom.), 54.7 (CH, C-6), 41.7 (CH, C-2), 31.2 (CH₂, C-5).


2.2 Synthesis of Chlorooxime (2)

S-3: Benzaldehyde (3.0 g, 30 mmol, 1.0 equiv.) was dissolved in MeOH (135 mL). Hydroxylamine hydrochloride (4.2 g, 60 mmol, 2.0 equiv.) was added to the stirred solution, followed by sodium acetate (4.9 g, 60 mmol, 2.0 equiv.) and 15 mL of water. The reaction was monitored by TLC (5 % EtOAc/Hexanes; Rₚ = 0.2, R₂ = 0.15 [corresponding to E/Z isomers]). After 2 h the reaction mixture was diluted with EtOAc (100 mL) and H₂O (100 mL). Phases were separated and the aqueous phase was extracted twice with EtOAc (50 mL). The combined organic phases were dried over MgSO₄, filtered and concentrated under reduced pressure. The product was obtained as a clear-yellowish oil in quantitative yield (3.63 g, 30 mmol).

**1H NMR** (300 MHz, CDCl₃, 25°C): δ/ppm = 8.17 (s, 1H; H-1, oxime), 7.57-7.60 (m, 2H; H-4/H-6), 7.39-7.40 (m, 3H; H-3/H-5/H-7); **13C NMR** (101 MHz, CDCl₃, 25°C): δ/ppm = 150.5 (CH, C-1), 132.1 (Cq, C-8), 130.2 (CH, C-5; arom.), 128.9 (CH, C-7/C-3; arom.), 127.2 (CH, C-4/C-6; arom.); **HRMS** (EI⁺): m/z : calcd for C₁₇H₁₄NO⁺: [M⁺]⁺ = 228.0861 ; found: 228.0875 and 226.0675 [M⁺-H₂O]⁺.
2: Oxime S-3 (1.21 g, 10 mmol, 1.0 equiv.) was dissolved in DMF (30 mL). N-chlorosuccinimide (1.47 g, 11 mmol, 1.1 equiv.) was added portionwise to the solution at r.t. and the reaction was stirred until consumption of starting material was observed (3 h) (TLC: 5% EtOAc/Hexanes; Rf = 0.41). The reaction mixture was diluted with 5% LiClaq (10 mL) and DCM (10 mL) phases were separated. The aqueous phase was extracted with dichloromethane until residual product was completely removed from the aqueous phase (TLC). The organic phase was washed with 5% LiClaq solution (10 x 10mL). The combined organic phases were washed with sat. NaCl solution, dried over MgSO4, filtered and concentrated in vacuo. The crude compound was purified on a short silica column (0.7 mL). The pure product was obtained as a white-yellow solid in 94 % yield (1.46 g, 9.4 mmol). The product should be stored in the fridge and kept dry due to risk of hydrolysis over time.

\[ \text{[M]}^+ = 155.01324; \text{found: 155.01293.} \]

2.3 Synthesis of Hydroxyimino Amide (3)

3: Z-(4-NC)-Lys-OBn (1) (19.0 mg, 50 μmol, 1.0 equiv.) was dissolved in 0.2 mL THF and chlorooxime 2 (9.3 mg, 60 μmol, 1.2 equiv.) was dissolved in a separate glass vial in 0.3 mL THF. PBS (1.0 mL, 0.05 M, pH = 7.4) was added to the isonitrile solution followed by the chlorooxime and the mixture was stirred at r.t. The reaction was monitored by TLC (1:1 EtOAc:Hexanes; Rf = 0.34). After complete consumption of starting material the solvent was removed under reduced pressure. The residual oil was dissolved in EtOAc and water. The phases were separated and the organic phase was washed with water twice. The combined organic phases were washed with brine, dried over MgSO4, filtered and concentrated in vacuo. The crude product (yellow oil) was purified by column chromatography (5 % – 100 % EtOAc/Hexanes). (Note: Upon purification on normal silica gel the hydroxyamino amide isomerizes into a mixture of E- and Z-isomer) The product was obtained as a white solid in 81 % yield (21 mg, 41 μmol).

\[ \text{[M]}^+ = 155.01324; \text{found: 155.01293.} \]
2.4 Synthesis of Mannosamine Derivatives 4 and 4a

\[
\begin{align*}
\text{HOONH}_2 &\xrightarrow{\text{Ac}_2\text{O}, \text{HCO}_2\text{H}} \text{HOOCNH} \xrightarrow{\text{DCC, PfpOH}} \text{POCl}_3, \text{Et}_3\text{N} &\xrightarrow{\text{THF}} \text{POCOO} \xrightarrow{\text{DCM}} \text{N} \\
\text{S-4} &\quad \text{S-5} &\quad \text{S-6}
\end{align*}
\]

S-4: 3-aminopropanoic acid (5.94 g, 66.7 mmol, 1.0 equiv.) was dissolved in formic acid (67 mL) and acetic anhydride (25.2 g, 246.4 mmol, 3.7 equiv., 23 mL) was added. The reaction was stirred at 50°C. After 2 h the reaction was diluted with water (200 mL) and concentrated under reduced pressure. The product was obtained in 90% yield (6.18 g, 60 mmol) and used without further purification.

\[^1\text{H} \text{NMR} \ (400 \text{ MHz, } \text{d}_6-\text{MeOD}, \ 25^\circ\text{C}): \delta/\text{ppm} = 7.93 \ (s, \ 1\text{H}, \ H-1), \ 3.37 \ (t, \ J = 6.4 \text{ Hz, } 2\text{H, H-4}), \ 2.43 \ (t, \ J = 6.4 \text{ Hz, } 2\text{H, H-3}); \]^1\text{C} \text{ NMR} \ (101 \text{ MHz, } \text{d}_6-\text{MeOD}, \ 25^\circ\text{C}): \delta/\text{ppm} = 175.2 \ (C_\text{O}, \ C-5), \ 163.9 \ (\text{CH}, \ C-1), \ 34.9 \ (\text{CH}_2, \ C-3), \ 34.7 \ (\text{CH}_2, \ C-4); \ HR-\text{MS} \ (\text{ESI}): \ m/z : \text{calcd for C}_4\text{H}_8\text{NO}_3: \ [M+H]^+ = 118.0499; \text{found: } 118.0496.\]

S-5: 3-N-formylpropanoicacid S-4 (1.17 g, 10 mmol, 1.0 equiv.) was suspended in anh. THF (60 mL). Pentfluorophenol (2.02 g, 11 mmol, 1.1 equiv.) and N,N-dicyclohexylcarbodiimide (2.27 g, 11 mmol, 1.1 equiv) were added and the reaction was stirred at r.t. for 3 h. The precipitate was filtered and washed with DCM twice. The product was obtained as a colorless oil in 84% yield (2.4 g, 8.4 mmol) and used immediately without further purification.

