Supporting information

Thio-linked UDP-peptide conjugates as O-GlcNAc transferase inhibitors

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Instrumentation and general methods

Solvents and reagents were purchased from Sigma Aldrich, Acros Organics, TCI UK, Carbosynth, Alfa Aesar, and VWR and used as delivered without additional purification.

Reactions required inert atmosphere were performed in oven dried glassware filled with Ar by three cycles of evacuation-filling of the reaction apparatus connected to the vacuum/ inert gas manifold while hot. Reactions were monitored by thin layer chromatography on TLC Silica gel 60 F_{254} aluminium sheets (Merck KGaA, Darmstadt, Germany) cut to 4 cm length, elution path 3.5 cm. The spots were visualised by dipping TLC plates into one of the reagents listed below and charring with a heat gun at 250-300 °C; cerium ammonium phosphomolibdate (4 g of ammonium pentamolybdate, 0.16 g of cerium (IV) sulfate in 100 mL of water-H_2SO_4 (9:1 v/v), orcinol (0.18 g of orcinol in 100 mL of EtOH-water (1:1)-H_2SO_4 (9:1 v/v), or acidic $KMnO_4$ (3 g $KMnO_4$, in 100 mL of 0.2 % H_2SO_4 in water). The generic extractive work-up to remove basic concomitants consisted of consecutive washing of the crude reaction mixtures diluted in ethyl acetate with water, 1M HCl solution, water, and a 1:1 mixture of brine and concentrated $NaHCO_3$ solution. For removal of acidic impurities 1M HCl wash was omitted. In case of emulsification, the layers were separated either by filtering through a pad of Celite, or centrifugation at 3g. The aqueous layers were back extracted with a single fresh portion of ethyl acetate. The organic layers were dried by filtration through a pad of anhydrous Na_2SO_4 and concentrated at rotary evaporator (bath temperature 40 °C. Flash column chromatography was performed on silica Redisep*Rf* cartridges (Teldyne Isco, USA) using appropriate gradients; solvent delivery system consisted of two C-601 pumps, pump manager C-615 (Buchi, Switzerland), and a Gilson 204 (Gison, USA) fraction collector. HPLC separations were performed on a Waters Peptide Separation Technology column, 5 µM, 130 Å, 19×100 mm (Waters, USA), using appropriate gradients. The automated HPLC system consisted of Gilson 321/322 gradient solvent delivery module, Gilson 156 UV/VIS detector, Gilson 506C system interface, and Gilson 203 fraction collector was controlled by Gilson Trilution LC software.

The LC-MS analysis was performed with an Agilent 1200 LC-MS system (Agilent Technologies, USA) fitted with a Max-Light Cartridge flow cell coupled to a 6130 Quadrupole spectrometer, controlled by MS Chemstation software. Agilent ZORBAX Eclipse Plus C18, 3.5 µm, 4.6×100 mm, or ZORBAX 300SB-C3, 5 µm, 2.1×150 mm columns were use appropriately. Elution was performed with a linear 5-95% gradient of buffer B (0.04 % trifluoroacetic acid (TFA) in MeCN)

in buffer A (0.05% TFA in water) at 0.3 mL/min. UV absorbance was monitored at 214 and 280 nm, MS acquisitions were carried out in positive ion modes.

NMR spectra were recorded on Bruker AVANCE II 500 or Bruker Ascend 400 spectrometers (Bruker Corporation, USA). Spectra were processed using Bruker Topspin 3.2 or Mnova 8.1 (Mestrelab research SL, Spain) software. Chemical shifts (δ) are reported in ppm. The multiplet splitting patterns are designated as follows: s, singlet; bs, broad singlet; d, doublet; t, triplet; m, multiplet.

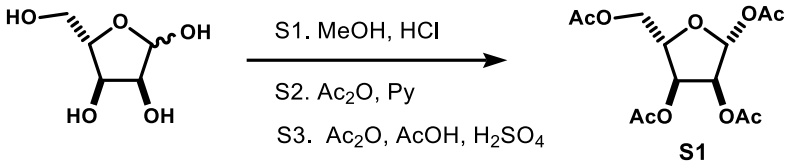
General procedure for Fmoc SP peptide synthesis

Microwave-assisted solid phase peptide synthesis was performed on CEM Liberty automated peptide synthesizer on Rink amide MBHA resin (Novabiochem). Standard amino building blocks and *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were supplied by Activotec. Instrument specific Fmoc chemistry protocols were used throughout. Fmoc deprotection was performed with 20% piperidine-DMF solution at 70 °C initially for 30 sec, and then with the fresh portion of the deprotection reagent for 3 min. Couplings were performed with 5 eqs of an amino acid and 5.5 eqs of HBTU relative to the theory resin load in the presence of 10 eq DIPEA (1M stock solution in N-methyl-2-pyrrolydone) at 70 °C for 5 min. Coupling of Ser and Cys blocks was performed at 50 °C for 10 min. After final Fmoc deprotection peptides were N-terminal acetylated with 20% Ac_2O-DMF at 70 °C for 5 min unless stated otherwise. The peptides were cleaved from the resin and deprotected with i-Pr_3SiH-H_2O-TFA 2.5:5:92.5 mixture for 2 h. The cleavage mixture was collected in a 50 mL Falcon tube and concentrated on a rotary evaporator down to 1-2 mL. Crude peptides were obtained after dilution of the reduced cleavage mixture with diethyl ether and centrifugation. Final purification was performed by reverse-phase HPLC in a linear gradient of buffer B (0.1% TFA in MeCN) in buffer A (0.1% TFA in water) typically 5% to 30% in 5 min, flow rate 20 mL min^{-1}. Detection was at double wavelength 214 and 220 nm. Appropriate fractions were pooled and freeze-dried to provide the target compounds as fluffy powders. Integrity and the purity of the peptides was confirmed by LC_MS ESI(+) analysis.

Photo thiol-ene conjugation reactions. General remarks.

The small scale reactions were performed in single use polypropylene spectrophotometer cuvettes (0.07-0.85 mL, Brandt) positioned immediately in front of a hand held 8W Camag 366 nm TLC reader lamp horizontally mounted on the bench and equipped with improvised aluminium foil blinds to prevent accidental UV light exposure. Larger scale reactions were performed using the same experimental layout in quartz (5 mL) cuvettes. The reaction mixtures were degassed immediately before the irradiation by bubbling argon via glass capillary inserted into the cuvette for 5 min. Depending on the solubility of the substrate peptide water, 0.1M acetate (pH 4), and 6MGn·HCl−0.2M phosphate (pH 7.5) buffer could be used as the reaction media.

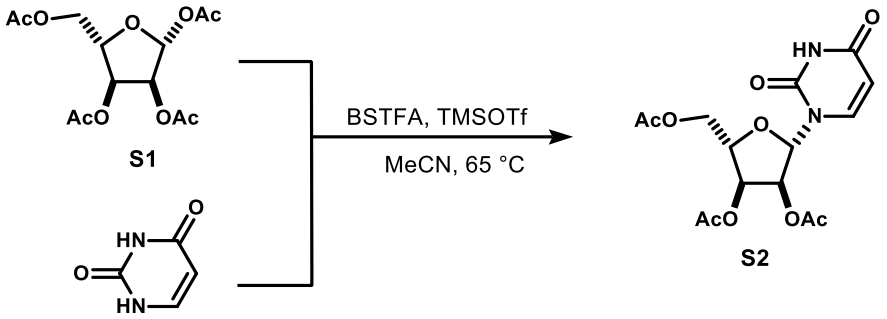
Synthetic procedures and spectral data for selected compounds



Scheme S1. Synthesis of 1,2,3,5-tetra-*O*-acetyl-β-*L*-ribofuranose (**S1**).

L-ribose (5.0 g, 33.33 mmol) was mixed with 0.2 M HCl-MeOH solution at 0 °C. The reaction was stirred at RT for 4 h and the clear solution was neutralized with Amberlite IRA-400 (OH^-) anion exchange resin. The resin was filtered off and the filtrate was concentrated. The residue was briefly dried in vacuum, dissolved in pyridine (25 mL) and solution was cooled down to 0 °C (ice-bath). Then, acetic anhydride (Ac_2O; 10 mL, 105 mmol) was added dropwise and the reaction was stirred at RT for 16 h. The reaction was quenched by addition of MeOH (1 mL) and stirred for 30 min. The reaction was concentrated and the residue was partitioned between EA (40 mL) and 1M HCl (40 mL). The layers were separated. The organic layer was successively washed with water and a mixture of $NaHCO_3$ saturated aqueous solution and brine. The aqueous layers were back extracted with EA (40 mL). The combined organic layer was dried and

concentrated to give 9.5 g of the crude intermediate product as a syrup. This was dissolved in a mixture of glacial acetic acid (30 mL) and Ac_2O (7.5 mL) and the solution was cooled down to 0 °C (ice-bath). Concentrated H_2SO_4 (2.1 mL) was then added dropwise and the reaction was stirred at room temperature for 16 h and poured on crushed ice (50 g). After ice melted, water (50 mL) and EA (50 mL) was added and the layers were separated. The organic layer was successively washed with water and a mixture of $NaHCO_3$ saturated aqueous solution and brine. The aqueous layers were back extracted with EA (40 mL). The combined organic layer was dried and concentrated to give 9.5 g of the crude product as a syrup. This was co-evaporated with toluene (2×20 mL) and dissolved in ethyl ether (20 mL). The solution was kept in a freezer at -18 °C for 16 h. The crystalline deposit was filtered off, washed with hexane-ether (2:1, 50 mL) and dried to give 5.5 g (17 mmol, 52%) of the target product **S1**. m.p. 81-83 °C.
Spectral data were in agreement with the published previously. *J. Med. Chem.* 2000, **43**, 1019



Scheme S2. Synthesis of 2′,3′,5′-O-triacetyl-*L*-uridine (**S2**)

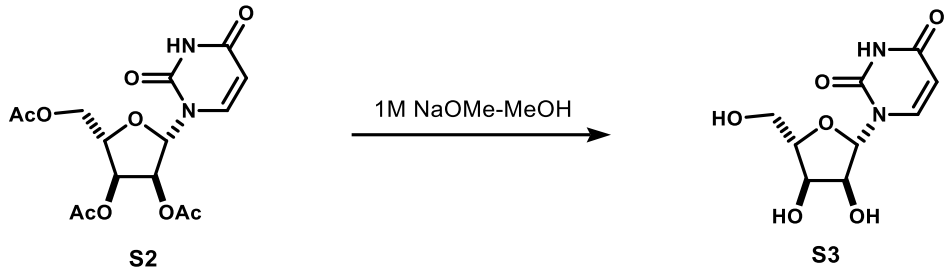
A mixture of 1,2,3,5-tetra-*O*-acetyl-β-*L*-ribofuranose (**S1**; 0.955 g, 2 mmol) and uracil (0.672 g, 6 mmol) was co-evaporated with MeCN (2×15 mL) and dissolved in a fresh portion of MeCN (30 mL). N,O-(bistrimethylsilyl)acetamide (1.48 mL, 6 mmol) was then added and the reaction flask was placed onto preheated (65 °C) oil-bath. The reaction was stirred for 45 min to result in formation of a clear solution. Trimethylsilyl trifluoromethanesulfonate (TMSOTf; 0.543 mL, 3 mmol) was then added and the reaction was stirred at 65 °C for 16 h. The reaction was cooled down, diluted with EA and washed successively with water and a mixture of saturated $NaHCO_3$ solution and brine. The aqueous layers were back extracted with EA. The combined organic layer was dried and concentrated. The residue was purified by flash column chromatography in

a gradient of [PE-DCM 4:1]-Me_2CO 5-30% to give 0.63 g (1.7 mmol, 85%) of the target compound as white amorphous solid.

