Site-Selective Conjugation of Thiols with Aziridine-2-Carboxylic Acid-Containing Peptides

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Supporting Information

General. All reactions were performed in dry modified Schlenk (Kjeldahl shape) flasks fitted with a glass stopper under a positive pressure of argon, unless otherwise noted. Air- and moisture-sensitive liquids were transferred via syringe. Organic solutions were concentrated by rotary evaporation below 30 °C at ca. 25 Torr. Flash column chromatography was performed employing 230-400 mesh silica gel. Thin-layer chromatography was performed using glass plates pre-coated to a depth of 0.25 mm with 230-400 mesh silica gel impregnated with a fluorescent indicator (254 nm). When necessary, solvents were degassed by the freeze-pump thaw method (>3 cycles).

Dichloromethane, toluene, acetonitrile, and tetrahydrofuran were purified by passage through two packed columns of neutral alumina under an argon atmosphere. Chloroform, pyridine, diisopropylethylamine and triethylamine were distilled from calcium hydride at 760 Torr. Methanol was distilled from magnesium oxide at 760 Torr. DMF was dried over activated 4 Å molecular sieves. Millipore water was used for reactions in an aqueous solvent or co-solvent.

Infrared (IR) spectra were obtained using a Perkin Elmer Spectrum BX spectrophotometer referenced to polystyrene standard. Data are presented as frequency of absorption (cm^{-1}). Proton and carbon-13 nuclear magnetic resonance (^1H NMR or ^{13}C NMR) spectra were recorded on a Varian 400, a Varian 500, a Varian 750 or a Varian Inova 500 NMR spectrometer; chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane and are referenced to residual protium in the NMR solvent (CHCl_3 : δ 7.27). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet and/or multiple resonances), and coupling constants in Hertz (Hz), and integration.

RP- HPLC purification and analyses were carried out on the Rainin HPLC system (Model SD 200), equipped with UV absorbance detector (Model UV 1), at a wavelength of 220 nm. The products were analyzed and purified on Vydac C18 columns (analytical: 250 x 4.6 mm and preparative: 250 x 22 mm). Products were eluted in 0.1% TFA in acetonitrile/water in a linear gradient optimized for every compound at flow rates of 1 mL min^{-1} for analytical and 8 mL min^{-1} on the preparative column.

TrtSerNHBn: Benzylamine (1.5 mL, 13.4 mmol, 3 equiv) was added dropwise to a solution of triethylammonium tritylserine (2.0 g, 4.46 mmol, 1 equiv), and BOP (3.07 g, 4.68 mmol, 1.05 equiv) in chloroform (45 mL) at 23 °C. The resulting solution was stirred at this temperature for 11 h, diluted with dichloromethane (1 L), and washed subsequently with 10 % aqueous citric acid (300 mL), saturated aqueous sodium bicarbonate (300 mL), and saturated aqueous sodium chloride solution (300 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica-gel flash chromatography (30 % ethyl acetate in toluene to 40 % ethyl acetate in toluene) to afford the amide (1.87 g, 96 %) as a white solid. M.p. 65 °C; R_f = 0.26 (40% ethyl acetate in toluene); ^1H NMR (400 MHz, CDCl_3): δ = 7.66 (t, J = 5.7 Hz, 1H), 7.43-7.17 (m, 20 H), 4.42 (dd, J = 14.9, 6.4 Hz, 1H), 4.30 (dd, J = 14.8, 5.6 Hz, 1H), 3.58 (ddd, J = 10.5, 5.4, 3.4 Hz, 1H), 3.34 (m, 1H), 2.99 (d, J = 6.9 Hz, 1H), 2.63 (pentet, J = 5.4 Hz, 1H), 2.16 ppm (t, J = 5.8 Hz, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ = 174.0, 145.9, 138.4, 129.0, 128.9, 128.5, 128.3, 127.8, 127.7, 127.1, 71.8, 63.6, 59.2, 43.6 ppm; FTIR (neat film): 3368, 1652, 1448, 731 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{29}\text{H}_{29}\text{N}_2\text{O}_2$ [$M+\text{H}$] $^+$: 437.2229; found 437.2187.