\[^1\text{H} \text{NMR} \ (500 \text{ MHz, } \text{d}_6-\text{DMSO}, \ 25^\circ\text{C}): \delta/\text{ppm} = 8.24 \ (bs, \ 1\text{H}, \ H-1), \ 8.04 \ (d, \ J = 1.1 \text{ Hz, } 1\text{H}; \ H-1), \ 3.45 \ (q, \ J = 6.4, 6.4, 6.3 \text{ Hz, } 2\text{H}; \ H-3), \ 2.99 \ (t, \ J = 6.6 \text{ Hz, } 2\text{H; H-4}); \]^1\text{C} \text{ NMR} \ (125 \text{ MHz, } \text{d}_6-\text{DMSO}, \ 25^\circ\text{C}): \delta/\text{ppm} = 167.9 \ (C_\text{O}, \ C-5), \ 161.4 \ (\text{CH}, \ C-1), \ 140.5 \ (\text{C}-\text{F}, \ m, \ ^1\text{J}_{C-F} = 250.4 \text{ Hz}), \ 138.9 \ (\text{C}-\text{F}, \ m, \ ^1\text{J}_{C-F} = 250.3 \text{ Hz}), \ 137.4 \ (\text{C}-\text{F}, \ m, \ ^1\text{J}_{C-F} = 250.3 \text{ Hz}), \ 124.4 \ (\text{C}_\text{O}, \ m, \ C-6), \ 33.6 \ (\text{CH}_2, \ C-3/C-4); \ ^{19}\text{F} \text{ (HDEC) NMR} \ (376 \text{ MHz, } \text{d}_6-\text{DMSO}, \ 25^\circ\text{C}): \delta/\text{ppm} = -162.7 \ (m, 2\text{F}), -158.0 \ (t, \ J = 23.2 \text{ Hz, } 1\text{F}), -153.1 \ (m, 2\text{F}); \ HRMS \ (\text{ESI}): \ m/z : \text{calcd for C}_{10}\text{H}_6\text{F}_5\text{NO}_3: 283.03 [M+H]^+; \text{not found, not stable.}\]

S-6: Formamide S-5 (4.9 g, 17.3 mmol, 1.0 equiv.) was dissolved in anh. THF (200 mL) in a flame-dried round bottom flask. The solution was cooled down to 0°C and Et$_3$N (4.4 g, 43.3 mmol, 2.5 equiv., 6 mL) was added, followed by POCl$_3$ (3.3 g, 21.6 mmol, 1.25 equiv., 2.02 mL). The reaction was stirred until the starting material was consumed (TLC 1:4 EtOAc:Hexanes, $R_f = 0.38$). The reaction mixture was diluted with DCM and quenched by addition of aqueous 2M NaHCO$_3$ solution. Phases were separated, the organic phase was washed with aqueous 2M NaHCO$_3$ solution (twice) and brine. The combined organic phases were dried over MgSO$_4$, filtered and concentrated under reduced pressure. The crude yellow crystalline product was further purified by column chromatography (1:4 EtOAc:Hexanes). The pure product was obtained as a white crystalline solid in 88% yield (4.02 g, 15.2 mmol).
**1H NMR** (400 MHz, CDCl$_3$, 25°C): $\delta$/ppm = 3.81 (t, $J$ = 6.7 Hz, 2H; H-2), 3.13 (t, $J$ = 6.7 Hz, 2H; H-3); **13C NMR** (75.4 MHz, CDCl$_3$, 25°C): $\delta$/ppm = 165.8 (C$_o$, C-4), 159.2 (C$_o$, C-1), 36.9 (CH$_2$, C-2), 33.6 (CH$_2$, C-3); **13C NMR** (125 MHz, d$_6$-DMSO, 25°C): $\delta$/ppm = 166.9 (C$_o$, C-4), 156.9 (C$_o$, t, C-1), 140.5 (C-F, m, $^1J_{CF}$ = 249.8 Hz ), 137.2 (C-F, m, $^1J_{CF}$ = 250.2 Hz), 139.1 (C-F, m, $^1J_{CF}$ = 250.4 Hz), 124.1 (C$_o$, m, C-5), 36.9 (CH$_2$, t, C-2), 32.8 (CH$_2$, C-3); **19F (HDEC) NMR** (376 MHz, CDCl$_3$, 25°C): $\delta$/ppm = 161.6 (d, 2F), 156.8 (t, 1F), 152.4 (m, 2F); **HRMS (ESI)**: $m$/z : calcd for C$_{10}$H$_5$F$_5$NO$_2$: [M+H]$^+$ = 265.02; found: not stable; **IR** (solid) = 2157, 1777, 1512, 1375, 1114, 983 cm$^{-1}$.

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4: To a solution of D-mannosamine hydrochloride (100 mg, 0.463 mmol, 1.0 equiv.) and Et$_3$N (0.13 mL, 0.972 mmol, 2.1 equiv.) in anhydrous MeOH (2 mL), was added S-6 (129 mg, 0.486 mmol, 1.05 equiv.) dissolved in 1 mL anhydrous CH$_2$Cl$_2$. Upon full consumption of starting material (TLC: CH$_2$Cl$_2$:MeOH 9:1, $R_f$ = 0.15; Seebach Stain) the crude mixture was concentrated under vacuum and used without further purification. The crude product was dissolved in pyridine (60 mL) and acetic anhydride (24 mL). The reaction was stirred at r.t. overnight. The reaction was diluted with EtOAc and water and the aqueous phase was extracted twice with EtOAc. The combined organic phases were washed with brine and dried over MgSO$_4$, filtered and concentrated in vacuo. The brown-yellow crude product was purified by column chromatography (Eluent: 0-100% EtOAc in Hexanes). Isonitrile 4 was obtained as a white solid in 42% yield (83 mg, 0.194 mmol). **1H NMR** (400 MHz, CDCl$_3$, 25°C): $\delta$/ppm = (major) 6.05 (d, $J$ = 1.8 Hz, 1H; H-2), 5.94 (d, $J$ = 7.8 Hz, NH; H-11), 5.35 (dd, $J$ = 10.2, 4.5 Hz, 1H; H-5), 5.18 (d, $J$ = 10 Hz, 1H; H-7), 4.67 (ddd, $J$ = 9.1, 4.6, 1.9 Hz, 1H; H-3), 4.30 (m, 1H; H-9), 4.12 (m, 1H; H-8), 3.73 (m, 2H; H-14), 2.68 (m, 2H; H-13), 2.19 (s, 3H, acetyl), 2.10 (s, 3H, acetyl), 2.07 (s, 3H, acetyl), 2.02 (s, 3H, acetyl); (minor) 5.87 (d, $J$ = 1.8 Hz, 0.5H; H-2’’), 5.92 (d, $J$ = 7.8 Hz, 0.5H; H-11’’), 5.13 (dd, $J$ = 9.9, 0.9 Hz, 0.5H; H-7’’), 5.09 (m, 0.5H; H-5’’), 4.82 (ddd, $J$ = 9.0, 3.8, 1.8 Hz, 0.5H; H-3’’), 4.30 (m, 0.5H, H-9’’), 3.82 (m, 0.5 H, H-9’’), 3.73 (m, 1H, H-14’’), 2.68 (m, 1H, H-13’’), 2.12 (s, 3H, acetyl), 2.11 (s, 3H, acetyl), 2.04 (s, 3H, acetyl), 2.01 (s, 3H, acetyl); **13C NMR** (101 MHz, CDCl$_3$, 25°C): $\delta$/ppm = (major) 170.2 (C$_o$, acetyl), 169.8 (C$_o$, acetyl), 168.6 (C$_o$, acetyl), 168.2 (C$_o$, acetyl), 157.9 (C$_o$, C-15), 91.6 (CH, C-2), 70.3 (CH, C-8), 68.8 (CH, C-5), 65.6 (CH, C-7), 62.2 (CH$_2$, C-9), 49.8 (CH, C-2), 35.9 (CH$_2$, C-14), 34.1 (CH$_2$, C-13); (minor) 170.7 (C$_q$, acetyl), 170.1 (C$_q$, acetyl), 169.2 (C$_q$, acetyl), 168.5 (C$_q$, acetyl), 90.6 (CH, C-2’’), 70.7 (CH, C-8’’), 71.3 (CH, C-5’’), 65.4 (CH, C-7’’), 62.0 (CH$_2$, C-9’’), 50.0 (CH, C-3’’), 35.9 (CH$_2$, C-14’’), 34.1 (CH$_2$, C-13’’), 21.0, 21.0, 20.9, 20.9, 20.9, 20.8, 20.8 (acetyl CH$_3$ overlapping major and minor); **HRMS (ESI)**: $m$/z : calcd for C$_{18}$H$_{24}$N$_2$NaO$_4$: [M+Na]$^+$ = 451.1323; found: 451.1328; **IR** (solid) = 2150, 1740, 1684, 1538, 1434, 1367, 1214, 1039, 972 cm$^{-1}$.

