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Supplementary information

Glucose-sensitive insulin with attenuation of hypoglycaemia

In the format provided by the authors and unedited

Methods

1. Preparation of O-succinimidyl pentyn-1-oxycarbonyl

A solution of bis(trichloromethyl)carbonate (52.9 g, 178 mmol) in dry tetrahydrofuran (215 mL) was added dropwise to a solution of pent-4-yn-1-ol (13.6 g, 162 mmol) in dry tetrahydrofuran (80 mL) over 15 minutes at 0 ºC. The reaction mixture was stirred for 1 hour under cooling and then overnight at room temperature. The solvent was removed under reduced pressure and the residue (yellow oil) was purified by vacuum distillation (p = 0.2 Torr, T = 80-95 ºC) to give pent-4-yn-1-yl carbonochloridate as off-white liquid. Yield: 16.3 g (69%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.44 (q, J=6.2 Hz, 2 H); 2.41-2.31 (m, 2 H); 2.04-1.90 (m, 3 H). To a solution of N-hydroxysuccinimide (12.8 g, 111 mmol) in dry tetrahydrofuran (450 mL) cooled at -5 ºC was added triethylamine (15.5 mL, 111 mmol, with forming of fine precipitate). A solution of the above chloroformate, pent-4-yn-1-yl carbonochloridate, (16.3 g, 111 mmol) in dry tetrahydrofuran (90 mL) was added dropwise over 10 min under intensive stirring (a precipitate triethylamine hydrochloride was gradually formed). The resulting suspension was stirred for 2 hours at -5 ºC and then overnight at room temperature. The white solid was filtered off and solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate (500 mL) and washed with 1 M aqueous solution of potassium hydrogen sulfate (2 x 300 mL), brine (3 x 300 mL) and dried over anhydrous sodium sulfate. After filtration, the solvent was removed under reduced pressure and the crude product was purified by column chromatography (Silica gel 60, 0.040-0.063 mm; eluent: cyclohexane/ethyl acetate 3:1-1:1) giving pure O-succinimidyl pentyn-1-oxycarbonyl (Extended data fig. 7a) as off-white liquid. Yield: 9.41 g (38%). RF (SiO_2, cyclohexane-ethyl acetate 1:1): 0.40. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.45 (t, J=6.3 Hz, 2 H, H-4); 2.83 (s, 4 H, H-1); 2.35 (td, J=6.9, 2.6 Hz, 2 H, H-6); 2.09-1.84 (m, 3 H, H-8 and H-5). ^{13}C NMR (126 MHz, $CDCl_3$) δ 168.6 (s, C-2), 151.5 (s, C-3), 82.1 (s, C-7), 69.7 (C-4), 69.6 (C-8), 27.2

(s, C-5), 25.4 (s, C-1), 14.8 (s, C-6). LCMS calculated for $C_{10}H_{11}NO_5$: 226.1, found: 226.4 $[M+H]^+$.