$[\alpha]_D$ = −13.3° *c* 1.0 $CHCl_3$

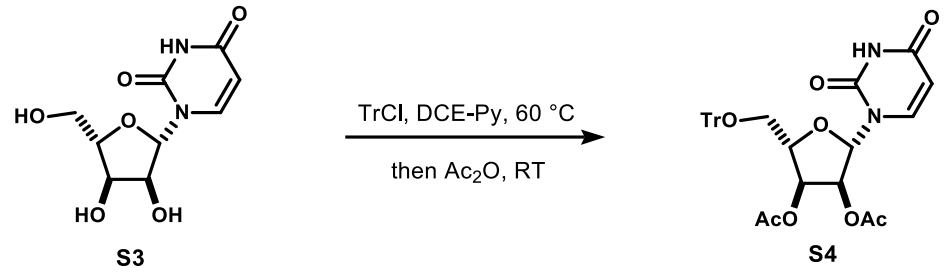
^{1}H NMR (500 MHz, $CDCl_3$) δ 9.16 (s, 1H), 7.41 (d, *J* = 8.2 Hz, 1H), 6.06 (d, *J* = 5.0 Hz, 1H), 5.81 (dd, *J* = 8.1, 2.1 Hz, 1H), 5.42 – 5.30 (m, 2H), 4.43 – 4.31 (m, 3H), 2.16 (s, 3H), 2.15 (s, 3H), 2.12 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 170.2, 169.68, 169.67, 163.1, 150.4, 139.4, 103.5, 87.5, 79.9, 72.7, 70.2, 63.2, 20.8, 20.5, 20.4.



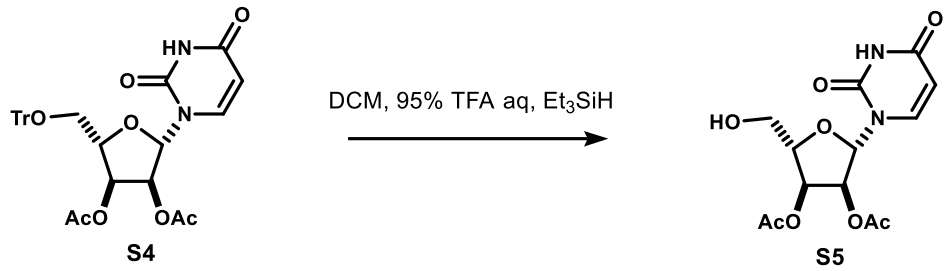
Scheme S3. Preparation of *L*-uridine (**S3**)

To a solution of 2′,3′,5′-O-triacetyl-*L*-uridine (**S2**; 0.616 g, 1.66 mmol) in MeOH (15 mL) freshly prepared 1M sodium methylate (NaOMe) solution in MeOH (0.17 mL, 0.17 mmol) was added at RT. The reaction was stirred for 2 h and neutralized with Dowex 8×50(H^+) ion exchange resin. The resin was filtered off and the filtrate was concentrated to give 0.375 g (1.5 mmol, 90%) of crude *L*-uridine (**S3**).



Scheme S4. Preparation of 2′,3′-O-diacetyl-5′-O-triphenylmethyl-*L*-uridine (**S4**)

A suspension of *L*-uridine (**S3**; 0.375 g, 1.5 mmol) and triphenylmethyl chloride (TrCl; 0.56 g, 2 mmol) in 1,2-dichloroethane/pyridine 1:1 (8 mL) was heated to 60 °C and stirred for 16 h. The reaction was cooled down to RT, then acetic anhydride (Ac_2O, 0.34 mL, 3.6 mmol) was added dropwise and the reaction was further stirred at RT for 16h. The reaction mixture was quenched by addition of MeOH and stirred for 1h. The reaction was concentrated, the residue was partitioned between EA and 1M HCl and the layers were separated. The organic layer was washed successively with water and a mixture of saturated $NaHCO_3$ solution and brine. The aqueous layers were back extracted with EA. The combined organic layer was dried and concentrated to give of the intermediate product **S4** as white foam.



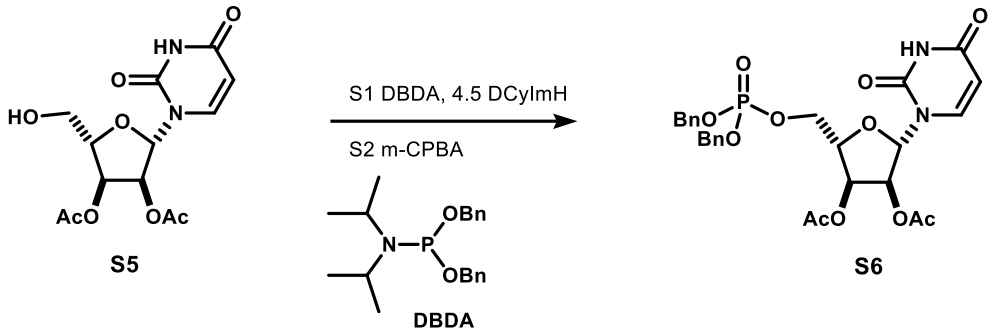
Scheme S5. Synthesis of 2′,3′-O-diacetyl-*L*-uridine (**S5**)

To a solution of the above residue in DCM (15 mL) 95% aqueous trifluoroacetic acid (TFA; 1 mL) was added at RT; the colour of solution turned dark yellow. Triethyl silane (Et_3SiH; 1 mL) was added dropwise over 5 min to result in mild exothermic reaction and produce very pale yellow solution at the end of addition. The reaction was stirred for 20 min, diluted with toluene and concentrated. The residue was dissolved in a mixture of $CHCl_3$ and toluene and concentrated to give off white amorphous residue. The residue was purified by flash chromatography in DCM-EA 0-100% gradient to give 0.488 g (1.48 mmol, nearly quant) of the target product as foam.

$[\alpha]_D$ = +6.5° *c* 1.0 $CHCl_3$

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.2 (s, 1H), 7.76 (d, 1H, J = 8.2 Hz), 6.1-6.05 (m, 1H), 5.78 (dd, 1H, J= 8.2, 1.8 Hz), 5.49-5.45 (m, 2H), 4.21 (q, 1H, J = 2.2 Hz), 3.94 (dd, 1H, J = 12, 2.2 Hz); 3.86 (dd, 1H, J = 12.1, 2.2 Hz), 2.13 (s, 3H), 2.08 (s, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3, 169.9, 163.3, 150.7, 141, 103.4, 87.7, 83.6, 73.1, 71.4, 62.0, 20.8, 20.6.



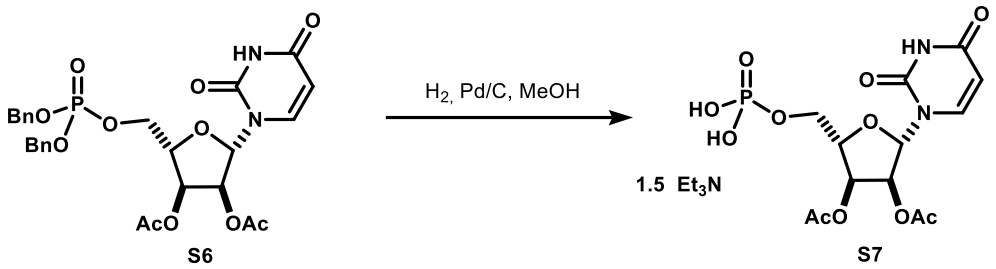
Scheme S5: Synthesis of 2′,3′-O-diacetyl-*L*-uridine 5'-dibenzyl phosphate (**S6**)

To a solution of 2′,3′-O-diacetyl-*L*-uridine (**S5**; 0.488 g, 1.48 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (0.625 g, 1.81 mmol) in MeCN (8 mL) 4,5-dicyanoimidazole (DCIm; 0.213 g, 1.81 mmol) was added at RT and the reaction was stirred at RT for 1 h. The reaction mixture was cooled down to 0 °C and 3-chloro perbenzoic acid (m-CPBA; 0.446 g; 1.81 mmol) was added. The reaction was further stirred for 30 min, quenched by addition of 20% solution of $Na_2S_2O_5$ (3 mL), stirred for 30 min, removed from the cooling bath and diluted with EA. The layers were separated; the organic layer was washed successively with water and a mixture of saturated $NaHCO_3$ solution and brine. The aqueous layers were back extracted with EA. The combined organic layer was dried and concentrated. The residue was purified by flash chromatography in a gradient of [PE-DCM 4:1]-EA 10-40% to give 0.81 g (1.38 mmol; 92%) of the target product as foam.