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![Diagram of reaction](image-url)
4a: α-azidoacetic acid (70 mg, 0.696 mmol, 1.5 equiv.), D-mannosamine hydrochloride (100 mg, 0.464 mmol, 1.0 equiv.) and Et$_3$N (0.13 mL, 0.928 mmol, 2.0 equiv.) were dissolved in anhydrous MeOH. The mixture was cooled to 0°C and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC-HCl; 177 mg, 0.928 mmol, 2.0 equiv.) and 1-hydroxybenzotriazole hydrate (HOBt; 70 mg, 0.464 mmol, 1.0 equiv.) were added in that order. The mixture was stirred at 0°C for 10 min and then overnight at rt. After complete consumption of starting material the solvent was removed under reduced pressure. The crude reaction mixture was purified by column chromatography (solid loading on celite, DCM:MeOH (from 1:0 to 5:1). The product was obtained in 45% yield as a white-yellow crystalline solid (54 mg, 0.21 mmol). The mannosamine derivative (54 mg, 0.21 mmol, 1.0 equiv.) was then dissolved in pyridine (4.5 mL). The mixture was cooled to 0°C and acetic anhydride (2.5 mL) and catalytic amounts of 4-dimethylaminopyridine (tip of spatula) were added. After 20 hrs the reaction mixture was diluted with DCM and the organic layer was washed three times with 1M aqueous HCl, and then once with sat. NaHCO$_3$, H$_2$O and brine. The combined organic phases were dried over MgSO$_4$, filtered and concentrated in vacuo to obtain the crude product as a yellow oil. The crude product was purified by column chromatography (Eluent: 30%–80% EtOAc) to give product 4a as a white solid in 47 % yield (44 mg, 0.10 mmol).

$^1$H NMR (400 MHz, CDCl$_3$, 25°C): δ/ppm = (major) 6.5 (d, $J = 9.3$ Hz , 1H; NH), 6.04 (d, $J = 1.9$ Hz, 1H; H-2), 5.33 (dd, $J = 10.1$, 4.2 Hz, 1H; H-5), 5.21 (dd, $J = 10.1$, 10.1 Hz, 1H; H-7), 4.61 (ddd, $J = 9.3$, 4.2, 1.9 Hz, 1H; H-3), 4.1 (m, 5.5H; H-9/H-9''/H-8/H-13/H-13''), solvent (EtOAc) impurity underneath), 2.06 (m, 18H, acetyl groups); (minor) 6.64 (d, $J = 9.0$ Hz, 0.5H; NH''), 5.88 (d, $J = 1.7$ Hz, 0.5H; H-2''), 5.15 (dd, $J = 9.8$, 9.8 Hz, 0.5H; H-7''), 5.05 (dd, $J = 9.9$, 3.9 Hz, 0.5H; H-5''), 4.72 (ddd, $J = 9.1$, 3.9, 1.7 Hz, 0.5H; H-3''), 3.82 (ddd, $J = 9.5$, 4.6, 2.4 Hz, 0.5H, H-8''), for H-9'' and H-13'' as well as acetyl groups see listing (major). $^{13}$C NMR (101 MHz, CDCl$_3$, 25°C): δ/ppm = (major) 170.6 (C$_q$, acetyl), 170.2 (C$_q$, acetyl), 169.6 (C$_q$, acetyl), 168.2 (C$_q$, acetyl), 166.9 (C$_q$, C-12), 91.4 (CH, C-2), 70.4 (CH, C-8), 68.9 (CH, C-5), 65.3 (CH, C-7), 61.9 (CH$_2$, C-9), 52.5 (CH, C-13), 49.4 (CH, C-3); (minor): 170.3 (C$_q$, acetyl), 169.7 (C$_q$, acetyl), 168.4 (C$_q$, acetyl), 168.5 (C$_q$, acetyl), 167.5 (C$_q$, C-12''), 90.4 (CH, C-2''), 73.5 (CH, C-8''), 71.5 (CH, C-5''), 65.1 (CH, C-7''), 61.8 (CH$_2$, C-9''), 52.7 (CH, C-13''), 49.8 (CH$_2$, C-3''), 21.1-20.6 (acetyl CH$_3$, overlapping major and mino). HR-MS (ESI): m/z : calcd for C$_{16}$H$_{22}$N$_4$NaO$_{10}$: [M+Na]$^+$= 453.1228; found: 453.1216.
In a round bottom flask equipped with a condensing funnel 4-carboxybenzaldehyde (2.2 g, 14.7 mmol, 1 equiv.), hydroxylamine hydrochloride (2.08 g, 29.33 mmol, 2 equiv.) and sodium acetate (6.04 g, 73.3 mmol, 5 equiv.) were mixed in THF (80 ml) and stirred under reflux. After 3.5 h full consumption of starting material was observed by TLC (eluent: EtOAc/MeOH 1:1). The solvent was removed in vacuo using a rotatory evaporator and ethyl acetate and water were added. The organic phase was washed twice with water, once with brine and then dried over Na$_2$SO$_4$. Evaporation of the solvent yielded the desired product in 83% yield (2.0 g, 12.2 mmol) as a white solid.

$^1$H NMR (400 MHz, d$_4$-MeOD, 25°C): $\delta$/ppm = 11.59 (bs, OH; H-1), 8.15 (s, 1H; H-2), 8.03 (d, $J = 8.1$ Hz, 2H; H-7/H-5), 7.70 (d, $J = 8.1$ Hz, 2H; H-6/H-4); $^{13}$C NMR (101 MHz, d$_4$-MeOD, 25°C): $\delta$/ppm = 169.3 (C$_9$, C-9), 149.1 (CH, C-2), 139.0 (C$_3$, C-3), 132.4 (C$_8$, C-8), 131.0 (CH, C-7/C-5), 127.6 (CH, C-6/C-4); HR-MS (ESI) : $m/z$ : calcd for C$_8$H$_8$NO$_3$: [M+H]$^+$ = 166.0499; found: 166.0499.
**S-8:** In a flame-dried round bottom flask carboxylic acid **S-7** (1.65 g, 9.9 mmol, 1 equiv.) was dissolved in THF (60 ml) under a nitrogen atmosphere. The stirred solution was cooled down to 0°C and N-hydroxysuccinimide (1.38 g, 11.9 mmol, 1.2 equiv.) and N,N′-dicyclohexycarbodiimide (2.07 g, 10 mmol, 1 equiv.) were added sequentially. The reaction was monitored by TLC (eluent: hexane/EtOAc 1:2; Rf = 0.7). After 8 h full consumption of the starting material was observed, the precipitate was removed by filtration and washed with diethylether. The volatiles of the filtrate were evaporated in vacuo. Combining the white solids provided the product in 89% yield (225 mg, 1.02 mmol).