2. Preparation of beta-carboxymethyl-oxyethyl-O-peracetyl-D-glucoside active ester

Alpha-bromo-O-peracetyl-D-glucoside (4.71 g, 11.5 mmol) was dissolved in anhydrous 1,4-dioxane (6 mL) and diethylene glycol (7.60 mL). Silver carbonate (3.47 g, 12.6 mmol) was added in one portion (gas evolution after short induction period). The resulting mixture was stirred at ambient temperature for 3 hours. Afterwards, it was diluted with ethyl acetate (80 mL), filtered through a celite pad, washed with ethyl acetate (3 x 30 mL) and the filtrate was evaporated. The oily residue was dissolved in water (80 mL) and extracted with dichloromethane (4 x 60 mL). Combined organic extracts were dried over anhydrous magnesium sulfate, filtered and evaporated *in vacuo*. The residue was purified by flash column chromatography (Silica gel 60, 0.040-0.063 mm; eluent: dichloromethane/ethyl acetate 1:1 to 0:1) to give beta-hydroxyethyl-oxyethyl-O-peracetyl-D-glucoside as a waxy white solid. Yield: 3.55 g (71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.22 (t, J=9.6 Hz, 1 H); 5.10 (t, J=9.6 Hz, 1 H); 5.00 (dd, J=9.4 and 7.9 Hz, 1 H); 4.62 (d, J=7.9 Hz, 1 H); 4.32-4.22 (m, 1 H); 4.20-4.11 (m, 1 H); 4.04-3.90 (m, 1 H); 3.80-3.55 (m, 8 H); 2.09 (s, 3 H); 2.06 (s, 3 H); 2.03 (s, 3 H); 2.01 (s, 3 H). LCMS calculated for $C_{18}H_{28}O_{12}$: 437.2, found: 437.1 $[M+H]^+$. To a well stirred solution of the given beta-hydroxyethyl-oxyethyl-O-peracetyl-D-glucoside (3.54 g, 8.11 mmol) and bis(acetoxy)iodobenzene (6.27 g, 19.5 mmol) in acetonitrile (40 mL) and water (10 mL) was added (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (51.0 mg, 0.32 mmol) in one portion. The reaction mixture was stirred overnight and concentrated *in vacuo*. The residue was treated with 10 % aqueous solution of sodium sulfite (40 mL) and stirred for 30 min. Afterwards, pH of the solution was adjusted with concentrated hydrochloric acid to pH = 2. The aqueous layer was extracted with dichloromethane (3 x 60 mL). The organic

extracts were combined, dried over anhydrous magnesium sulfate, filtered and evaporated. The residue was purified by flash column chromatography (Silica gel 60, 0.040-0.063 mm; eluent: acetonitrile/water 30:1 + 0.5 % of formic acid). After freeze-drying, the residue was treated with dry diethyl ether (60 mL). A white solid was filtered, washed with dry diethyl ether (3 x 20 mL) and dried *in vacuo* affording beta-carboxymethyl-oxyethyl-O-peracetyl-D-glucoside as a white solid. Yield: 2.93 g (80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.22 (t, J=9.5 Hz, 1 H); 5.10 (t, J=9.6 Hz, 1 H); 5.01 (dd, J=9.5 and 8.0 Hz, 1 H); 4.63 (d, J=7.9 Hz, 1 H); 4.30-4.24 (m, 1 H); 4.22-4.12 (m, 3 H); 4.05-3.93 (m, 1 H); 3.85-3.65 (m, 4 H); 2.09 (s, 3 H); 2.06 (s, 3 H); 2.03 (s, 3 H); 2.01 (s, 3 H). LCMS calculated for $C_{18}H_{26}O_{13}$: 449.1, found: 449.2 $[M-H]^-$. The given beta-carboxymethyl-oxyethyl-O-peracetyl-D-glucoside (2.77 g, 6.15 mmol) was dissolved in dry dichloromethane (30 mL), and triethylamine (2.60 mL, 18.5 mmol) was added. The mixture was cooled to 0 °C and 5-bromo-2-hydroxy-3-(trifluoromethyl)benzenesulfonyl chloride (4, 2.50 g, 7.38 mmol) was added in three portions over 10 min. The mixture was stirred under cooling for 30 min, then at room temperature overnight. Solvent was evaporated *in vacuo*. To the residue, ethyl acetate (40 mL) was added. The resulting suspension was filtered through celite pad, and filtrate was evaporated. The residue was purified by flash column chromatography (Silica gel 60, 0.040-0.063 mm; eluent: dichloromethane/2-propanol 100:0 to 90:10 + 0.5% of triethylamine) giving beta-carboxymethyl-oxyethyl-O-peracetyl-D-glucoside active ester (Extended data fig. 7b) as white foam. Yield: 3.34 g (63%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.86 (bs, 1 H, NH of Et3N); 8.11 (d, J=2.4 Hz, 1 H, H-4*); 7.99 (d, J=2.4 Hz, 1 H, H-2*); 5.25 (t, J=9.6 Hz, 1 H, H-3); 4.95-4.83 (m, 2 H, H-4 and H-1); 4.76 (dd, J=9.7 and 8.1 Hz, 1 H, H-2); 4.35 (d, J=7.1 Hz, 2 H, OCH2C=O); 4.18 (dd, J=12.3 and 5.0 Hz, 1 H, H-6); 4.06-3.94 (m, 2 H, H-6 and H-5); 3.87-3.78 (m, 1 H, OCH2CH2O); 3.73-3.58 (m, 3 H, OCH2CH2O and OCH2CH2O); 3.08 (q, J=7.2 Hz, 6 H, Et3N); 2.02 (s, 3 H); 1.99 (s, 3 H); 1.98 (s, 3 H); 1.93 (s, 3 H); 1.17 (t,