^{1}H NMR (500 MHz, $CDCl_3$) δ 9.30 (s, 1H), 7.45 (d, *J* = 8.1 Hz, 1H), 7.43 – 7.31 (m, 9H), 6.15 (d, *J* = 6.5 Hz, 1H), 5.53 (d, *J* = 8.2 Hz, 1H), 5.34 (dd, *J* = 5.9, 3.2 Hz, 1H), 5.22 (t, *J* = 6.2 Hz, 1H), 5.18 – 4.99 (m, 4H), 4.31 – 4.14 (m, 3H), 2.14 (s, 3H), 2.10 (s, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ 169.7, 162.8, 150.5, 139.3, 135.2, 129, 128.8, 128.2, 128.1, 103.5, 86.1, 80.84 (d, $J_{C\text{-}P}$ = 8.8 Hz), 77.3, 77.1, 72.4, 70.00 (d, $J_{C\text{-}P}$ = 5.3 Hz), 69.97, 66.30 (d, $J_{C\text{-}P}$ = 5.3 Hz), 20.5, 20.4.

^{31}P NMR (202 MHz, $CDCl_3$) δ -0.92.



Scheme S6. Synthesis of 2′,3′-O-diacetyl-*L*-uridine 5'-phosphate (**S7**)

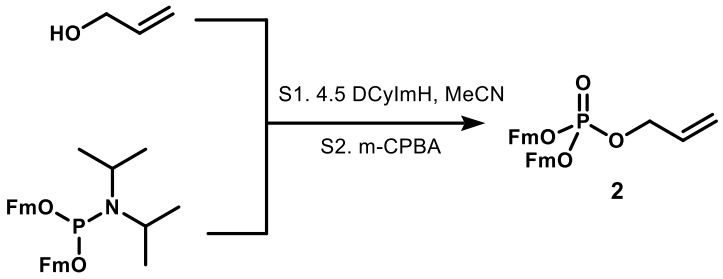
A solution of 2′,3′-O-diacetyl-*L*-uridine 5'-dibenzyl phosphate (**S6**; 0.285 g, 0.48 mmol) in MeOH (6 mL) was stirred under slight overpressure of hydrogen gas (H_2) in the presence of 10% palladium on charcoal (0.06 g) for 1h at RT. The reaction mixture was transferred into a centrifuge tube (MeOH, 15 mL total volume) and centrifuged for 5 min. The supernatant was withdrawn by the syringe and filtered through a syringe filter into a receiving flask. The sediment was re-suspended in MeOH (7 mL) and centrifuged again. The supernatant was withdrawn and filtered. Triethylamine (Et_3N; 0.5 mL) was added to the receiver flask and the combined filtrate was concentrated and dried in vacuum to give 0.256 g (0.46 mmol, 95%) of the target product as hard foam, assumed 1.3×Et_3N salt.

^{1}H NMR (500 MHz MeOD-d_4) δ 8.42 (s, 1H), 7.98 (d, *J* = 8.2 Hz, 1H), 6.21 (d, *J* = 7.0 Hz, 1H), 5.81 (d, *J* = 8.1 Hz, 1H), 5.45 (dd, *J* = 5.5, 2.3 Hz, 1H), 5.34 (dd, *J* = 7.0, 5.5 Hz, 1H), 4.11 (ddd, *J* = 11.6, 4.3, 2.3 Hz, 1H), 4.06 (ddd, *J* = 11.6, 4.9, 2.2 Hz, 1H), 2.11 (s, 3H), 2.03 (s, 3H).

There were additional signals at δ 3.06 (q, *J* = 7.3 Hz, 8H) and 1.28 (t, *J* = 7.3 Hz, 12H) corresponding to 1.3 molecule of Et_3N present in the dried compound.

^{13}C NMR (500 MHz MeOD-d_4) δ 171.5, 171.2, 166.0, 142.5, 103.9, 87.5, 83.9, 83.8, 74.6, 73.1, 65.8, 20.7, 20.4.

Additional signals from Et_3N δ 48.6, 10.3

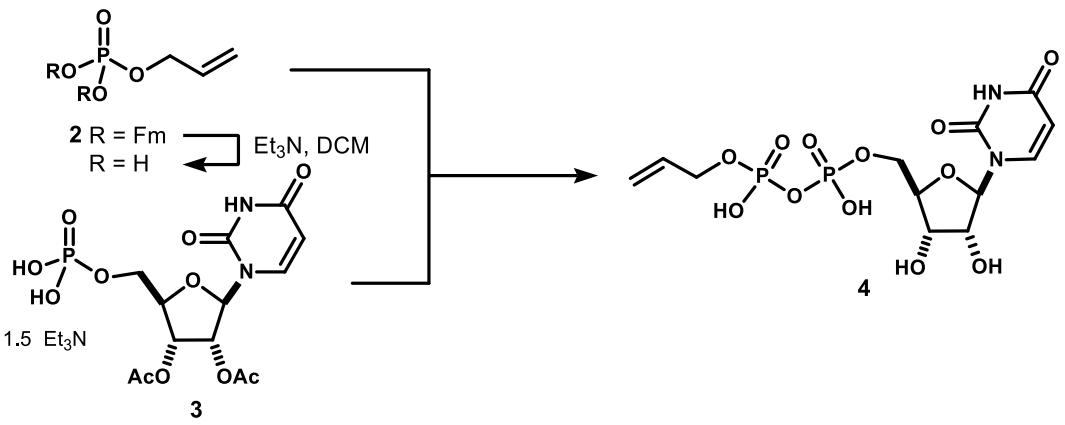


Scheme S7. Synthesis of allyl bis-fluorenemethyl phosphate **2.**

To a solution of allyl alcohol (0.184 mL, 2.54 mmol) and bis((9H-fluoren-9-yl)methyl) diisopropylphosphoramidite (0.71 g, 1.36 mmol) in MeCN (15 mL) 4,5-dicyanoimidazole (0.16 g, 1.36 mmol) was added at RT in one portion and reaction was stirred for 20 min. The reaction mixture was cooled down to 0 °C and 3-chloro perbenzoic acid (0.335 g; 1.36 mmol) was added in one portion. The reaction was further stirred for 30 min. The reaction was quenched by addition of aqueous 10% $Na_2S_2O_5$ solution (20 mL) and vigorously stirred for 30 min. The reaction was mixed with EA and transferred into a separating funnel; the layers were separated. The organic layer was successively washed with water and a mixture of saturated aqueous $NaHCO_3$ solution and brine. The aqueous layers were back extracted with EA. The combined organic layer was dried and concentrated. The residue was purified by flash column chromatography in [PE-DCM 4:1]-EA 10-25% to give 0.576 g (1.17 mmol, 85%) of the target product as clear oil.

LC_MS ESI(+) m/z = 495.35, $C_{31}H_{28}O_4P$; expected for $[M+H]^+$ m/z = 495.1725

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (ddt, *J* = 8.8, 8.0, 0.9 Hz, 4H), 7.56 (ddq, *J* = 19.8, 7.6, 0.9 Hz, 4H), 7.47 – 7.34 (m, 4H), 7.34 – 7.22 (m, 5H), 5.93 – 5.76 (m, 1H), 5.33 – 5.14 (m, 2H), 4.42 (ddt, *J* = 8.5, 5.6, 1.4 Hz, 2H), 4.31 (dd, *J* = 6.8, 5.9 Hz, 4H), 4.18 (t, *J* = 7.1 Hz, 2H).
^{13}C NMR (101 MHz, $CDCl_3$) δ 143.14, 143.08, 141.38, 132.34 (d, *J* = 7.4 Hz), 127.9, 127.1, 125.14, 120.0, 118.4, 69.25 (d, *J* = 7.3 Hz), 68.18 (d, *J* = 7.3 Hz), 47.9 (d, *J* = 7.3 Hz).
^{31}P NMR (202 MHz, $CDCl_3$) δ −2.30.



Scheme S8. Synthesis of allyl-UDP **4**.

Step 1: A solution of compound **2** (0.44 g, 0.89 mmol) in DCM: Et_3N 4:1 (10 mL) was kept at RT for 16 h; *tlc* [PE-DCM 4:1]-Me_2CO 20% revealed complete consumption of the starting material and formation of very polar new product. The reaction was concentrated and briefly dried in vacuum to give crude allyl phosphoric acid bis triethylammonium salt, which was used in the Step 3 (see below) without purification.

Step 2. To a cold (0 °C, ice-bath) solution of compound **3** (0.422 g, 0.76 mmol), *N*,*N*-dimethylaniline (0.192 mL, 1.52 mmol), and Et_3N (0.211 mL, 1.52 mmol) in anhydrous MeCN (4.5 mL) a solution of trifluoroacetic anhydride (0.349 mL, 2.5 mmol) in MeCN (3.5 mL) was added dropwise *via* cannula. The reaction mixture was stirred for a few minutes at RT. The reaction was concentrated to remove an excess of trifluoroacetic anhydride and the residue was re-dissolved in a fresh portion of MeCN (2.5 mL).The solution was cooled to 0 °C and a solution of *N*-methylimidazole (0.335 mL, 4.2 mmol) and Et_3N (0.585 mL, 4.2 mmol) in anhydrous MeCN (3.2 mL) was then added dropwise *via* cannula. The reaction mixture was further stirred for 15 min at RT.

Step 3. Thus prepared solution of *N*-methyl phosphoimidazolide was added *via* cannula to a flask containing crude product from the Step1 in MeCN (3 mL) at 0 °C. The reaction was further stirred at RT for 3 h. The reaction was quenched by addition of cold $Et_3NH{\times}HCO_3$ buffer (1 mL) and stirred for 20 min. The reaction was concentrated; the residue was dissolved in a 5:2:1 mixture of MeOH:H_2O:Et_3N (16 mL) and kept at RT for 16 h. The reaction mixture was concentrated, diluted with aqueous 0.25 M NH_4HCO_3 (15 mL) and extracted with $CHCl_3$ (5 mL).

The layers were separated by centrifugation for 10 min at 4 °C; the aqueous layer was collected. The extraction was repeated two times more. The combined aqueous layer was purified by size exclusion chromatography (Bio-Gel P2 fine; column 2.6×100 cm; flow rate 1 mL/min; elution with 0.25 M NH_4HCO_3). Appropriate fractions were pooled and freeze dried to give 0.22 g (0.46 mmol, 60%) of the crude product as amorphous solid.

The crude product was purified by HPLC on Waters Peptide Separation Technology C18 column (5 µm, 19×100, flow rate 10 mL/min) using linear gradient 2-15% of buffer B (0.1 mM Et_3NHOAc in 50% MeCN-water) in buffer A (0.1 mM Et_3NHOAc in water) over 15 min. Appropriate fractions were pooled and freeze dried; the residue was desalted by size exclusion chromatography as above to give 0.16 g (0.33 mmol, 44%) of the target compound as white powder.