TrtAzyNHBn: Triethylamine (0.9 mL, 6.4 mmol, 1.5 equiv) was added dropwise to a solution of TrtSerNHBn (1.87 g, 4.3 mmol, 1 equiv) and methanesulfonyl chloride (365 μL , 4.7 mmol, 1.1 equiv) in dichloromethane (21 mL) at 0 °C. The resulting white slurry was stirred at this temperature for 1 h 15 min, diluted with dichloromethane (1 L) and washed subsequently with 10 % aqueous citric acid (200 mL), and water (200 mL). The organic layer was dried (sodium sulfate) and concentrated. The residue was suspended in THF (18 mL), and triethylamine (1.2 mL, 8.6 mmol, 2 equiv) was added dropwise. The resulting solution was heated to 70 °C for 1 h 20 min, cooled to 23 °C, diluted with dichloromethane (1 L), and washed subsequently with 10 % aqueous citric acid (200 mL), saturated aqueous sodium bicarbonate (200 mL), and water (200 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica gel flash chromatography (10 % ethyl acetate in toluene to 12 % ethyl acetate in toluene) to afford the aziridine (1.50 g, 84 %) as a white solid. M.p. 88 °C; R_f = 0.21 (10 % ethyl acetate in toluene); ^1H NMR (500 MHz, CDCl_3): δ = 7.39-7.11 (m, 21 H), 4.78 (dd, J = 14.9, 7.6 Hz, 1H), 4.34 (dd, J = 15.1, 5.0 Hz, 1H), 2.09 (dd, J = 6.6, 2.7 Hz, 1H), 2.02 (d, J = 2.6 Hz, 1H), 1.51 ppm (d, J = 6.7 Hz, 1H); ^{13}C NMR (126 MHz, CDCl_3): δ = 171, 143.4, 138.7, 129.5, 129.0, 128.0, 127.9, 127.7, 127.4, 74.7, 43.1, 34.3, 30.0 ppm; FTIR (neat film): 3307, 1660, 1526, 731 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{29}\text{H}_{27}\text{N}_2\text{O}$ [$M+\text{H}$] $^+$: 419.2123; found 419.2124.

HAzyNHBn: Trifluoroacetic acid (2.3 mL, 28.6 mmol, 20 equiv) was added dropwise to a solution of TrtAzyNHBn (586 mg, 1.4 mmol, 1equiv) in a mixture of chloroform (2.2 mL) and methanol (1.8 mL) at 0 °C. The resulting solution was stirred at this temperature for 17.5 h, transferred to a flask with cold MeOH, and concentrated by rotary evaporation (bath ~ 10 °C). The residue was partitioned between ethyl acetate (50 mL) and water (3 x 20 mL). Combined aqueous fractions were neutralized with sodium bicarbonate (s), and extracted with dichloromethane (5 x 80 mL). The organic layer was dried (sodium sulfate), filtered and concentrated to afford the free aziridine (221 mg, 90 %) as a white solid. M.p. 68 °C; R_f = 0.24 (10 % methanol in dichloromethane); ^1H NMR (400 MHz, CDCl_3): δ = 7.31-7.19 (m, 5 H), 7.10 (br s, 1H), 4.36 (d, J = 5.9 Hz, 2H), 2.40 (dd, J = 5.7, 2.9 Hz, 1H), 1.79 (d, J = 5.8 Hz, 1H), 1.76 (d, J = 1.9 Hz, 1H), 1.24 ppm (br s, 1H); ^{13}C NMR (100 MHz, CDCl_3): δ = 171.0, 138.0, 128.7, 127.7, 127.6, 43.5, 30.4, 26.4 ppm; FTIR (neat film): 3272, 1652, 1558, 1253 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{10}\text{H}_{13}\text{N}_2\text{O}$ [$M+\text{H}$] $^+$: 177.1028; found 177.1028.