J=7.2 Hz, 9 H, Et3N). ^{13}C NMR (126 MHz, DMSO-d_6) δ 170.5 (s, COCH3 of C-6 acetyl), 170.0 (s, COCH3 of C-3 acetyl), 169.7 (s, COCH3 of C-4 acetyl), 169.6 (s, COCH3 of C-2 acetyl), 168.4 (s, OCH2C=O), 144.4 (s, C-5*), 144.2 (q, J(C-F)=1.9 Hz, C-6*), 135.9 (s, C-4*), 130.5 (q, J(C-F)=4.7 Hz, C-2*), 125.7 (q, J(C-F)=31.3 Hz C-1*), 122.4 (q, J(C-F)=273.9 Hz, CF3), 118.7 (s, C-3*), 99.9 (s, C-1), 72.6 (s, C-3), 71.3 (s, C-2), 71.0 (s, C-5), 70.1 (s, OCH2CH2O), 68.7 (s, OCH2CH2O), 68.7 (s, C-4), 68.3 (s, OCH2C=O), 62.2 (s, C-6), 46.3 (s, CH2 of Et3N), 20.9 (s, COCH3), 20.8 (s, COCH3), 20.8 (s, COCH3), 20.7 (s, COCH3), 9.2 (s, CH3 of Et3N). ^{19}F NMR (282 MHz, DMSO-d_6) δ -59.98. LCMS calculated for $C_{25}H_{28}BrF_3O_{16}S$: 752.4, found: 753.3 $[M+H]^-$. HRMS $[M+H]^+$ calculated for $C_{25}H_{28}BrF_3O_{16}S$ requires: 751.0161, found: 751.0164.

3. Preparation of the macrocycle roof propyl azide

a. Synthesis of the trisbromomethyl acid

3-(3,5-dimethylphenyl)propanoic acid (5.00 g, 28.1 mmol, 1 eq.) was dissolved in HBr/AcOH (33%, 53 mL). Paraformaldehyde (8.84 g, 295 mmol, 10.5 eq.) and $ZnBr_2$ (10.1 g, 44.9 mmol, 1.6 eq.) were added and the reaction mixture was heated to 100 °C for 18 hours. The reaction was cooled, and the precipitate isolated by filtration, washing with acetic acid (50 mL) and water (800 mL) to give the product (Extended data fig. 8a) (12.2 g, 26.6 mmol, 95%) as white crystals. ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.75 (d, J = 1.5 Hz, 6H), 3.14 – 2.96 (m, 2H), 2.58 (d, J = 18.5 Hz, 2H), 2.40 (d, J = 1.1 Hz, 6H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.44, 140.36, 138.81, 134.06, 132.99, 31.54, 31.22, 24.39, 15.21. LCMS (electrospray) $[M+Na]^+$ calculated for $C_{14}H_{17}Br_3NaO_2$ requires: 478.9, 480.9 found: 478.9, 480.9. HRMS $[M+H]^+$ calculated for $C_{14}H_{17}Br_2O_2$ requires: 376.9569, 374.9590, 378.9549, found: 376.9568, 374.9592, 387.9548 (trisbromomethyl acid fragments to the bisbromide and shows several isotopes).