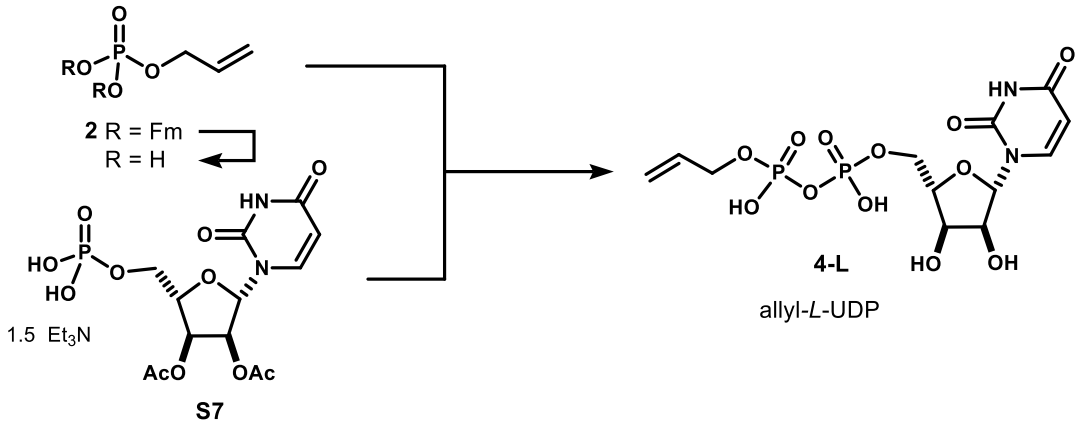
$[\alpha]_D$ = -4° *c* 0.3 H_2O

^{1}H NMR (400 MHz, D_2O) δ 7.87 (d, *J* = 8.1 Hz, 1H), 5.99 – 5.82 (m, 3H), 5.28 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.14 (dq, *J* = 10.5, 1.5 Hz, 1H), 4.37 (ddt, *J* = 8.2, 5.3, 1.5 Hz, 2H), 4.33 – 4.25 (m, 2H), 4.22 – 4.18 (m, 1H), 4.18 (ddd, *J* = 11.8, 5.4, 2.8 Hz, 1H), 4.10 (ddd, *J* = 11.8, 5.4, 2.8 Hz, 1H).

^{13}C NMR (101 MHz, D_2O) δ 166.26, 151.83, 141.60, 133.79 (d, *J* = 7.4 Hz), 116.90, 102.63, 88.31, 83.23 (d, *J* = 9.1 Hz), 73.71, 67.02 (d, *J* = 5.8 Hz), 66.99, 64.83 (d, *J* = 5.6 Hz).

^{31}P NMR (202 MHz, D_2O) δ -10.94 (d, *J* = 21.2 Hz), -11.48 (d, *J* = 21.2 Hz).

Compound **4-L** was obtained from compound **2** and *L*-UMP **(S7)** using the protocol described for the synthesis of allyl-UDP.



$[\alpha]_D$ = 4° *c* 0.28 H_2O

Scheme S9. Synthesis of allyl-*L*-UDP **4-L**.

Compound 6: Procedure for the preparation of compound **6** represents the general protocol for the preparative photo thiol-ene conjugation reaction.

A solution of compound **4** (0.024 g; 0.05 mmol, 20 mM), peptide **5** (0.037 g; 0.05 mmol, 20 mM), and LAP (0.0015 g; 0.005 mmol, 2 mM) in water (2.5 mL) contained in a 5mL quartz spectrophotometer cuvette was irradiated at 366 nm for 10 min. The reaction was diluted with the buffer A (0.1 % TFA-water) and purified by HPLC. The appropriate fractions were pooled, and freeze-dried to give 0.035 g (0.03 mmol, 60%) of the target product as fluffy powder.

LC_MS ESI(+) m/z =1174.65

^{1}H NMR (400 MHz, D_2O) δ = 7.90 (d, *J*=8.1, 1H), 5.95 – 5.83 (m, 2H), 4.54 (dd, *J*=8.6, 6.6, 2H), 4.38 (dd, *J*=8.3, 6.1, 1H), 4.32 – 4.00 (m, 13H), 3.98 – 3.86 (m, 2H), 3.83 (dt, *J*=10.9, 6.4, 1H), 3.65 (dt, *J*=10.2, 7.1, 1H), 2.92 (dd, *J*=13.7, 7.3, 1H), 2.84 (dd, *J*=13.7, 7.2, 1H), 2.31 – 2.16 (m, 1H), 1.96 (s, 3H), 2.05 – 1.89 (m, 3H), 1.89 – 1.76 (m, 3H), 1.33 (d, *J*=7.2, 3H), 1.18 (d, *J*=6.4, 3H), 1.13 (d, *J*=6.4, 3H), 0.91 – 0.82 (m, 9H).

^{13}C NMR (101 MHz, D_2O) δ = 177.41, 174.35, 173.94, 173.90, 173.22, 172.45, 171.23, 169.96, 166.14, 151.75, 141.66, 102.64, 88.31, 83.21, 73.78, 69.60, 66.92, 66.82, 65.04, 64.85, 64.82, 60.35, 59.50, 59.42, 59.14, 57.04, 52.92, 49.52, 48.47, 32.32, 30.12, 30.02, 29.82, 29.49, 27.87, 24.67, 21.58, 18.67, 18.39, 18.32, 17.80, 17.71, 17.54, 16.71.

^{31}P NMR (202 MHz, D_2O) δ -10.94 (bs), -11.48 (bs)

Compounds **11** and **12**

The peptidyl resin H-ahx-RQIKIWFQNRRMKWKKVTPVCTA-MBHA-Rink amide was synthesized according to the general protocol starting with 0.1 g of the Rink amide MBHA resin (Novabiochem, 0.52 mmol/g theory load) with manual addition of 6-(Fmoc-amino)hexanoic acid (Fmoc-ahx-COOH; Novabiochem) block. After the final deprotection, the resin was returned and split into two equal portions.

Peptide 8: One-half of the resin was treated with 20% Ac_2O in DMF for 30 min, drained, filtered, and washed 3 times with DMF and 3 times with DCM; the peptide was cleaved, from the resin and purified in a usual way, to give 0.03 g (0.01mmol, 40%) of the target product.

LC_MS ESI(+) m/z = 3073.01 expected for $C_{141}H_{232}N_{43}O_{30}S_2$ $[M+H]^+$ m/z: 3072.7425

Conjugate 11: A solution of the peptide **8** (0.015 g, 0.005 mmol), allyl-UDP **4** (0.0025 g, 0.005 mmol) and LAP (0.0003 g, 0.001 mmol) in water (0.5 mL) contained in a 1mL polypropylene spectrophotometer cuvette was irradiated at 366 nm for 10 min. The reaction was diluted with the buffer A (0.1 % TFA-water) and purified by HPLC. The appropriate fractions were pooled, and freeze-dried to give 0.0084 g (0.0024 mmol, 47%) of the target product as fluffy powder.

LC_MS ESI(+) m/z = 3515.52 expected for $C_{153}H_{251}N_{45}O_{42}P_2S_2$ $[M+H]^+$ m/z: 3516.7802

Peptide 9: The second half of the resin was treated with a solution of 5-fluorescein isothiocyanate (FITC; 0.05 g) in DMF (2 mL) in the presence of DIPEA (0.1 mL) for 16h. The resin was drained, washed 3 times with DMF and 3 times with DCM. The peptide was cleaved from the resin and purified in a usual way to give 0.023 g (0.007 mmol, 28%) of the target product;

LC_MS ESI(+) m/z = 3420.12 expected for $C_{160}H_{241}N_{44}O_{34}S_3$ $[M+H]^+$ m/z =3419.7678.

Conjugate 12: A solution of peptide **9** (0.0115 g, 0.0034 mmol), allyl-UDP 4 (0.0016 g, 0.0034 mmol) and LAP (0.0002 g, 0.0007 mmol) in water (0.35 mL) contained in a 1mL polypropylene spectrophotometer cuvette was irradiated at 366 nm for 10 min. The reaction was diluted with the buffer A (0.1 % TFA-water) and purified by HPLC. The appropriate fractions were pooled, and freeze-dried to give 0.0075 g (0.002 mmol, 57%) of the target product **12** as fluffy orange powder.

LC_MS ESI(+) m/z =3864.34 expected for $C_{172}H_{259}N_{46}O_{46}P_2S_3$ $[M+H]^+$ m/z= 3863.8013.

Compound **13**

Peptide 10: The requisite peptide Ac-YGRKKRRQRRR-*adex*-VTPVCTA was synthesized according to the general protocol starting with 0.11 g of the Rink amide MBHA resin (Novabiochem, 0.52 mmol/g theory load) with manual addition of 8-(Fmoc-amino)-3,6-dioxaoctanoic acid (Fmoc-adex-COOH; IRIS Biotech GmbH) block, and automated acetylation at the N-terminus. The peptide was cleaved from the resin and purified in a usual way, to give 0.044 g (0.018 mmol, 32%) of the target product;

LC_MS ESI(+) m/z = 2419.01 expected for $C_{101}H_{182}N_{41}O_{26}S$ $[M+H]^+$ m/z: 2418.3934

Conjugate 13: A solution of peptide **10** (0.027 g, 0.011 mmol), allyl-UDP **4** (0.006 g, 0.012 mmol) and LAP (0.00045 g, 0.0015 mmol) in 0.2 M pH 6.2 phosphate buffer (1.1 mL) contained in a 2 mL polypropylene spectrophotometer cuvette was irradiated at 366 nm for 10 min. The cloudy reaction mixture was diluted with the buffer A (0.1 % TFA-water) and purified by HPLC. The appropriate fractions were pooled, and freeze-dried to give 0.014 g (0.005 mmol, 45%) of the target product as fluffy powder.

LC_MS ESI(+) m/z = 2863.31 expected for $C_{113}H_{200}N_{43}O_{38}P_2S$ $[M+H]^+$ m/z: 2862.4269

Compound **17**

Peptide **14**: The requisite peptide H-ahx-VTPVCTA was synthesized according to the general protocol starting with 0.125 g of the rink Amide MBHA resin (Novabiochem, 0.78 mmol/g theory load) with manual addition of Fmoc-Ahx-COOH block. After the final deprotection, the resin was returned and the peptide was cleaved and purified following standard protocol to give 0.036 g (0.045 mmol, 50%) of the target product.
LC_MS ESI(+) m/z = 803.13 expected for $C_{35}H_{64}N_9O_{10}S$ $[M+H]^+$ m/z: 802.4497

Conjugate **15**: A solution of **4** (0.01 g; 0.0224 mmol, 10 mM), peptide **14** (0.018 g; 0.0224 mmol, 10 mM), and LAP (0.0007 g; 0.0023 mmol, 0.1 mM) in water (2.25 mL) was irradiated at 366 nm for 10 min. The reaction was analyzed and finally purified by HPLC to give 0.013 g (0.011 mmol, 50%) of the target product.