$^1$H NMR (400 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 11.78 (s, 1H, OH; H-1), 8.29 (s, 1H; H-2), 8.11 (d, J = 8.1 Hz, 2H; H-7/H-5), 7.85 (d, J = 8.1 Hz, 1H; H-6/H-4), 2.90 (s, 4H; H-11/H-12); $^{13}$C NMR (101 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 170.3 (C$_6$, C-10/C-13), 161.5 (C$_6$, C-9), 147.3 (CH, C-2), 139.7 (C$_6$, C-8), 130.5 (CH, C-7/C-5), 127.2 (CH, C-6/C-4), 124.5 (C$_6$, C-3), 25.6 (CH$_2$, C-11/C-12); HR-MS: m/z : calcd for C$_{11}$H$_{16}$N$_3$O$_2$: [M+H]$^+$ = 222.1237; not found due to decomposition.

**S-9:** To a vigorously stirred solution of 1,3-diaminopropane (600 mg, 8 mmol, 8 equiv.) in THF (9 ml) was added a solution of **S-8** (300 mg, 1.14 mmol, 1 equiv.) in THF (12 ml). A white precipitate formed immediately and was filtered off after 10 min. Diethylether was added to the recovered solution to induce the formation of a second precipitate (**S-9**), which was filtered off and further dried under vacuum. Combining the white solids provided the product in 89% yield (225 mg, 1.02 mmol).

$^1$H NMR (400 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 11.44 (bs, 1H, OH; H-1), 8.56 (t, J = 5.6 Hz, 1H, NH; H-10), 8.16 (m, 1H, NH; H-2), 7.84 (d, J = 8.1 Hz, 2H; H-6/H-4), 7.66 (d, J = 8.1 Hz, 1H; H-7/H-5), 3.31 (td, J = 6.54, 6.53 Hz, 4H; H-11/H-14(NH$_2$)), 2.58 (t, J = 6.62 Hz, 2H; H-13), 1.58 (q, J = 6.75 Hz, 2H; H-12); $^{13}$C NMR (101 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 165.6 (C$_6$, C-9), 147.5 (CH, C-2), 135.5 (C$_6$, C-3), 135.1 (C$_6$, C-8), 127.5 (CH, C-7/C-5), 126.2 (CH, C-6/C-4), 39.8 (CH$_2$, C-13), 37.2 (CH$_2$, C-11), 32.9 (CH$_2$, C-12); HR-MS (ESI): m/z : calcd for C$_{11}$H$_{16}$N$_3$O$_2$: [M+H]$^+$ = 222.1237; found: 222.1238.

**S-10:** (The following procedure was adapted from Sachon et al. *Anal. Chem*. 2003, 75, 6536.) In a round bottom flask hydrogen peroxide (2ml, 35% in water) was added to a suspension of biotin (500 mg, 2.04 mmol, 1 equiv.) in acetic acid (6 ml) and the reaction was stirred at room temperature. After 30 min dissolution of the starting material was observed and after prolonged stirring biotin sulfone **S-10** slowly precipitated. After 3 days the precipitate was
filtered off, washed with diethyl ether and dried in vacuo. Sulfone S-10 was obtained in 89% yield (502 mg, 1.82 mmol) as a white solid.

^1H NMR (400 MHz, d_6-DM SO, 25°C): δ/ ppm = 12.00 (bs, 1H, OH), 6.69 (s, 1H, NH; H-11), 6.59 (s, 1H, NH; H-9), 4.46-4.30 (m, 2H; H-12/H-8), 3.38-3.26 (m, 1H; H-13'), 3.22-3.12 (m, 1H; H-7), 3.02 (d, J = 14.2 Hz, 1H, H-13''), 2.22 (t, J = 7.25 Hz, 2H; H-3), 1.74-1.34 (m, 6H; H-4/H-5/H-6); 13C NMR (150 MHz, d_6-DM SO, 25°C): δ/ ppm = 174.3 (C_9, C-2), 161.6 (C_9, C-10), 60.2 (CH, C-7), 54.2 (CH_2, C-13), 53.5 (CH, C-12), 49.0 (CH, C-8), 33.4 (CH_2, C-3), 25.5 (CH_2, C-5), 24.4 (CH_2, C-4), 21.1 (CH_2, C-6); HR-MS (ESI): m/z : calcd for C_{10}H_{17}N_{2}O_{5}S: [M+H]^+ = 277.0853; found: 277.0855.

S-11: In a flame-dried round bottom flask biotin sulfone S-10 (200 mg, 0.724 mmol, 1 equiv.), pentafluorophenol (160 mg, 0.869 mmol, 1.2 equiv.) and EDC-HCl (153 mg, 0.796 mmol, 1.1 equiv.) were dissolved in a mixture of THF:CH_2Cl_2:DMF (1:5:1:5, 23 ml) and the solution was stirred under nitrogen. The formation of the new product S-11 was monitored by TLC (eluent: EtOAc/MeOH 9:1). After 20 h ethyl acetate was added to the reaction and the white precipitate slowly started to form in the organic phase. The suspension was filtered and the white solid obtained was washed with cold ethyl acetate. Product S-11 was obtained in 69% yield (217 mg, 0.49 mmol).

^1H NMR (400 MHz, d_6-DM SO, 25°C): δ/ ppm = 6.71 (s, 1H; H-16), 6.61 (s, 1H; H-14), 4.46-4.36 (m, 2H; H-17/H-23), 3.38-3.26 (m, 1H; H-18'), 3.21 (td, J = 7.21, 7.11 Hz, 1H; H-12), 3.03 (d, J = 13.9 Hz, 1H; H-18''), 2.81 (t, J = 7.41 Hz, 2H; H-8), 1.80-1.61 (m, 4H; H-9/H-11), 1.58-1.46 (m, 2H; H-10); ^19F-NMR (376 MHz, d_6-DM SO): δ/ ppm = -153.3 (m, 2F, F-1/F-5), -158.1 (t, J = 23.1 Hz, 1F, F-3), -162.6 (dd, J = 23.4, 19.2 Hz, 2F, F-2/F-4); 13C NMR (125 MHz, d_6-DM SO, 25°C): δ/ ppm = 169.3 (C_9, C-7), 161.4 (C_9, C-15), 139.4 (C-F, J_{CF} = 250.4 Hz), 138.04 (C-F, J_{CF} = 250.2 Hz), 137.2 (C-F, J_{CF} = 250.4 Hz), 124.3 (C-O), 60.0 (CH, C-12), 54.0 (CH_2, C-18), 53.3 (CH, C-17), 48.4 (CH, C-13), 32.0 (CH_2, C-8), 25.0 (CH_2, C-10), 24.0 (CH_2, C-11), 20.9 (CH_2, C-9); HR-MS (ESI): m/z : calcd for C_{16}H_{15}F_5N_{2}O_{5}S: [M+H]^+ = 454.0853; found: 454.0853.

S-12: In a round bottom flask a solution of triethylamine (53 mg, 0.52 mmol, 1.1 equiv) in DMF (2 ml) was added to a solution of Pfp-ester S-11 (199 mg, 0.45 mmol, 1 equiv.) in DMF (3 ml). A solution of oxime S-9 (104 mg, 0.47 mmol, 1.05 equiv.) in DMF (3ml) was added to this mixture and the solution was stirred at room temperature. The reaction was monitored by TLC (eluent: EtOAc/MeOH 1:1, Rf = 0.7, KMnO_4). After 1.5 h
addition of ethyl acetate resulted in the formation of a white precipitate (8), which was filtered off and washed with cold ethyl acetate and cold acetone. The product was obtained as a white solid in 97% yield (0.44 mmol, 209 mg).