b. Synthesis of the carboxy tris-azide

The trisbromomethyl acid (14.0 g, 30.6 mmol, 1 eq.) was dissolved in anhydrous DMF (12.0 mL), and NaN_3 (11.9 g, 184 mmol, 6 eq.) was added in 4 portions over 20 min. The reaction mixture was left to stir for 40 hours and then quenched with 1 M HCl (400 mL). The resultant suspension was extracted with EtOAc (3 × 1.5 L) and the combined organics washed with water (5 × 400 mL), brine (400 mL) and dried (Na_2SO_4). The solvents were evaporated under reduced pressure to an oil which crystallised upon standing to give the product (Extended data fig. 8b) (9.89 g, 28.8 mmol, 94%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.54 (s, 4H), 4.51 (s, 2H), 3.25 – 3.10 (m, 2H), 2.72 – 2.57 (m, 2H), 2.48 (d, J = 0.7 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.51, 140.23, 139.05, 132.37, 130.94, 49.04, 48.79, 35.37, 25.18, 16.73. LCMS (electrospray) $[M+Na]^+$ calculated for $C_{14}H_{17}N_9NaO_2$ requires: 366.1 found: 366.0. HRMS $[M+H]^+$ calculated for $C_{14}H_{17}N_7NaO_2$ requires: 366.1397, found: 366.1397.

c. Synthesis of the alcohol tris-azide

The carboxy tris-azide (9.89 g, 28.8 mmol, 1 eq.) was dissolved in anhydrous THF (30 mL), and 1 M BH_3.THF in THF (58 mL, 58.0 mmol, 2 eq.) was added dropwise. After stirring for 5 hours, further 1 M BH_3.THF in THF (20 mL, 20.0 mmol, 0.7 eq.) was added and the reaction was stirred for 16 hours. The reaction was quenched with saturated aqueous $NaHCO_3$ (60 mL). The THF was removed under a stream of nitrogen and the remnants extracted with EtOAc (100 mL). The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by flash chromatography (petroleum ether 40/60 with increasing EtOAc). The fractions containing the product were combined and the solvent removed under vacuum to yield the product (Extended data fig. 8c) (4.42 g,

13.4 mmol, 47%) as white crystals. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.53 (s, 4H), 4.50 (s, 2H), 3.77 (t, J = 5.9 Hz, 2H), 2.99 – 2.88 (m, 2H), 2.46 (s, 6H), 1.86 – 1.74 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 142.13, 138.85, 131.67, 130.75, 62.24, 49.07, 48.75, 34.56, 26.59, 16.67. LCMS (electrospray) $[M+Na]^+$ calculated for $C_{14}H_{19}N_9NaO$ requires: 352.1 found: 352.1. HRMS $[M+H]^+$ calculated for $C_{14}H_{19}N_9NaO$ requires: 352.1605, found: 352.1604.

d. Synthesis of the alcohol tris-Boc

The alcohol tris-azide (1.40 g, 4.25 mmol, 1 eq.) and Boc_2O (4.18 g, 19.1 mmol, 4.5 eq.) were dissolved in anhydrous THF (80 mL). A slurry of Pd/C (200 mg, 10% w/w) in DCM was added, followed by Et_3N (1.80 mL, 12.8 mmol, 3 eq.). The reaction was placed under an atmosphere of hydrogen and stirred overnight. The reaction mixture was centrifuged, and the supernatant was concentrated under reduced pressure before being redissolved in EtOAc (100 mL) and washed with 5% aqueous $KHSO_4$ (100 mL), saturated aqueous $NaHCO_3$ (100 mL) and brine (100 mL), then dried (Na_2SO_4). The organic solvents were removed *in vacuo* and the crude product was purified by flash chromatography (petroleum ether 40/60 with increasing EtOAc). The fractions containing the product were combined and the solvent removed under vacuum to yield the product (Extended data fig. 8d) (2.02 g, 3.64 mmol, 86%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.37 (d, J = 11.0 Hz, 7H), 3.72 (t, J = 5.7 Hz, 2H), 2.94 – 2.77 (m, 2H), 2.36 (s, 6H), 1.76 – 1.65 (m, 2H), 1.44 (d, J = 2.4 Hz, 27H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.77, 155.69, 140.84, 137.19, 134.01, 133.44, 79.92, 79.70, 62.47, 39.40, 35.11, 28.50, 26.53, 21.17, 16.02. LCMS $[2M+H\text{-}Boc]^+$ calculated for $C_{53}H_{91}N_6O_{12}$ requires: 1003.7, found: 1003.6. HRMS $[M+H]^+$ calculated for $C_{29}H_{49}N_3O_7$ requires: 552.3643, found: 552.3633.