LC_MS ESI(+) m/z = 1246.93 expected for $C_{47}H_{82}N_{11}O_{22}P_2S$ $[M+H]^+$ m/z: 1246.4832

Conjugate **17**: To a stirred solution of **15** (0.011 g, 0.009 mmol) in a mixture of DMF (2 mL) and 0.15 M $NaHCO_3$ buffer (0.4 mL) a solution of 5(6)-carboxyfluorescein N-hydroxysuccinimide ester (FloNHS; 7.4 mg, 0.015 mmol) in DMF (0.1 mL) was added at RT. The reaction was stirred at RT for 3 h. The reaction was purified by HPLC, the appropriate fractions were pooled and freeze-dried to give 0.012 g (0.0075 mmol, 85%) of the target peptide as yellow fluffy material.

LC_MS ESI(+) m/z =1604.01; expected for $C_{68}H_{92}N_{11}O_{28}P_2S$ $[M+H]^+$ m/z: 1604.5309

Table S1. Chemical yields and observed m/z ESI (+) of S-UDP-peptide conjugates **6**, **7**, **29-38**

	Peptide sequence		Yield	m/z
1	VTPVC(*S*-propyl-UDP)TA	**6**	60%	1174.49
2	VTPVC(*S*-propyl-*L*-UDP)TA	**7**	51%	1174.51
3	VPTVC(*S*-propyl-UDP)TA	**29**	53%*	1174.51
4	SVPYC(*S*-propyl-UDP)SA	**30**	56%	1210.40
5	VTPVC(*S*-propyl-UDP)SA	**31**	62%	1148.51
6	VTPVC(*S*-propyl-UDP)RA	**32**	47%	1229.63
7	PVFTC(*S*-propyl-UDP)RS	**33**	77%	1293.70
8	VTPVC(*S*-propyl-UDP)TATH	**34**	52%	1412.83
9	SVPYC(*S*-propyl-UDP)SAQS	**35**	53%	1425.62
10	PVFTC(*S*-propyl-UDP)RSAA	**36**	56%	1435.85
11	PVC(*S*-propyl-UDP)TATHSLSRLH	**37**	77%	1906.37
12	KENSPAVTPVC(*S*-propyl-UDP)TA	**38**	57%	1800.75

*Reaction was performed in 6MGn·HCl−0.2M phosphate buffer (pH 7.5)