AcAzyNHBn (1): Acetylchloride (26 μL , 0.34 mmol, 1.05 equiv) was added dropwise to a solution of HazyNHBn (58 mg, 0.34 mmol, 1.0 equiv) and triethylamine (52 μL , 0.38 mmol, 1.1 equiv) in dichloromethane (2.0 mL) at 0 °C. The resulting solution was stirred at this temperature for 18 min, diluted with dichloromethane (100 mL), and washed sequentially with 1 M aqueous sodium dihydrogen phosphate (75 mL), water (75 mL), 5 % aqueous sodium bicarbonate (75 mL), and saturated aqueous sodium chloride (75 mL). The organic layer was dried (sodium sulfate), filtered and concentrated. The residue was purified by silica gel flash chromatography (66 % ethyl acetate in dichloromethane) to afford the AcAzyNHBn **1** (60 mg, 83 %) as a white solid. M.p. 72 °C; R_f = 0.23 (66 % ethylacetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.37-7.22 (m, 5 H), 6.43 (br s, 1H), 4.47 (A of ABX, J = 14.7, 6.5 Hz, 1H), 4.43 (B of ABX, J = 14.7, 5.0 Hz, 1H), 3.08 (dd, J = 6.5, 3.0 Hz, 1H), 2.58 (dd, J = 6.4, 1.0 Hz, 1H), 2.36 (dd, J = 3.0, 0.9 Hz, 1H), 2.18 ppm (s, 3H); ^{13}C NMR (100 MHz, CD_3OD): δ = 183.8, 169.5, 139.7, 130.0, 128.8, 128.5, 44.4, 37.4, 31.2, 23.9 ppm; FTIR (neat film): 3306, 1699, 1655, 2365, 1182 cm^{-1} ; HRMS (ESI) : m/z calcd for $\text{C}_{12}\text{H}_{15}\text{N}_2\text{O}_2$ [$M+\text{H}$] $^+$: 219.1134; found 219.1123.

Reaction of AcAzyNHBn with EtSH: DBU (1.2 μL , 0.008 mmol, 0.1 equiv) was added to a solution of AcAzyNHBn **1** (18 mg, 0.08 mmol, 1 equiv), and ethanethiol (12 μL , 0.16 mmol, 2.0 equiv) in chloroform (500 μL) at 23 °C. The resulting solution was stirred at this temperature for 3 d, and then concentrated in vacuo. The residue was purified by silica-gel flash chromatography (50 % to 66 % ethyl acetate in dichloromethane, followed by 6 % to 10 % methanol in ethyl acetate) to afford cysteine derivative **2** (14 mg, 61 %), as a white solid and β^2 -amino acid adduct **3** (0.8 mg, 4 %), as a white solid.

AcCys(Et)NHBn (2): M. p. = 126 °C ; R_f = 0.32 (80 % ethyl acetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.35-7.27 (m, 5 H), 6.98 (br s, 1H), 6.59 (d, J = 7.0 Hz, 1H), 4.55 (dt, J = 7.5, 5.1 Hz, 1H), 4.45 (d, J = 5.7 Hz, 2H), 2.98 (dd, J = 13.7, 5.1 Hz, 1H), 2.80 (dd, J = 13.9, 8.0 Hz, 1H), 2.59 (m, 2H), 2.00 (s, 3H), 1.26 ppm (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ = 170.5, 170.4, 137.8, 128.9, 127.9, 127.8, 52.9, 43.9, 33.9, 26.7, 23.4, 14.9 ppm; FTIR (neat film): 3289, 1645, 1533 cm^{-1} ; HRMS (ESI) : m/z calcd for $\text{C}_{14}\text{H}_{21}\text{N}_2\text{O}_2\text{S}$ $[M+\text{H}]^+$: 281.1324; found 281.1335.