$^1$H NMR (400 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 11.43 (s, 1H; H-1), 8.48 (t, $J = 5.66$ Hz, 1H, NH; H-10), 8.19 (s, 1H; H-2), 7.88-7.70 (m, 3H; H-4/H-6/H-10), 7.69-7.63 (m, 2H; H-5/H-7), 6.69 (s, 1H, NH; H-24), 6.59 (s, 1H, NH; H-22), 4.46-4.34 (m, 2H; H-25/H-21), 3.34-3.21 (m, 3H; H-26'/H-11), 3.17 (td, $J = 6.95$, 6.73 Hz, 1H; H-20), 3.10 (td, $J = 6.65$, 6.64 Hz, 2H; H-13), 3.02 (d, $J = 13.86$ Hz, 1H; H-26’’), 2.09 (t, $J = 7.36$ Hz, 2H; H-16), 1.74-1.58 (m, 4H, H-12/H-19), 1.57-1.49 (m, 2H; H-17), 1.47-1.33 (m, 2H; H-18); $^{13}$C NMR (101 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 170.2 (C$_{ar}$, C-15), 165.6 (C$_{ar}$, C-23), 161.6 (C$_{ar}$, C-9), 147.5 (CH, C-2), 135.5 (C$_{ar}$, C-8), 135.0 (C$_{ar}$, C-3), 127.5 (CH, C-4/C-6), 126.1 (CH, C-5/C-7), 60.2 (C-H, C-20), 54.1 (CH$_2$, C-26), 53.4 (CH, C-25), 48.9 (CH, C-21), 37.0 (CH$_2$, C-11), 36.3 (CH$_2$, C-13), 35.0 (CH$_2$, C-16), 29.3 (CH$_2$, C-12), 25.2 (CH$_2$, C-18), 21.0 (CH$_2$, C-19); HR-MS (ESI): m/z : calcd for C$_{21}$H$_{29}$N$_5$O$_6$S: [M+Na]$^+$ = 502.1731; found: 502.1728.

5: In a flame-dried round bottom flask a solution of NCS (30.5 mg, 0.229 mmol, 1.1 equiv.) in DMF (1 ml) was added to a stirred solution of oxime S-12 (100 mg, 0.208 mmol, 1 equiv.) in DMF (1.5 ml) and the temperature was raised to 55°C. After 30 min the reaction was cooled to room temperature, ethyl acetate was added and a white precipitate was formed. The precipitate was filtered off, washed with cold ethyl acetate and provided chlorooxime 5 as a white solid in 96% yield (0.199 mmol, 103 mg).

$^1$H NMR (400 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 12.58 (s, 1H, OH; H-1), 8.57 (m, 1H, NH; H-10), 7.82-7.95 (m, 5H; H-4/H-8/H-14), 6.69 (s, 1H, NH; H-24), 6.59 (bs, 1H, NH; H-22), 4.48-4.25 (m, 2H; H-25/H-21), 3.32 (m, 1H; H-26’), 3.28 (t, $J = 6.47$ Hz, 2H; H-11), 3.17 (td, $J = 6.81$, 6.74 Hz, 1H; H-20), 3.10 (td, $J = 6.61$, 6.59 Hz, 2H; H-13), 3.02 (d, $J = 14.9$ Hz, 1H; H-26’’), 2.09 (t, $J = 7.35$ Hz, 2H; H-16), 1.74-1.60 (m, 4H; H-12/H-19), 1.59-1.47 (m, 2H; H-17), 1.45-1.32 (m, 2H; H-18); $^{13}$C NMR (101 MHz, $d_6$-DMSO, 25°C): $\delta$/ppm = 165.3 (C$_{ar}$, C-15), 162.3 (C$_{ar}$, C-23), 161.5 (C$_{ar}$, C-9), 136.0 (C-Cl, C-2), 134.9 (C$_{ar}$, C-8), 134.8 (C$_{ar}$, C-3), 127.5 (CH$_{ar}$, C-4/C-6), 126.4 (CH$_{ar}$, C-5/C-7), 60.2 (CH, C-20), 54.1 (CH$_2$, C-26), 53.1 (CH, C-25), 48.9 (CH, C-21), 37.0 (CH$_2$, C-11), 36.2 (CH$_2$, C-13), 35.0 (CH$_2$, C-16), 29.2 (CH$_2$, C-12), 25.1 (CH$_2$, C-18), 21.0 (CH$_2$, C-19); HR-MS (ESI): m/z : calcd for C$_{21}$H$_{28}$ClN$_5$O$_6$S: [M+Na]$^+$ = 536.1341; found: 536.1345; FTIR (solid): $\nu = 3286, 3070, 2985, 2854, 1695, 1652, 1564, 1465, 1327, 1129$ cm$^{-1}$. 
In a round bottom flask a solution of triethylamine (7.5 mg, 7.5 µmol, 1.1 equiv) in DMF (0.28 ml) was added to a solution of Pfp-ester S-11 (30 mg, 6.8 µmol, 1 equiv) in DMF (0.45 ml). A solution of dibenzocyclooctyne-amino (DBCO-NH₂) (20 mg, 7.1 µmol, 1.05 equiv) in DMF (0.43 ml) was added to this mixture and the solution was stirred at room temperature. The reaction was monitored by RPLC-MS. After completion, the crude mixture was concentrated in vacuo and purified by reverse phase chromatography (30 % – 7 % Acetonitrile in H₂O, 30 min). The product was obtained as a white solid in 81 % yield (5.4 µmol, 29 mg).

**H NMR** (400 MHz, d₆-DMSO, 25°C): δ/ppm = 7.64-7.30 (m, 9H; H₉), 6.66 (bs, 1H; NH-H-4), 6.59 (bs, 1H, NH; H-1), 5.04 (d, J = 14.1Hz, 1H; H-17), 4.43-4.32 (m, 2H; H-2/H-3), 3.63 (d, J = 14.1 Hz, 1H; H-17'), 3.30 (dd, J = 14.1, 6.9 Hz, 1H; H-6), 3.15-3.07 (m, 2H; H-7/H-8'), 3.01 (d, J = 14.2 Hz, 1H; H-6'), 2.96-2.88 (m, 1H; H-8), 2.41 (ddd, J = 15.1, 8.2, 6.2 Hz, 1H; H-9'), 1.93 (m, 2H; H-14), 1.83 (m, 1H; H-9), 1.64-1.52 (m, 2H; H-10), 1.45-1.37 (m, 1H; H-15), 1.34 (m, 1H; H-11); **C NMR** (101 MHz, d₆-DMSO, 25°C): δ/ppm = 171.7 (Cq, C-12), 170.2 (Cq, C-16), 161.6 (Cq, C-5), 151.4 (Cq, C-31), 148.4 (Cq, C-18) , 132.3 (CH₉), 129.5 (CH₉), 128.9 (CH₉), 128.2 (CH₉), 128.1 (CH₉), 127.7 (CH₉), 126.8 (CH₉), 125.2 (CH₉), 122.4 (CH₉), 121.4 (CH₉), 114.3 (Cq, C-26), 108.1 (Cq, C-23), 60.2 (CH, C-7), 54.8 (CH₂, C-17), 54.2 (CH₂, C-6), 53.4 (CH, C-3), 48.9 (CH, C-2), 34.9 (CH₂, C-14), 34.8 (CH₂, C-9), 34.2 (CH₂, C-8), 25.6 (CH₂, C-11), 24.9 (CH₂, C-15), 20.9 (CH₂, C-10); **HR-MS** (ESI): m/z : calcd for C₂₈H₃₈N₄NaO₅S: [M+Na]+ = 557.1829; found: 557.1825;
3. Chemoselectivity Studies