e. Synthesis of the O-mesylate tris-Boc

The alcohol tris-Boc (4.41 g, 7.99 mmol, 1 eq.) was dissolved in anhydrous DCM (16 mL). Et_3N (2.80 mL, 20.0 mmol, 2.5 eq.) was added and the reaction cooled to 0°C, followed by addition of MsCl (0.62 mL, 8.00 mmol, 1 eq.) over a period of 10 min. The reaction was left to stir at room temperature for 3 hours, then cooled to 0°C and more MsCl (0.40 ml, 5.17 mmol, 0.65 eq.) was added. After 3 hours stirring at room temperature, the reaction mixture was diluted with DCM (60 mL) and partitioned with brine (60 mL). The organics were dried (Na_2SO_4) and concentrated under reduced pressure. The crude product was purified by flash chromatography (petroleum ether 40/60 with increasing EtOAc). The fractions containing the product were combined and the solvent removed under vacuum to yield the product (Extended data fig. 8e) (4.85 g, 7.70 mmol, 96%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.38 – 4.29 (m, 9H), 3.11 (s, 3H), 2.99 – 2.78 (m, 2H), 2.36 (s, 6H), 1.89 (dq, J = 11.8, 5.9 Hz, 2H), 1.43 (s, 27H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.67, 155.54, 137.50, 133.20, 79.81, 68.57, 39.90, 39.52, 37.66, 31.57, 28.50, 26.19, 16.01. LCMS (electrospray) $[M+Na]^+$ calculated for $C_{30}H_{51}N_3NaO_9S$ requires: 652.3, found: 652.3. HRMS $[M+H]^+$ calculated for $C_{30}H_{51}N_3O_9S$ requires: 630.3419, found: 630.3407.

f. Synthesis of the azide tris-Boc

The O-mesylate tris-Boc (1.31 g, 2.08 mmol, 1 eq.) was dissolved in anhydrous DMF (21 mL), NaN_3 (676 mg, 10.4 mmol, 5 eq.) was added and the reaction stirred at room temperature for 22 hours. The reaction mixture was diluted with water (100 mL) and extracted with Et2O (5 × 50 mL). The organic layer was dried ($MgSO_4$) and concentrated under reduced pressure. The crude oil was purified by reverse phase flash chromatography. The fractions containing the product were combined and the solvent removed under vacuum to yield the product (Extended data fig. 8f) (800 mg, 1.39 mmol, 67%) as a white solid. ^{1}H

NMR (400 MHz, $CDCl_3$) δ 4.50 – 4.19 (m, 9H), 3.43 (t, J = 6.7 Hz, 2H), 2.86 – 2.72 (m, 2H), 2.37 (s, 6H), 1.79 – 1.68 (m, 2H), 1.45 (s, 27H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 155.59, 155.47, 139.80, 137.35, 134.24, 133.10, 79.63, 79.57, 51.42, 39.81, 39.45, 31.51, 28.41, 27.17, 15.91. LCMS (electrospray) $[2M+H\text{-}Boc]^+$ calculated for $C_{53}H_{89}N_{12}O_{10}$ requires: 1053.7, found: 1053.6. HRMS $[M+H]^+$ calculated for $C_{29}H_{48}N_6O_6$ requires: 577.3708, found: 577.3698.