Acβ²EtS-HGlyNHBn (3): R_f = 0.17 (80 % ethyl acetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.38-7.29 (m, 5 H), 6.81 (br s, 1H), 6.27 (br s, 1H), 4.49 (A of ABX, J = 13.6, 4.0, 1H), 4.47 (B of ABX, J = 13.6, 4.0, 1H), 3.80 (ddd, J = 13.2, 7.4, 4.5 Hz, 1H), 3.51 (m, 2H), 2.62 (m, 2H), 2.1 (s, 3H), 1.25 ppm (t, J = 7.4 Hz, 3H); FTIR (neat film): 3290, 1652, 1548 cm^{-1} ; HRMS (ESI) : m/z calcd for $\text{C}_{14}\text{H}_{21}\text{N}_2\text{O}_2\text{S}$ $[M+\text{H}]^+$: 281.1324; found 281.1309.

TrtSerAlaOBn: *N,N*-diisopropylethylamine (2.1 mL, 12.2 mmol, 1.5 equiv) was added dropwise to a solution of triethylammonium tritylserine (4.0 g, 8.92 mmol, 1.1 equiv), alanine benzyl ester tosylate (2.85 g, 8.11 mmol, 1.0 equiv), and BOP (3.95 g, 8.92 mmol, 1.1 equiv) in chloroform (164 mL) at 23 °C. The resulting solution was stirred at this temperature for 12 h, diluted with chloroform (1 L), and washed subsequently with 10 % aqueous citric acid (400 mL), saturated aqueous sodium bicarbonate (400 mL), and saturated aqueous sodium chloride solution (400 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica-gel flash chromatography (25 % ethyl acetate in toluene to 30 % ethyl acetate in toluene) to afford the dipeptide (3.81 g, 92 %) as a white solid. M.p. 61 °C; R_f = 0.30 (30 % ethyl acetate in toluene); ^1H NMR (500 MHz, CDCl_3): δ = 7.49-7.17 (m, 20 H), 7.57 (d, J = 7.1 Hz, 1H), 5.17 (ABq, J_{AB} = 12.1 Hz, 2H), 4.34 (pentet, J = 7.3 Hz, 1H), 3.65 (ddd, J = 10.7, 6.9, 3.7 Hz, 1H), 3.25 (br s, 1H), 2.96 (br s, 1H), 2.88 (m, 1H), 2.30 (m, 1H), 1.35 ppm (d, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ = 174.0, 173.1, 146.1, 135.6, 129.3, 129.1, 128.9, 128.7, 128.5, 128.5, 128.3, 126.9, 71.8, 67.5, 64.5, 59.3, 48.5, 18.1 ppm; FTIR (neat film): 3368, 1740, 1656, 1448, 1209, 1153, 733 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{32}\text{H}_{33}\text{N}_2\text{O}_4$ $[M+\text{H}]^+$: 509.2440; found 509.2440.

TrtAzyAlaOBn: Triethylamine (1.55 mL, 11.2 mmol, 1.5 equiv) was added to a solution of TrtSerAlaOBn (3.8 g, 7.5 mmol, 1 equiv) and methanesulfonyl chloride (690

μL , 9.0 mmol, 1.2 equiv) in dichloromethane (20 mL) at 0 °C. The resulting white slurry was stirred at this temperature for 22 min, diluted with dichloromethane (1.3 L) and washed subsequently with 10 % aqueous citric acid (350 mL), and water (350 mL). The organic layer was dried (sodium sulfate) and concentrated. The residue was dissolved in THF (18 mL), and triethylamine (2.1 mL, 14.9 mmol, 2 equiv) was added dropwise. The resulting solution was heated to 70 °C for 2 h, cooled to room temperature, diluted with dichloromethane (1 L), and washed subsequently with 10 % aqueous citric acid (275 mL), saturated aqueous sodium bicarbonate (275 mL), and water (275 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica gel flash chromatography (6 % ethyl acetate in toluene to 7 % ethyl acetate in toluene) to afford the aziridine dipeptide (3.34 g, 91 %) as a white solid. M.p. 60 °C; R_f = 0.28 (7 % ethyl acetate in toluene); ^1H NMR (500 MHz, CDCl_3): δ = 7.46-7.17 (m, 20 H), 5.25 (ABq, J_{AB} = 12.3 Hz, 2H), 4.74 (pentet, J = 7.2 Hz, 1H), 2.36 (br s, 1H), 2.00 (m, 2H), 1.48 ppm (d, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ = 172.9, 170.7, 143.5, 135.6, 129.6, 129.3, 128.9, 128.7, 128.6, 128.4, 128.4, 128.0, 127.3, 125.5, 74.8, 67.4, 47.8, 34.2, 30.0, 18.8 ppm; FTIR (neat film): 3370, 1744, 1668, 1516, 1157, 906 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{32}\text{H}_{31}\text{N}_2\text{O}_3$ [$M+\text{H}$] $^+$: 491.2335; found 491.2335.