Chemoselectivity studies were performed with isonitrile 1 (1.0 equiv., 0.53 mM) and chlorooxime 2 (1.2 equiv.) in the presence of different additives (1.0 equiv.) in a 2:3 mixture of THF:PBS (0.005 M, pH 7.4) at room temperature. (Note, the solubility of the reactants did not allow for using a higher amount of water). 1 and the additive were mixed in the appropriate amount of the THF:PBS mixture and 2 was then added. For 1 and 2 stock solutions in THF (3 mM) and for the additives stock solutions in buffer (3 mM) were used. Conversion to product 3 was determined using reverse phase HPLC analysis (UV detection at 214 nm) after three hours by measuring the consumption of isonitrile 1 (red) and the formation of the hydroxyimino amide product 3 (blue) (Figures S1–S13, chlorooxime (green)). Butylhydroxytoluene (BHT) served as an internal standard. HPLC chromatograms were recorded with a gradient from 90-to-45(1min)-to-35(6min) of H2O in acetonitrile at 50°C. Note, chlorooxime 2 decomposes over time in the THF/PBS mixture (Figures S2 and S3).

![Figure S1. Isonitrile 1](image1)

![Figure S2. Chlorooxime 2](image2)

![Figure S3. Chlorooxime 2, after 24h.](image3)

![Figure S4. Crude reaction mixture without additives at pH 7.4.](image4)

![Figure S5. Crude reaction mixture with Ac-Gln-OH at pH 7.4.](image5)

![Figure S6. Crude reaction mixture with H-Trp-OH at pH 7.4.](image6)
**Figure S7.** Crude reaction mixture with H-Ser-OH at pH 7.4.

**Figure S8.** Crude reaction mixture with Boc-Hyp-OMe at pH 7.4.

**Figure S9.** Crude reaction mixture with H-Lys-OH at pH 7.4.

**Figure S10.** Crude reaction mixture with H-His-OH at pH 7.4.

**Figure S11.** Crude reaction mixture with GSH at pH 7.4.

**Figure S12.** Crude reaction mixture with GSH (1.0 equiv) at pH 5.

**Figure S13.** Crude reaction mixture with GSH (5mM) at pH 5.

**Figure S14.** Crude reaction mixture with palmitoleic acid at pH 7.4.
4. Mechanistic Investigations

4.1. Kinetic Studies

The kinetic studies were performed according to the reaction progress kinetic analysis by Blackmond *Angew. Chem. Int. Ed.* 2005, 44, 4302. Isonitrile 1 and chlorooxime 2 were dissolved in a THF/PBS (1:2) and the formation of product 3 was monitored by HPLC analysis of the crude reaction mixture with data points collected every 10 min. The conversion was plotted as a function of time and a 9th order polynomial function was used to fit the data (Figure 2a). Differentiation of this function provided the rate, which was subsequently plotted as rate/[2] against [1], followed by a linear fit to obtain the rate constant (k). The data analyses were performed with OriginLab and Microsoft Excel (16.16.4).

**Experiment A**
- [1] = 1.06 mM
- [2] = 2.24 mM
- THF/PBS = 1:1.5
- 25°C

**Experiment B**
- [1] = 525 μM
- [2] = 1.21 mM
- THF/PBS = 1:2.1
- 25°C

**Experiment C**
- [1] = 525 μM
- [2] = 1.21 mM
- THF/PBS = 1:2.1
- 37°C

*Figure S-15. Top: Conversion to 3 measured by HPLC at different conditions (Experiment A-C). Data points (blue) were fitted with a 9th order polynomial (red line). Bottom, left: Concentration of 1 plotted against the ratio of the reaction rate to the concentration of 2 (Rate/[oxime]) Bottom, right: calculated rate constants for each experiment (A-C).*
Experiment D
Kinetic experiments were also performed in citric buffer (50mM) at pH 5, similarly to entry 13 in Table 1 in the manuscript. These results confirm that the kinetic profile of our reaction is not changed under these conditions.

Experiment D

\[ [1] = 525 \mu M \]
\[ [2] = 121 \text{ mM} \]
THF/citric buffer = 1:1.5
25°C

Exp D: \( k = 0.90 \text{ M}^{-1}\text{s}^{-1} \)

*Figure S-16.* Left: Conversion to 3 measured by HPLC at pH 5 (Experiment D). Data points (blue) were fitted with a 9th order polynomial (red line). Right: Concentration of 1 plotted against the ratio of the reaction rate to the concentration of 2 (Rate/[oxime]).
4.2 Studies on the Nitrile Oxide Intermediate

4.2.1 Trapping Experiments

The formation of the nitrile oxide as an intermediate was probed by [3+2] cycloaddition reaction with a strained alkene and a strained alkyne as shown below.

1. Reaction with Norbornene

\[ \text{N-Hydroxy-4-nitrobenzimidoyl chloride I (1.0 equiv.) and norbornene II (10 equiv.) were dissolved in DMSO:PBS (1:10). The reaction was stirred at r.t. for 2 h. The reaction mixture was diluted with EtOAc and phases were separated. The aqueous phase was extracted once with EtOAc. The combined org. phases were dried over MgSO}_4 \text{ and concentrated in vacuo. The cyclized product III was obtained in quantitative yield and identified by } ^1\text{H- and } ^13\text{C-NMR.} \]

\[ ^1\text{H NMR (400 MHz, CDCl}_3, 25^\circ\text{C): } \delta / \text{ppm} = 8.24 (d, J = 8.9 \text{ Hz, 2H}), 7.87 (d, J = 9.0 \text{ Hz, 2H}), 4.73 (dt, J = 8.4, 1.3, 1.3 \text{ Hz, 1H}), 3.49 (dt, J = 8.4, 1.0, 1.0 \text{ Hz, 1H}), 2.67 (m, 1H), 2.50 (m, 1H), 1.62 (m, 2H), 1.49 (m, 1H), 1.39 (m, 1H), 1.23 (m, 2H); ^13\text{C NMR (101 MHz, CDCl}_3, 25^\circ\text{C): } \delta / \text{ppm} = 155.7 (C_q), 148.3 (C_q), 135.8 (C_q), 127.6 (CH_{ar}), 124.1(CH_{ar}), 89.2 (CH), 56.4 (CH), 43.1 (CH), 39.4 (CH), 32.5 (CH$_2$), 27.5 (CH$_2$), 22.8 (CH$_2$). See spectra in chapter 7.}\]

2. Competition Experiment with Cyclooctyne S-13

This experiment was performed to validate that the formation of the nitrile oxide species is kinetically compatible with the ligation mechanism, both at pH 5 and pH 7.

a) Reaction between 2 and S-13: Chlorooxime 2 (1.0 equiv.) was dissolved in THF and cyclooctyne S-13 (1.0 equiv.) was added subsequently. To this solution was then added PBS (10 mM, pH 7.4) or citric buffer (50 mM, pH 5) in a ratio of 2:1 (buffer:THF). The crude reaction mixture was analyzed by LC-MS.
b) **Competition experiment:** Isonitrile 1 (1.0 equiv.) was dissolved in THF and cyclooctyne S-13 (1.0 equiv.) was added subsequently, followed by chlorooxime 2 (1.0 equiv.). To this solution was then added PBS (5 mM, pH 7.4) or citric buffer (50 mM, pH 5) in a ratio of 2:1 (buffer:THF). The crude reaction mixture was analyzed by LC-MS.