g. Synthesis of the propyl azide tris-isocyanate

The azide tris-Boc (350 mg, 0.617 mmol, 1 eq.) was dissolved in anhydrous dichloromethane (6 mL), followed by addition of anhydrous 2-chloropyridine (0.51 mL, 5.46 mmol, 9 eq.). Triflic anhydride (0.46 mL, 2.73 mmol, 4.5 eq.) was added dropwise to the reaction mixture and the reaction stirred at room temperature for a further 30 min after addition was complete. The solvent was removed under vacuum to give a crude solid. The product was extracted with boiling petroleum ether 40/60 3 times, the extracts combined and concentrated under vacuum until a precipitate was observed. This suspension was then reheated to the boiling point until a solution was obtained, which was then cooled in a freezer at -18 °C overnight. The product crystals were then obtained by filtration, washing with cold petroleum ether 40/60 and drying under high vacuum to yield the product (Extended data fig. 8g) (170 mg, 0.480 mmol, 79%). ^1H NMR (400 MHz, $CDCl_3$) δ 4.50 (s, 6H), 3.49 (t, J = 6.2 Hz, 2H), 2.92 – 2.76 (m, 2H), 2.48 (s, 6H), 1.79 (dq, J = 11.9, 6.1 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 139.12, 137.06, 134.30, 133.01, 123.54, 51.34, 41.51, 41.26, 31.23, 27.19, 15.87. LCMS (electrospray) $[M+Na]^+$ calculated for $C_{17}H_{18}N_6NaO_3$ requires: 377.1 found: 377.2.

h. Synthesis of the macrocycle roof propyl azide

The half macrocycle was synthesised according to Tromans et al.[4,40] (Extended data fig. 8h). The half macrocycle (315 mg, 0.363 mmol, 1 eq.) was dissolved in anhydrous DMF (26 mL) and anhydrous pyridine (60 mL) and heated to 45ºC. The propyl azide tris-isocyanate (Extended data fig. 8g) (154 mg, 0.435 mmol, 1.2 eq.) was then added as a solution in anhydrous toluene (2.5 mL) over 10 hours and the reaction mixture left to stir for a further 6 hours. The reaction mixture was then concentrated under vacuum and the crude residue precipitated with 1 M aqueous HCI (1000 mL), filtered, washed with water and dried. The filtered material was dissolved in EtOH/water 1:1 mixture (30 mL) and NaOH (144 mg, 3.60 mmol, 10 eq.) was added. The reaction was stirred at 40 ºC for 6 hours. The reaction was cooled to room temperature and the organic solvent removed under vacuum. The crude product was then precipitated with 1 M aqueous HCI (400 mL), filtered, washed with 1 M aqueous HCI and dried. The crude solid was then dissolved in acetone/water, dry loaded onto C18 column and purified by reverse phase flash chromatography. The fractions containing the product were combined and the solvent removed under vacuum to yield the product (Extended data fig. 8i) (278 mg, 0.244 mmol, 68%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (s, 3 H, H-11), 8.06 (m, 3 H, H-8), 7.92 (m, 3 H, NH), 7.59 (d, J=9.2 Hz, 3 H, H-9), 7.49 (s, 3 H, NH), 6.60-6.35 (m, 6 H, NH), 4.35 (m, 12 H, H-5 and H-15), 3.46 (m, 2 H, H-23), 2.89 (m, 2 H, H-21), 2.67 (m, 6 H, H-2), 2.39 (s, 6 H, H-18), 1.67 (m, 2 H, H-22), 1.15 (t, J=7.2 Hz, 9 H, H-1). ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.37 (C-13), 156.11 (C-14), 155.93 (C-14), 154.67 (C-6), 142.57 (C-3), 139.05 (C-20), 136.82, 136.66, 136.47, 135.06, 133.96 (unassigned aryl – C-7, C-16, C-17 and C-19), 132.99 (C-4), 132.92 (C-4), 128.24 (C-10 or C-12), 125.75 (C-9 and C-11), 125.51 (C-9 and C-11), 125.41 (C-9 and C-11), 123.95 (C-10 or C-12), 119.56 (C-8), 50.96 (C-23), 38.43 (C-5 or C-15), 37.89 (C-5 or C-15), 37.01 (C-5 or C-15), 31.18 (C-22), 26.57 (C-21), 22.54 (C-2), 16.52 (C-1), 16.47 (C-

1), 16.01 (C-18). LCMS (electrospray), $[M+H]^+$ calculated for $C_{56}H_{63}N_{15}O_{12}$ requires:1138.5, found: 1138.4. HRMS $[M+H]^+$ calculated for $C_{56}H_{63}N_{15}O_{12}$ requires: 1138.4853, found: 1138.4815.