Copies of NMR spectra for selected compounds

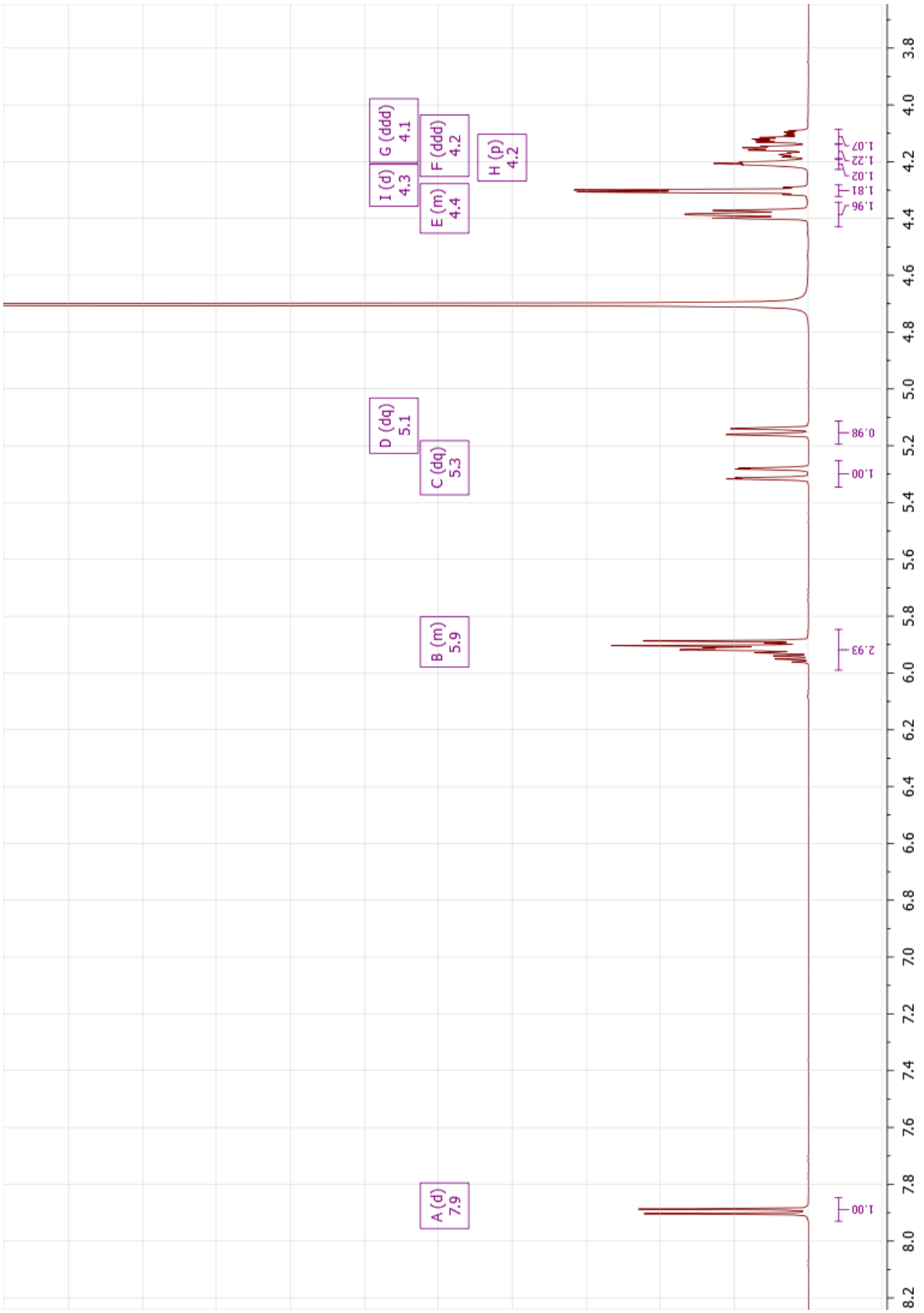


Figure S1. ^{1}H NMR spectrum of compound **4**

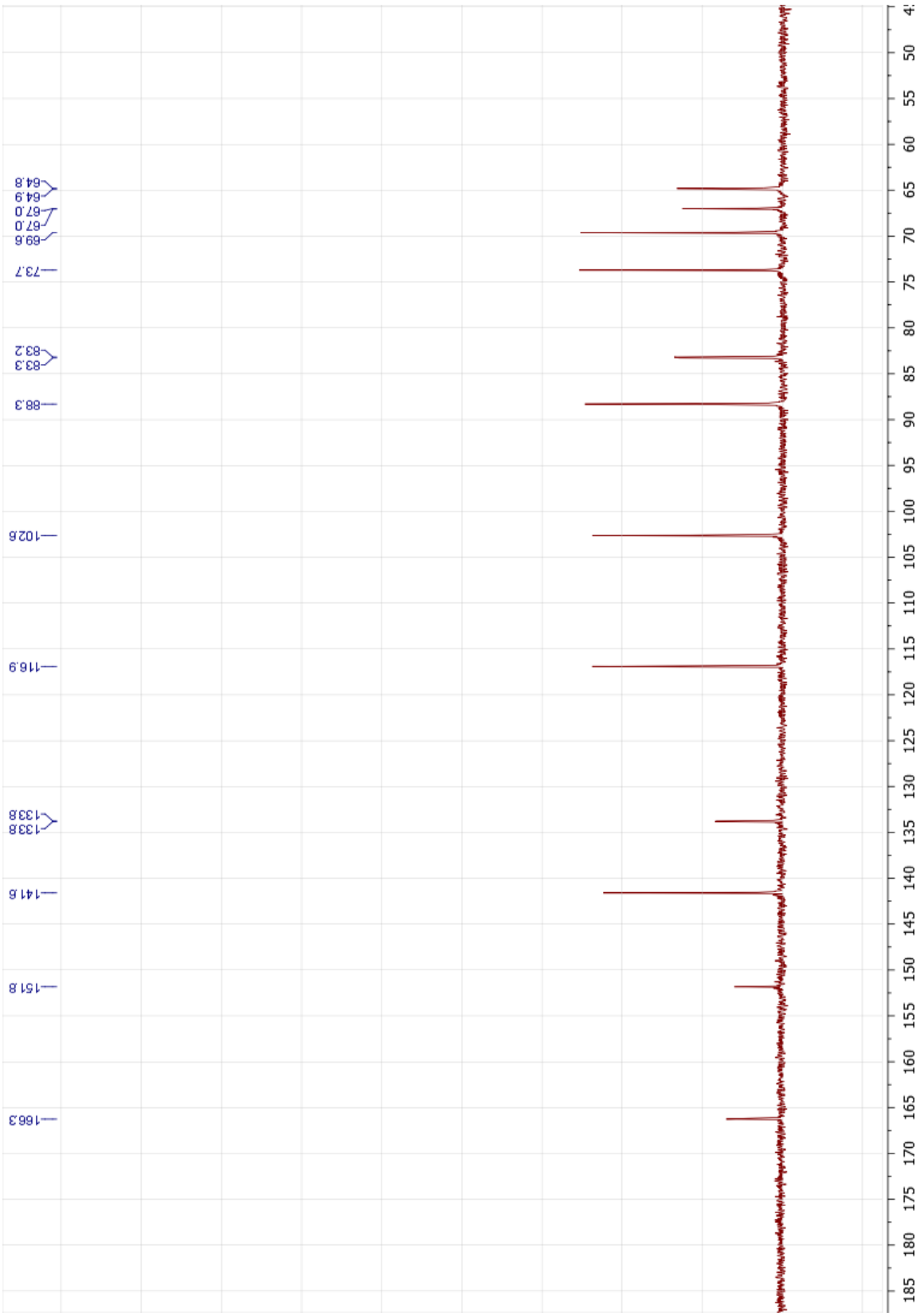


Figure S2. ^{13}C NMR spectrum of compound **4**

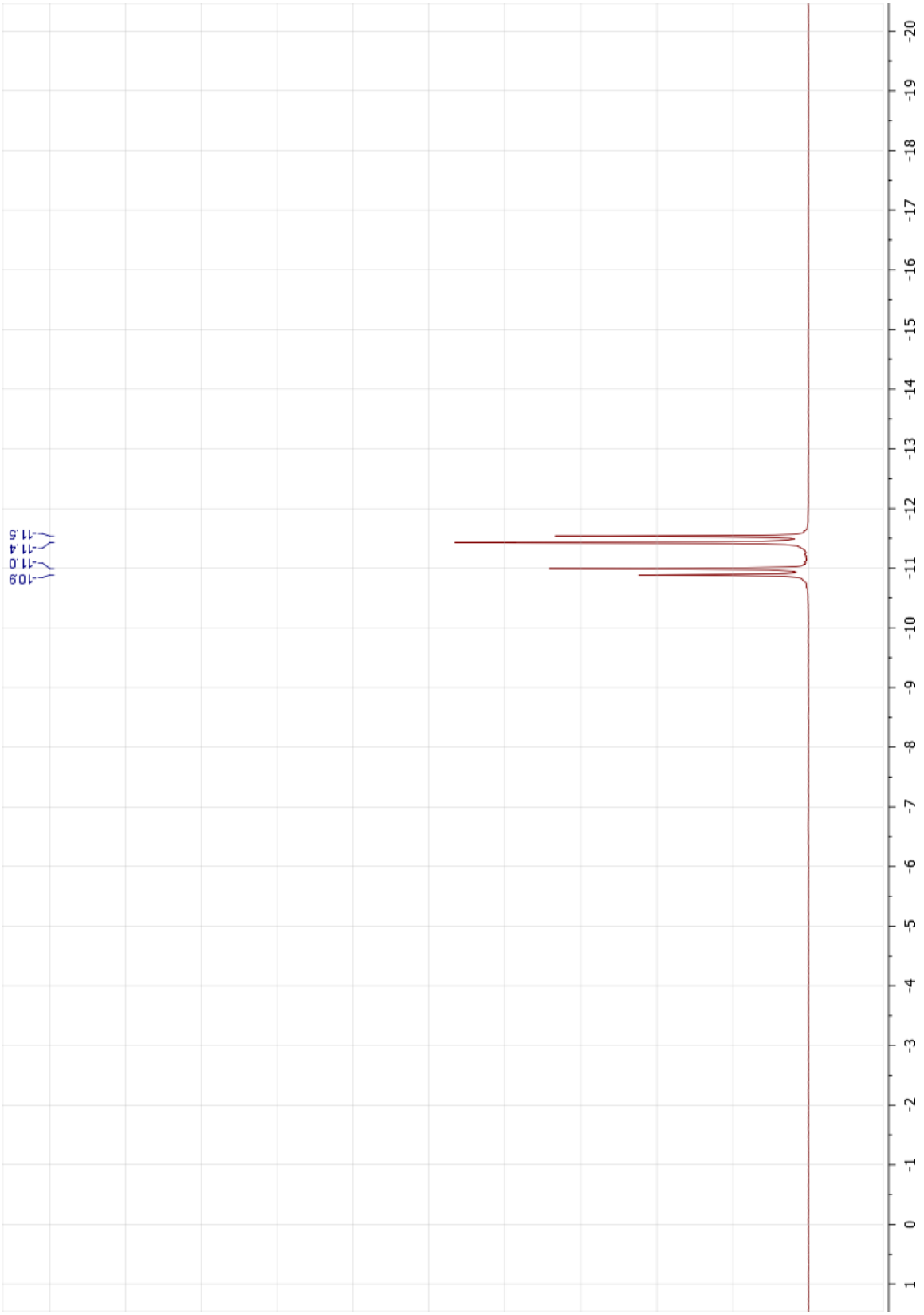


Figure S3. ^{31}P NMR spectrum of compound **4**

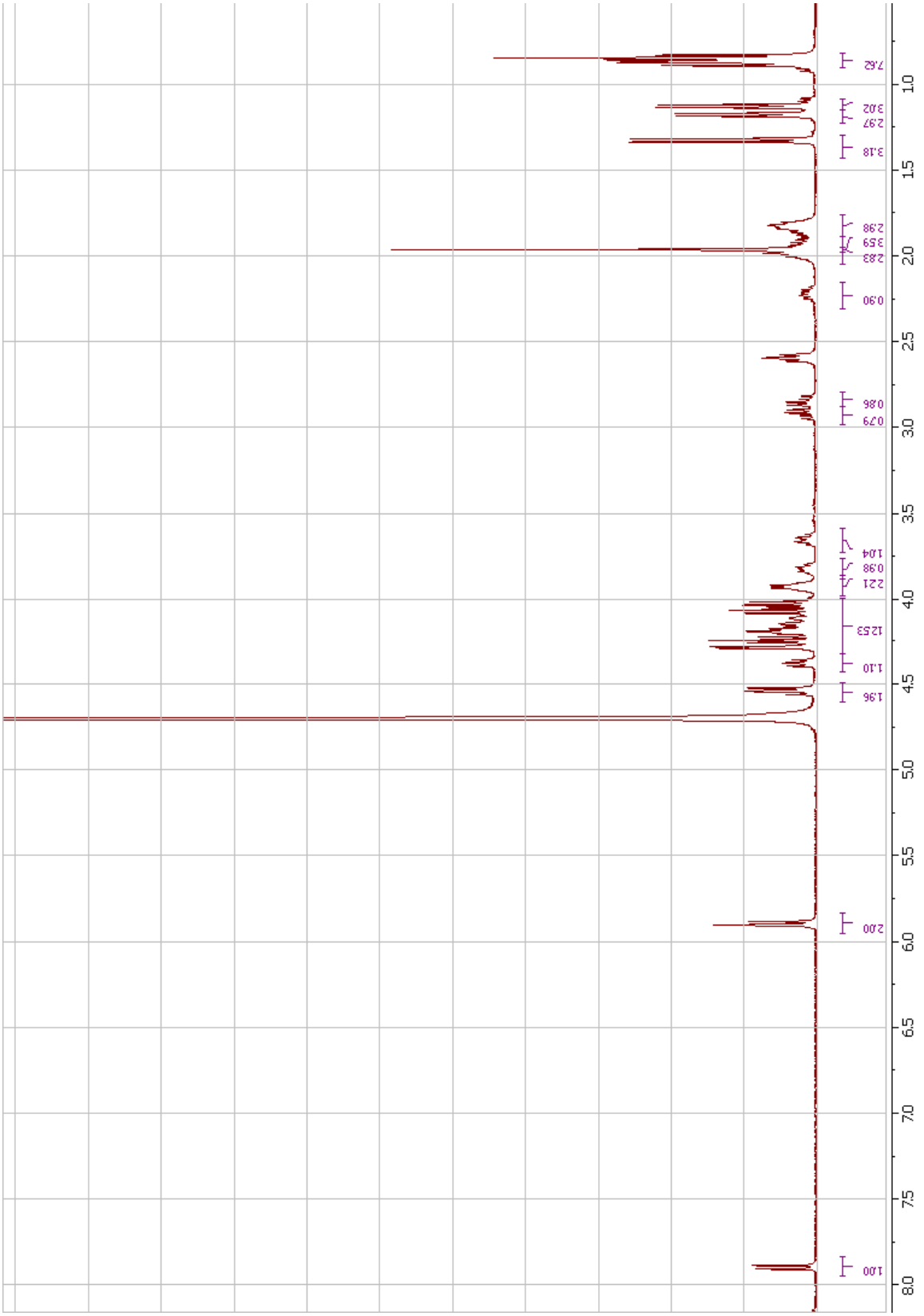


Figure S4. ^{1}H NMR for compound **6.**

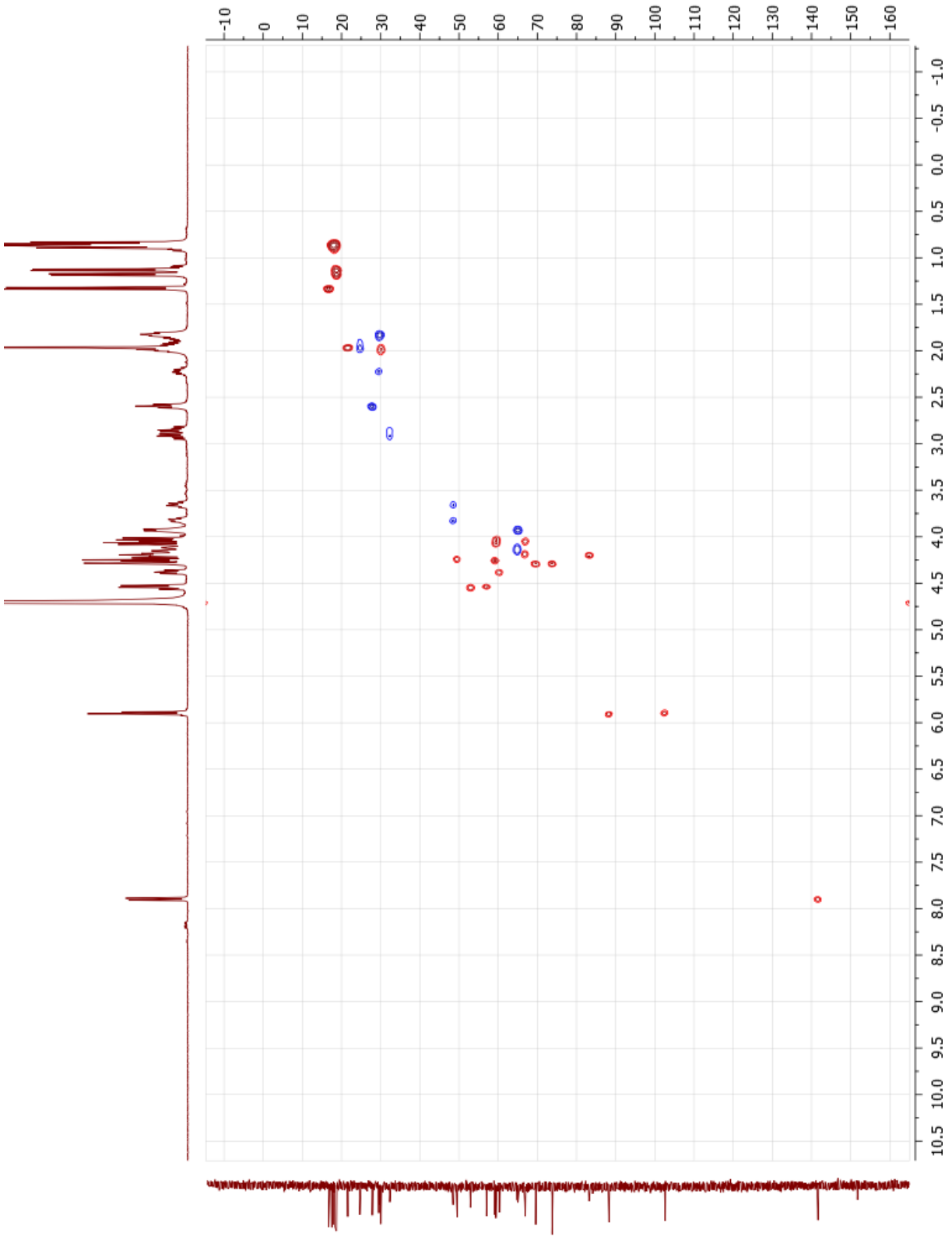


Figure S5. HSQC spectrum for compound **6.**

Crystallography and structure solution

Human OGT (312-1031) was recombinantly expressed and purified as a cleavable GST-fusion protein, as described previously[1]. A solution of 8 mg/mL hOGT and 1 mM **1** or **6** in 25 mM Tris-HCl pH 8.0, 20 mM NaCl, 0.5 mM TCEP was incubated on ice for 15 minutes. Hanging-drop vapour diffusion crystallisation experiments were performed by mixing drops in a 2:1 ratio of protein-ligand:reservoir solution with the hOGT:**1** complex grown in [1.3 M DL-Malic acid, 0.1 M Bis-Tris propane pH 6.4] supplemented with hOGT crystal seeds, grown in the same condition and the hOGT:**6** complex grown in [1.45 M K_2HPO_4, 8 mM EDTA, 1% xylitol] supplemented with 0.5 M $(NH_4)_2SO_4$ and seeds generated from the same condition as used for hOGT:**1** complex. Crystals grew O/N and were cryo-protected by short immersion in 2.5 M sodium malonate pH 7.0, supplemented with 1 mM **1** or **6** and flash-frozen in liquid nitrogen. Data were collected at the European Synchrotron Radiation Facility (ESRF) on beamline ID30A-1/MASSIF-1 (**1**) and ID29 (**6**), were processed with XDS[2] and scaled to 1.68 Å (**1**) and 1.85 Å (**6**) using aimless[3]. 5% of total reflections were set aside as an R_{free} test set. Crystals belonged to space group F222 with one molecule per asymmetric unit, a solvent content of 63% and a Matthews coefficient of 3.33 for both crystals. The structures were solved with MOLREP[4], using chain A of PDB 3PE4[5] as a search model. The structure was fully refined using iterative cycles of Refmac5[6] and COOT[7]. Ligand topology was generated using PRODRG[8]. Data collection and refinement statistics can be found in Supplementary Table 2.

hOGT activity measurement

Human OGT (312-1031) was recombinantly expressed as a cleavable His_6-fusion protein and was purified as described previously[9]. OGT activity was determined by setting up reactions containing 5 nM His_6-hOGT in 50 mM Tris-HCl pH 7.5, 0.1 mg/mL BSA, 10 µM sodium dithionate and 10 µM of peptide (KKENSPAVTPVSTA) in a total volume of 100 µL. Reaction mixtures were pre-incubated with varying concentrations of **6** and **7** (0.01 – 300 µM) for 15 min and initiated by addition of UDP-GlcNAc to a final concentration of 3.2 µM. The reaction was stopped after 2 h at 21 °C by addition of 200 µL of 25 mM HEPES pH 7.4, 10 mM NaCl, 75 µM pyrocatechol violet, 50% (v/v) MeOH and 15 µM fluorophore[9, 10]. UDP formation was detected fluorometrically on a Gemini EM fluorescence Microplate reader (Molecular Devices) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Data analysis was performed using GraphPad Prism 7. Turnover did not exceed 10% for either substrate.

Fluorescence polarimetry measurements

The binding affinity of **17** and **18** were determined by titrating hOGT at a fixed concentration of probe (125 nM) in 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5 mM TCEP and 5% DMSO in a total volume of 25 µL and incubated in the dark for 30 min before read-out.

Binding affinities for ligands were determined by **17** displacement. Reactions contained 0.8 µM hOGT and 0.75 µM **17** in 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, 0.5 mM TCEP and 5% DMSO in a total volume of 25 µL. Reaction mixtures were incubated with varying concentrations of inhibitor for 30 min in the dark. Fluorescence polarisation was measured on a PHERAStar plate reader (BMG LABTECH). Data analysis was performed in GraphPad Prism 7 and binding constants for the labelled **17** and **18** were determined by fitting a one-site – Total binding curve. Binding constants for ligands in a displacement experiment were determined using GraphPad Prism7 and the equation reported by Nikolovska-Coleska *et al.*[11].