![Chemical structures and reactions](image)

**Figure S17.** Left: UV-LCMS chromatograms of purified product 3 (top), reaction of chlorooxime 2 with cyclooctyne S-13 at pH 7 (middle) and reaction of chlorooxime 2 with cyclooctyne S-13 and isonitrile 1 at pH 7 (bottom). Right: UV-LCMS traces of purified product 3 (top), reaction of chlorooxime 2 with cyclooctyne S-13 at pH 5 (middle) and reaction of chlorooxime 2 with cyclooctyne S-13 and isonitrile 1 at pH 5 (bottom).
4.2.3 Study of the Half-life of the Chlorooxime under Reaction Conditions

A series of experiments was conducted by adding the isonitrile 1 (1.0 equiv.) at different time points to a premixed solution of chlorooxime 2 (1.0 equiv.) in THF/PBS.

<table>
<thead>
<tr>
<th>time point</th>
<th>conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 min</td>
<td>&gt;95</td>
</tr>
<tr>
<td>120 min</td>
<td>90</td>
</tr>
<tr>
<td>240 min</td>
<td>81</td>
</tr>
<tr>
<td>360 min</td>
<td>74</td>
</tr>
<tr>
<td>480 min</td>
<td>62</td>
</tr>
<tr>
<td>600 min</td>
<td>44</td>
</tr>
</tbody>
</table>

The conversion to the ligation product 3 depended on the delay of the addition of the isonitrile 1. These results further support the expected instability of the nitrile oxide intermediate over a longer period of time.

4.3 Stability of the Isonitrile 1 in the Presence of Glutathione

We tested the stability of isonitrile 1 in the presence of glutathione by mixing 1 with 1.0 equiv. GSH in THF:PBS (2:3 (HPLC), 1:1 (NMR)) at pH 7.4 and monitored the reaction mixture by HPLC analysis and $^1$H NMR spectroscopy at different time points (5 min – 12 h). The HPLC measurements were performed in the absence and in the presence of BHT. The obtained results show that isonitrile 1 does not react with glutathione and that BHT does not influence the stability of isonitriles in the presence of redox active species such as GSH.

![Stacked $^1$H NMR spectra (D$_2$O:THF-d$_8$ (1:1)) of glutathione (top), isonitrile 1 (middle) and a 1:1 mixture of glutathione and isonitrile 1 (bottom), after 5 h. Right: HPLC chromatogram of a 1:1 mixture of GSH and isonitrile 1 after 5 min (left) and 12 h (right), in the absence (top) and in the presence (bottom) of BHT. NMR spectra are referenced to THF-d$_8$ indicated by the asterix.](image)

Figure S18. Left: Stacked $^1$H NMR spectra (D$_2$O:THF-d$_8$ (1:1)) of glutathione (top), isonitrile 1 (middle) and a 1:1 mixture of glutathione and isonitrile 1 (bottom), after 5 h. Right: HPLC chromatogram of a 1:1 mixture of GSH and isonitrile 1 after 5 min (left) and 12 h (right), in the absence (top) and in the presence (bottom) of BHT. NMR spectra are referenced to THF-d$_8$ indicated by the asterix.
5. **Cell Experiments**

5.1 **Flow Cytometry**: CHO-K1 cells were seeded at a density of 100’000 cells/well in 24-well plates and grown in the presence of sugar 4 (0–300 μM) for 3 days in F-12K medium (1 ml, 10% FCS) at 37 °C. Cells were then washed with PBS (3 x 250 μL) and incubated with 10 μM probe solution in PBS (500 μL) for 5 min at 25 °C. Afterwards cells were washed with PBS (3 x 250 μL) and incubated with Avidin-Alexa Fluor 488 (20 μg/ml) for 20 min at 4 °C. The cells were then washed with PBS (3 x 250 μL) and treated with trypsin-EDTA (100 μL, 0.05%/0.02%) for 5 min at 37°C. Subsequently PBS (300 μL, 4 °C) was added, cells were gently suspended and centrifuged at 1’500 rpm for 5 min. The supernatant was carefully removed and the pellet re-suspended in solution of 1.5 μM propidium iodide (PI) and 2 mM ethylenediaminetetraacetic acid (EDTA) in PBS (400 μL) at 4 °C. The cell suspensions were placed in FACS tubes and kept on ice prior to analysis. Alexa Fluor 488 was excited at 488 nm and monitored with 530/30 bandpass filters on a BDLSFRortessa flow cytometer. Events corresponding to cellular debris were removed by gating on forward and side scatter. Each sample was run in triplicate (3 x 10'000 events) at the day of measurement and the entire experiment was repeated at least three times.

5.2 **Labeling with 4**: Prior to each measurement, the sugar solution was freshly prepared as a 1 mM stock solution in F-12K medium. The probe solution was prepared as a 5 mM stock solution in DMSO. CHO-K1 cells were seeded at a density of 10’000 cells/well in ibidi 8-well plates and grown in the presence of sugar 4 (0–300 μM) for 3 days in F-12K medium (10% FCS) at 37 °C. Afterwards cells were washed with PBS (3 x 250 μL) and a solution of probe 5 in PBS (10 μM, 200 μL) was added and incubated for 5 min at 25 °C. Cells were then washed with PBS (3 x 200 μL) and incubated with Hoechst33342 (100 μL, 2 μM in DMEM) for 5 min at 37 °C. Cells were washed with PBS (3 x 200 μL) and incubated with Avidin-Alexa Fluor 488 (20 μg/ml in DMEM) for 20 min at 4 °C. Afterwards, cells were washed with PBS (3 x 200 μL) and live cell imaging solution (200 μL) was added. The live cells were then immediately examined on the confocal microscope (Vistron Spinning Disk).

5.3 **Dual Labeling with 4 and 4a**: CHO-K1 cells were seeded at a density of 10’000 cells/well in ibidi 8-well plates and grown in the presence of the isonitrile sugar 4 (300 μM) and azide sugar 4a (50 μM) for 3 days in F-12K medium (10% FCS) at 37 °C. Afterwards cells were washed with PBS (3 x 200 μL) and incubated with DIBO-Alkyne 647 (10 μM in DMEM) for 30 min at 37 °C. Cells were then washed with PBS (3 x 200 μL) and incubated with a solution of probe 5 in PBS (10 μM, 200 μL) for 5 min at 25°C. Cells were then washed with PBS (3 x 200 μL) and incubated with Hoechst33342 (100 μL, 2 μM in DMEM) for 5 min at 37°C. Cells were washed with PBS (3 x 200 μL) and incubated with Avidin-Alexa Fluor 488 (20 μg/ml in DMEM) for 20 min at 4 °C. Afterwards, cells were washed with PBS (3 x 200 μL) and live cell imaging solution (200 μL) was added. The live cells were then immediately examined on the confocal microscope (Vistron Spinning Disk).

5.4 **MTT assay**: One day before the experiment ~10’000 cells per well, suspended in F-12K medium (10% FCS), were seeded into 96-well plates and allowed to adhere overnight. Cells were washed once with PBS (200 μL) and incubated with 4 (stock solution in DMEM, 1 mM) or 5 (stock solution in DMSO, 40 mM, V = 129 μL) at 0–1000 μM in F-12K medium (10% FCS) for 3 d at 37°C (4) or 1 h at 37°C (5). Afterwards, cells were washed with PBS (3 x 100 μL) and the MTT solution (100 μL, 5mg/ml in PBS) was added. The cells were incubated for 3 h at 37°C.
The supernatant was discarded and DMSO (200 μL) was added. Samples were agitated and the absorbance measured after 30 min with the microplate reader at 570 nm. Each measurement was repeated in triplicate.