4. Preparation of insulin conjugate NNC2215

DesB30 human insulin (1.96 g, 343 mmol) was dissolved in 150 mM K_2CO_3 buffer (50 mL) and pH was checked to be 10.5. O-Succinimidyl pentyn-1-oxycarbonyl (Extended data fig. 7a) (116 mg, 515 mmol) was dissolved in DMSO (1 mL) and added to the insulin solution. After 1 hour, the reaction was acidified using TFA, and the B29-alkyne was purified by RP-HPLC on C18 column using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The product was isolated by lyophilisation, 0.75 g (38% yield). LCMS measured 1454.9, calculated 1455.1 for $[M+4H]^{4+}$.

The given B29-alkyne insulin (255 mg, 44 mmol) was dissolved in 40 mM phosphate buffer (30 mL) at pH 7.5 and mixed with a solution of beta-carboxymethyl-oxyethyl-O-peracetyl-D-glucoside active ester (Extended data fig. 7b) (66 mg, 88 mmol) in DMSO (1 mL). The mixture was gently stirred for 2 days, while the reaction was monitored by LCMS analysis. The reaction mixture was then acidified using TFA, and the B1-O-peracetyl-glycoside B29-alkyne insulin was purified by RP-HPLC on C18 column using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The product was isolated by lyophilisation, 72 mg (26% yield). LCMS measured 1563.1, calculated 1563.2 for $[M+4H]^{4+}$.

The given B1-O-peracetyl-glycoside B29-alkyne insulin (78 mg, 12 mmol) was dissolved in 400 mM Na_2CO_3 buffer (20 mL) at pH 10.5. Saponification of the O-acetyl esters were monitored by LCMS analysis. After 6 hours, the reaction mixture was acidified using TFA,

and the B1-glycoside B29-alkyne insulin was purified by RP-HPLC on C18 column using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The product was isolated by lyophilisation, 45 mg (62% yield). LCMS measured 1520.9, calculated 1521.2 for $[M+4H]^{4+}$.

The given B1-glycoside B29-alkyne insulin (20 mg, 3 mmol) and macrocycle roof propyl azide (Extended data fig. 8i) were dissolved in a mixture of DMSO (1.5 mL) and 2 M triethanolamine acetate (1.5 mL). Tris(3-hydroxypropyltriazolylmethyl)amine was added (1 mg, 1.9 umol), and the reaction mixture was degassed, flushed with argon and kept under argon atmosphere. CuI was added (1 mg, 5.2 umol) and click reaction was monitored by LCMS analysis. After 2 hours, the reaction mixture was acidified using TFA and NNC2215 (Fig. 2a) was purified by RP-HPLC on C18 column using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The product was isolated by lyophilisation, 12.8 mg (60% yield). LCMS measured 1805.5 and 1444.6, calculated 1805.8 for $[M+4H]^{4+}$ and 1444.8 for $[M+5H]^{5+}$. High-resolution MS: 7214.1821, calculated 7214.1894 for $C_{325}H_{461}N_{79}O_{97}S_6$. An analytical sample of NNC2215 was treated with trypsin followed by TCEP. LCMS of the reaction mixture showed the macrocycle attached to the B23-B29 fragment: 1054.5, calculated 1054.0 for $[M+2H]^{2+}$, and the glucoside attached to the B1-B22 fragment: 1376.6, calculated 1376.2 for $[M+2H]^{2+}$, along with free A-chain 1192.5, calculated 1192.0 for $[M+2H]^{2+}$.