OGT inhibition in cell extracts

HeLa cells were grown in DMEM medium supplemented with 10% FBS, 2 mM L-Glutamine and 1% Penicillin/Streptomycin (100 U/ml and 100 µg/ml respectively) at 37 °C and 5% CO2. Cells were lysed for 10 min on ice in a buffer containing 50 mM Tris-HCl, 0.1 mM EGTA, 1 mM EDTA, 1% Triton-X 100, 1 mM Na_3VO_4, 50 mM, 5 mM $Na_4P_2O_7$, 0.27 M sucrose, 1 mM β-mercaptoethanol and protease inhibitor cocktail. Lysates were spun down at 13400 rpm for 10 min, supernatants were transferred into fresh tubes and protein concentration was quantified using Bradford assay. To strip O-GlcNAc from proteins cell lysates were treated with *Cp*OGA[12], a promiscuous bacterial O-GlcNAc hydrolase, (85 µg per 1 mg of lysate) for 1 h at 37 °C. GlcNAcstatin G (Dorfmueller et al., 2006) was added to a final concentration of 10 µM to inhibit *Cp*OGA and endogenous OGA. Thiol-linked bisubstrate conjugates **6** and **7** were added to a final concentration of 250, 500 and 1000 µM with 1 µM OGT and pre-incubated for 25 min prior to addition of UDP-GlcNAc to a final concentration of 1 mM. As a positive control cell lysate was treated with a known OGT inhibitor UDP-5S-GlcNAc at a final concentration of 500 µM. Reactions were incubated for 1.5 h at 37 °C. Protein samples were prepared using Laemmli buffer. Samples were loaded on an SDS PAGE gel and run at 180 V until the dye front ran off. Proteins were then transferred onto nitrocellulose membrane at 35 V for 1.5 h. Membranes were stained with Ponceau-S to check for successful transfer, washed with 0.2% TBS-Tween and blocked in 5% BSA in 0.2% TBS-Tween. Membranes were incubated with primary antibodies overnight at 4°C. After washing with 0.2% TBS-Tween, membranes were incubated with secondary antibodies for 1 h at room temperature. Antibodies used: anti-O-GlcNAc RL2 (Abcam, Cat#: ab2739) - 1:1000, HSP60 (Cell Signaling Technology, Cat#: 4870) - 1:1000. Secondary goat anti-mouse IRdye® 680 and donkey anti-rabbit IRDye® 800 antibodies were purchased from LI-COR.

HeLa cell microscopy

HeLa cells were plated at a density of 5 x 10^5 cells/well in 6-well plates containing coverslips (VWR) and grown over night in DMEM medium (Gibco), supplemented with 10% foetal bovine serum (FBS), L-glutamine, penicillin and streptomycin, at 37 °C in a humidified 5% CO_2 incubator. Fluorescent bisubstrate conjugate **12** was added to the medium 2 h before fixation. Cells were fixed for 20 min in 4% paraformaldehyde, stained with 2 µg/mL DAPI (Sigma-Aldrich) and mounted onto microscopy slides (VWR) using a fluorescent mounting medium (Dako). Images were acquired and analyzed using a DeltaVision system (GE Healthcare).

Cell culture and western blot analysis

HeLa/SH-SY5Y cells were grown to 80-90% confluency before addition of **11** or **13** and incubated for 24 h. Cells were washed in ice-cold PBS and subsequently incubated in lysis buffer (50 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.27 M sucrose, 0.1% 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethane sulfonyl fluoride, 5 µM leupeptine) for 10 minutes on ice. Cell lysates were centrifuged at 15,000g for 10 min at 4 °C. Supernatants were collected and heated in Laemmli-buffer for 5 min at 95 °C. Protein concentration was determined by Bradford assay. Samples were resolved by SDS-PAGE followed by Western blot with RL2 (anti-O-GlcNAc, Abcam), actin (Sigma-Aldrich) and tubulin (Cell Signaling Technology) primary antibodies. Anti-mouse and anti-rabbit fluorescent secondary antibodies were used and the blots were scanned using the LI-COR Odyssey Image system.

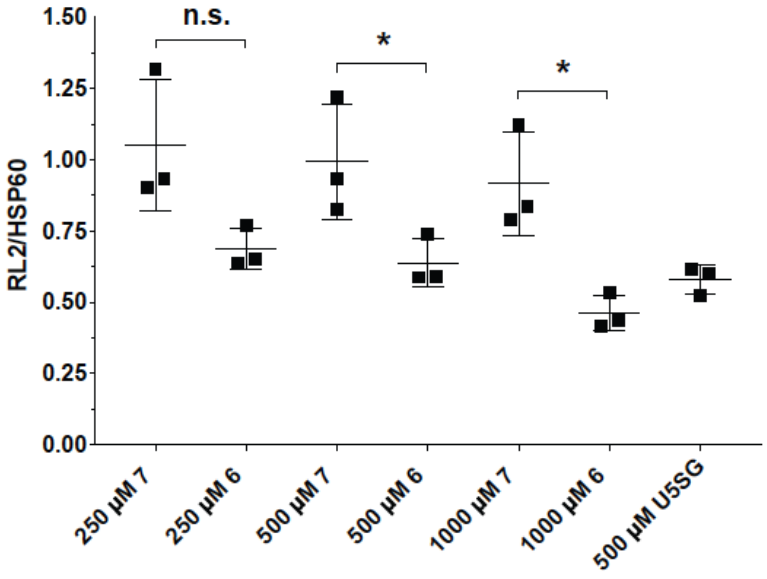


Figure S6. Relative levels of global O-GlcNAc levels from HeLa lysate treated with recombinant OGT in the presence and absence of increasing concentrations **6** or **7**. Fluorescent signal from the Western blot analysis was quantified using a LICOR Odyssey system and normalized to the HSP60 loading control.