Figure S1.9. Cell viability of CHO K1 cells in the presence of 5 (0-1000 μM) after 1h at 37°C. Error bars represent standard deviation of three repeats.

5.5 Residual Reactivity of Probe 5:

To evaluate the origin of the residual background labeling of the cell membrane in the absence of isonitrile-containing glycoproteins, we performed cell experiments to investigate the possible reactivity of extracellular thiols with the chlorooxime probe 5.

The labeling experiments were done either at acidic pH (citric buffer, pH 5) or at neutral pH after a capping protocol using Ellman’s reagent.

a) Ellman’s reagent: Prior to the experiment, CHO-K1 cells were seeded at a density of 100’000 cells/well in 24-well plate and incubated for 3 days in F-12K medium (1 ml, 10% FCS) at 37 °C. Cells were washed with PBS (3 x 250 μL) and incubated with Ellman’s reagent (5,5-dithio-bis(2-nitrobenzoic acid, 1.2 mM) in 500 μL DMEM for 30 minutes. Afterwards, cells were washed with PBS (3 x 250 μL) and incubated with 10 μM probe 5 solution in PBS (500 μL) for 5 min at 25 °C. Afterwards cells were washed with PBS (3x 250 μL) and incubated with Avidin-Alexa Fluor 488 (20 μg/ml) for 20 min at 4 °C. The cells were then washed with PBS (3 x 250 μL) and subjected to the procedure described for flow cytometry.

b) pH 5: Prior to the experiment, CHO-K1 cells were seeded at a density of 100’000 cells/well in 24-well plate and incubated for 3 days in F-12K medium (1 ml, 10% FCS) at 37 °C. Cells were washed with PBS (3 x 250 μL) and incubated with 10 μM probe 5 solution in pH 5.0 citric buffer (500 μL) for 5 min at 25 °C. Afterwards cells were washed with PBS (3x 250 μL) and incubated with Avidin-Alexa Fluor 488 (20 μg/ml) for 20 min at 4 °C. The cells were then washed with PBS (3 x 250 μL) and subjected to the procedure described for flow cytometry.

Figure S20. Flow cytometry results comparing unspecific background labeling when treating CHO K1 cells with probe 5 either at acidic pH (citric buffer, light grey), at neutral pH after a capping protocol using Ellman’s reagent (black) or at standard conditions (dark grey).

As shown in the graph above, both conditions reduced the background labeling. This finding suggests that extracellular thiolates are likely the nucleophilic species causing the background labeling.

5.6 Comparison to SPAAC

While the SPAAC ligation has been proved to be an excellent tool for cell membrane imaging, the isonitrile-chlorooxime ligation presented within this work offers a new, complementary possibility for these purposes. The experiment below aimed at defining the relative efficiency in labeling and imaging of glycoproteins using the two different protocols.

Figure S21. Flow cytometry data comparing imaging glycoproteins of cell membranes with SPAAC (grey) and isonitrile-chlorooxime ligation (black).

CHO-K1 cells were seeded at a density of 100’000 cells/well in 24-well plates and grown in the presence of isonitrile sugar 4 (0–300 μM) or azide sugar 4a (50 μM) for 3 days in F-12K medium (1 ml, 10% FCS) at 37 °C. Cells were then washed with PBS (3 x 250 μL) and incubated either with 10 μM probe 5 solution in PBS (500 μL) or 10 μM biotin-alkyne S-13 solution in PBS for 5 min at 25 °C. Afterwards cells were washed with PBS (3x 250 μL) and incubated with Avidin-Alexa Fluor 488 (20 μg/ml) for 20 min at 4 °C. The cells were then subjected to the procedure described for flow cytometry.
6. Additional Confocal Microscopy Images of Dual Labeling Experiment

*Figure S22*: Confocal microscopy images of CHO K1 cells grown in medium containing 4 (300 μM)/4a (50 μM) for 3 days, labeled with first DIBO-AlexaFluor647 (red, middle) and then probe 5/avidin AlexaFluro488 (green, left) and overlay (right). All cell images are overlaid with the blue channel (Hoechst 33342 nuclei stain). Scale bar 20 μm.

*Figure S23*: Confocal microscopy images of CHO K1 cells grown in medium in the absence of 4 and 4a for 3 days and treated the same as those shown in Figure S14 with DIBO-AlexaFluor647 (middle) and probe 5/avidin AlexaFluro488 (left) and overlay (right). All cell images are overlaid with the blue channel (Hoechst 33342 nuclei stain). Scale bar 20 μm.
7. Western Blot Analysis of Cell Lysates

CHO-K1 cells were seeded at a density of 200'000 cells/well in 24-well plates and grown in the presence of sugar 4 (300 µM) or without additional sugar for 3 days in F-12K medium (1 ml, 10 % FCS) at 37 °C. Cells were then washed with PBS (3 x 250 µL), trypsinated and re-suspended in PBS (400 µL) and pelleted by centrifugation (5 min, 1500 rpm). The supernatant was discarded and the pellet re-suspended in PBS (400 µL) and the resulting suspension pelleted by centrifugation (5 min, 1500 rpm). The cells were lysed in lysis buffer (400 µL; stock: Nuclei Pure Lysis Buffer (10 mL) containing 100 µL TRITON X-100 (10%) and 10 µL dithiothreitol (1 M)) and incubated at 4°C for 30 min. The lysate was cleared by centrifugation (10000 rpm, 10 min, 4°C). Biotinsulfon probe 5 (5 mM in DMSO) was added to the cleared supernatant (1600 µL) to a final concentration of 150 µM. The samples were incubated for 5 min at 25°C. Five different dilutions (V_\text{tot} = 10 µL) were prepared from both the sample without and with sugar 4 (0.15 (A), 0.3125 (B), 0.625 (C), 1.25 (D), 2.5 (E) µM). To these dilution series, 2 X SDS sample buffer (10 µL) was added, and each sample was boiled at 90°C for 15 min. Proteins were separated using SDS-polyacrylamide gel electrophoresis using 8-16 % polyacrylamide gels (BioRad) and transferred to nitrocellulose membranes (BioRad). The membranes were washed with TBS-T and incubated with blocking buffer (5 % Skin milk Powder in TBS-T containing 1 % Triton X-100) for 1h at rt, followed by avidin AlexaFluor 488 (5 mL; 0.5 µg/mL in TBS-T with 1 % BSA) for at least 1h at rt. The nitrocellulose membranes were subsequently washed with TBS-T (3x 10min) and imaged using BioRad fluorescence imaging system ChemiDoc.

![Western Blot Analysis of Cell Lysates](image)

**Figure S24:** Western blot analysis of cell lysate containing 4 (300 µM) (right) and without 4 (left). A-E: different concentrations (0.15 (A), 0.3125 (B), 0.625 (C), 1.25 (D), 2.5 (E) µM) of 5, along with an increasing cell lysate concentration (left-right). The first lane shows the protein marker.

Labeling was observed in the samples containing sugar 4 (indicated by arrows) and also those not containing 4 (left, column C-E). The decreased intensity in case of the cell lysate containing 4 is likely due to an overall smaller number of collected cells after trypsination.
8. $^1$H NMR, $^{19}$F NMR and $^{13}$C NMR Spectra

S-2

[Chemical structure and spectra]
$S-6\ (^{13}C\ NMR,\ d_6\text{-DMSO})$
$S-7$