5. Preparation of control compound NNC2215a (without glucoside)

NNC2215a (Extended data fig. 9) was prepared as described for NNC2215, except the glycoside conjugation step to B1 was omitted. Accordingly, the B29-alkyne of desB30 human insulin was prepared as described above in Section 4. The resulting insulin alkyne

(10 mg, 1.7 µmol) was dissolved in 1:1 DMSO and 2 M aqueous triethylamine (1 mL). Tris(3-hydroxypropyltriazolylmethyl)amine was added (0.2 mg, 0.52 µmol) followed by macrocycle roof propyl azide (22.3 mg, 2.0 µmol; Extended data fig. 8i) and CuI (spatula tip). The reaction was followed by LCMS, and was completed after 45 min. The reaction mixture was acidified using TFA, and NNC2215a was purified by RP-HPLC on C18 column using 0.1% TFA in water as buffer A and 0.1% TFA in acetonitrile as buffer B. The product was isolated by lyophilisation, 5 mg (42% yield). LCMS measured 1739.6 and 1391.8, calculated 1739.7 for $[M+4H]^{4+}$ and 1392.0 for $[M+5H]^{5+}$. High-resolution MS: 6950.1033, calculated 6950.1048 for $C_{315}H_{445}N_{79}O_{89}S_6$. An analytical sample of NNC2215a was treated with trypsin. LCMS of the reaction mixture showed the macrocycle attached to the B23-B29 fragment: 1054.5, calculated 1054.0 for $[M+2H]^{2+}$, along with the B1-B22 fragment connected to the A-chain: 1622.80, calculated 1622.84 $[M+3H]^{3+}$.

6. Pharmacokinetic/pharmacodynamic models

The relationship between plasma pharmacokinetics of NNC2215 and the plasma glucose in pigs was evaluated using non-linear mixed effects pharmacokinetic/pharmacodynamic modelling using the Phoenix NLME® software (Certara, NJ, USA). First, the plasma pharmacokinetic data were fitted to a standard 2-compartment model with first order elimination, resulting in simulated plasma pharmacokinetics for each pig. Next, the simulated plasma concentrations were used in the minimal model for insulin effect on glucose[32].

The key equations of the minimal model were the following:

$$dX/dt = -p2 \cdot X + p3 \cdot \mathrm{ConcIns} \qquad \text{Eq. 1a}$$

which can be rewritten as

$$dX/dt = -p2 \cdot (X - SI \cdot \mathrm{ConcIns}) \qquad \text{Eq. 1b}$$

and

$$dG/dt = -(SG + X) \cdot G + SG \cdot GB + GIR \qquad \text{Eq. 2}$$

where X is the insulin concentration in the remote insulin action compartment, p2 is the rate constant for loss of insulin action, p3 is the rate constant for gain of insulin action from the central compartment, ConcIns is the (simulated) concentration of insulin in the central compartment, SI is given by the ratio p3/p2 and is interpreted as the potency of a given insulin, G is plasma glucose (in mmol/kg), SG is the rate constant for insulin-independent glucose production and elimination, GB is the steady-state glucose level and GIR is the glucose input (in mmol/h/kg). The plasma D-glucose concentration, DGC, is derived as G/VG, where VG is the volume of distribution for glucose (in l/kg). For NNC2215, the glucose sensitivity of SI was estimated as a saturable Michaelis-Menten-like equation:

$$\mathrm{SI} = \mathrm{SI}_{max} \cdot \mathrm{DGC}/(\mathrm{SIEC50} + \mathrm{DGC}) \qquad \text{Eq. 3}$$

where SI_{max} is the maximum SI and SIEC50 is the glucose concentration giving 50% of the SImax. To ease the interpretation of the SI, an SI index was calculated as the percentage of the SI at 5.5 mM D-glucose:

$$\text{SI index} = 100\% \cdot \mathrm{SI}/(\text{SI at 5.5 mM D-glucose}) \qquad \text{Eq. 4}$$