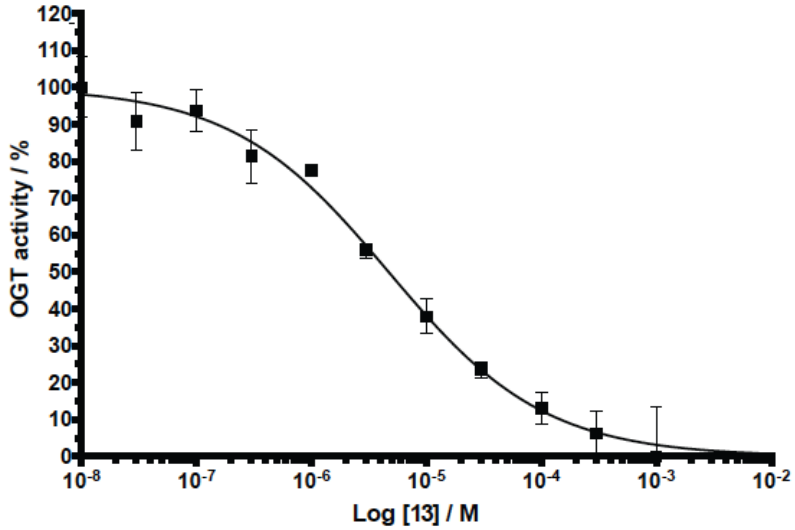


Figure S7. Dose-response curves of inhibition of OGT activity in the presence of increasing concentrations of **13**. Errors shown represent the s.e.m. of three replicates.

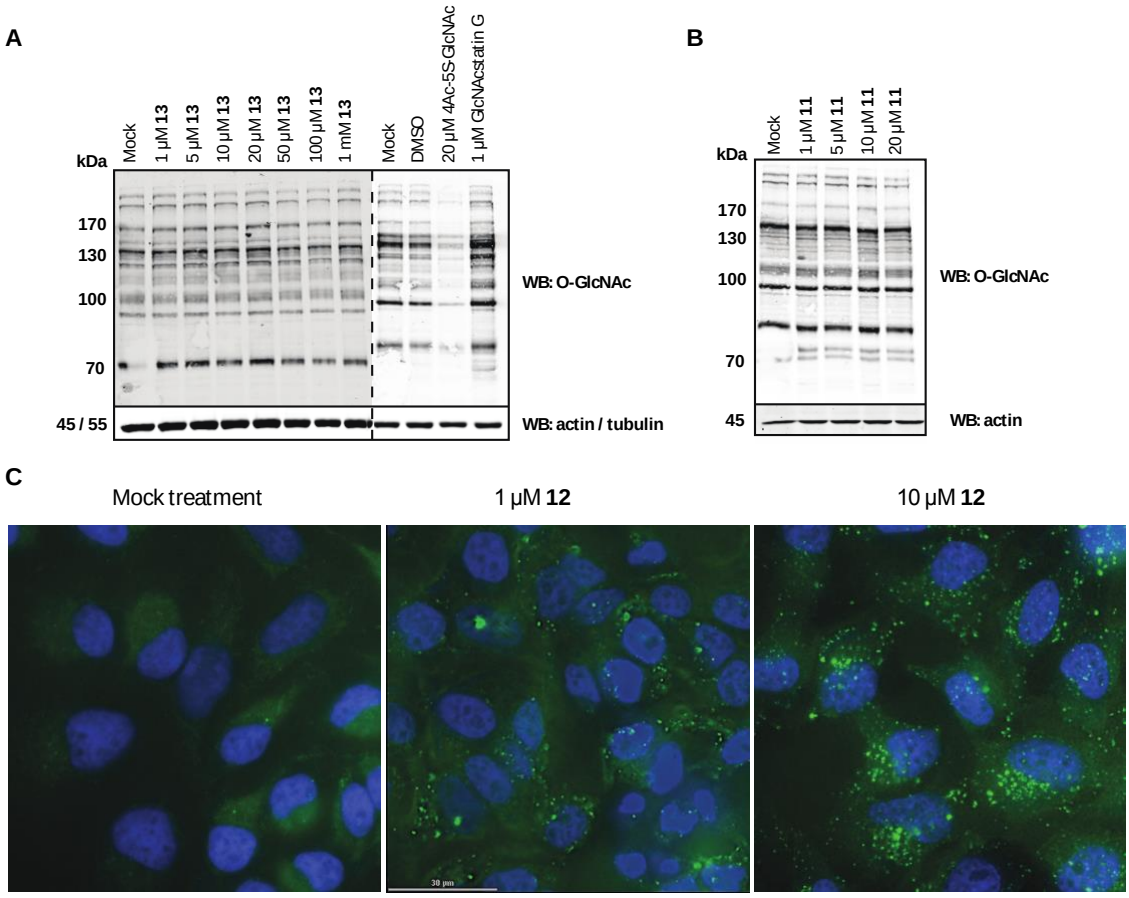


Figure S8.

(A) Western blot analysis of SH-SY5Y cells treated with varying concentrations of **13.** Global O-GlcNAc levels were visualized by anti-O-GlcNAc antibody RL2 (Abcam, Cat#: ab2739) and actin (Sigma-Aldrich, Cat#: A2066) or tubulin (Cell Signaling Technology, Cat#:2125S) as loading controls. To show dynamic modulation of global O-GlcNAc levels known inhibitors of OGT and OGA were treated with 20 µM UDP-5S-GlcNAc (administered as 4Ac-5S-GlcNAc) and 1 µM GlcNAcstatin G [13, 14], respectively.

(B) Western blot analysis of HeLa cells treated with varying concentrations of **11.** Global O-GlcNAc levels were visualized by anti-O-GlcNAc antibody RL2 (Abcam, Cat#: ab2739) and actin as loading controls (Sigma-Aldrich, Cat#: A2066).

(C) Deconvolution microscopy images of HeLa cells treated with vehicle (left), 1 µM **12** (middle) and 10 µM **12** (right). FITC signal is shown in green and DAPI signal in blue.

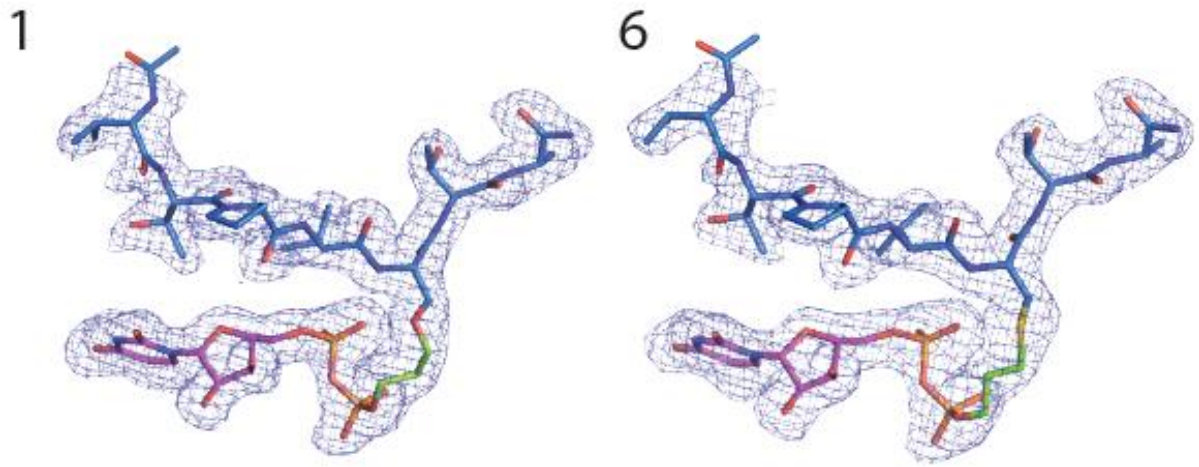


Figure S9. Unbiased $|F_o|-|F_c|$ electron density maps shown as a blue mesh for bisubstrate conjugates **1** and **6**, contoured to 2.25 σ. The peptide part is colored in blue, the linker/sugar in green and the UDP moiety in magenta.

Table S2. Data collection and refinement statistics. High-resolution shell values shown in parenthesis.

	hOGT + **1**	hOGT + **6**
Data collection		
Beamline, wavelength	ID30-A1, 0.98 Å	ID29, 0.976 Å
Space group	*F222*	*F*222
Cell dimensions (Å)	*a*=137.87*, b*=151.33*, c*=200.48,□α=β=γ=90	*a*=138.04*, b*=150.95*, c*=200.54,□α=β=γ=90
Resolution (Å)	46.10-1.68 (1.71-1.68)	46.01-1.85 (1.88-1.85)
R_{merge}	0.05 (1.285)	0.06 (0.871)
$I/\sigma I$	13 (1.0)	9.7 (1.3)
$CC_{1/2}$	0.99 (0.45)	0.99 (0.65)
R_{meas}	0.06 (1.624)	0.078 (1.202)
R_{pim}	0.04 (0.979)	0.048 (0.711)
Completeness (%)	99.3 (99.9)	99.5 (99.8)
Redundancy	4.7 (4.8)	4.0 (4.1)
Refinement	46.01-1.68	46.01—1.85Å
No. total reflections	547394	349113
No. unique reflections	117257	88158
R_{work}, R_{free}	0.193 / 0.222	0.215 / 0.247
No. atoms		
Protein	5508	5478
Ligand	78	78
Water	478	362
B-factor average		
Protein	36.8	46.78
Ligand	32.5	38.65
R.m.s. deviations		
Bond lengths (Å)	0.0077	0.0115
Bond angles (°)	1.248	1.5
PDB ID	5NPS	5NPR

Supplementary References

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