HazyAlaOBn: Trifluoroacetic acid (1.4 mL, 16.8 mmol, 5.5 equiv) was added dropwise to a solution of TrtAzyAlaOBn (1.5 g, 3.1 mmol, 1 equiv) in a mixture of chloroform (6.3 mL) and methanol (4.2 mL) at 0 °C. The resulting solution was stirred at this temperature for 2.5 h, transferred to a flask with cold MeOH, and concentrated by rotary evaporation (bath ~ 10 °C). The residue was partitioned between ethyl acetate (60 mL) and water (35 mL). The organic layer was reextracted with water (2 x 20 mL). The combined aqueous fractions were neutralized with sodium bicarbonate (s), and extracted with dichloromethane (5 x 200 mL). The organic layer was dried (sodium sulfate), filtered and concentrated to afford the free aziridine dipeptide (0.5 g, 66 %) as a white solid. M.p. 99 °C; R_f = 0.13 (ethyl acetate); ^1H NMR (500 MHz, CDCl_3): δ = 7.36 (m, 5 H), 6.74 (br s, 1H), 5.19 (ABq, J_{AB} = 12.3 Hz, 2H), 4.65 (pentet, J = 7.4 Hz, 1H), 2.50 (br s, 1H), 1.92 (br s, 1H), 1.82 (br s, 1H), 1.42 ppm (d, J = 7.2 Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ = 172.8, 170.8, 135.4, 128.8, 128.6, 128.3, 67.3, 48.2, 630.4, 26.6, 18.4 ppm; FTIR (neat film): 3372, 1738, 1659, 1549, 1147 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{13}\text{H}_{17}\text{N}_2\text{O}_3$ [$M+\text{H}$] $^+$: 249.1239; found 249.1238.

BocAlaAzyAlaOBn (4a). *N,N*-Diisopropylethylamine (475 μL , 2.1 mmol, 2.0 equiv) was added dropwise to a solution of Boc-alanine (393 mg, 1.6 mmol, 1.5 equiv), Hazy-Ala-OBn (259 mg, 1 mmol, 1.0 equiv), and BOP (912 mg, 1.6 mmol, 1.5 equiv) in chloroform (25 mL) at 23 °C. The resulting solution was stirred at this temperature for 40 min, diluted with dichloromethane (350 mL), and washed subsequently with 10 % aqueous citric acid (100 mL), saturated aqueous sodium bicarbonate (100 mL), and saturated aqueous sodium chloride (100 mL). The organic layer was dried (sodium

sulfate), filtered, and concentrated. The residue was purified by silica gel flash chromatography (20 % ethyl acetate in dichloromethane to 25 % ethyl acetate in dichloromethane) to afford the tripeptide **4a** (384 mg, 88 %) as a colorless oil. R_f = 0.19 (20 % ethyl acetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.37 (m, 5 H), 6.87 (d, J = 7.6 Hz, 1H), 5.20 (ABq, J_{AB} = 12.2 Hz, 2H), 5.10 (d, J = 7.2 Hz, 1H), 4.65 (pentet, J = 7.3 Hz, 1H), 4.30 (pentet, J = 7.5 Hz, 1H), 3.13 (dd, J = 6.6, 3.3 Hz, 1H), 2.76 (d, J = 6.7 Hz, 1H), 2.38 (d, J = 2.8 Hz, 1H), 1.46 (d, J = 7.2 Hz, 3H), 1.43 (d, J = 7.2 Hz, 3H), 1.42 ppm (s, 9H); ^{13}C NMR (100 MHz, CDCl_3): δ = 185.2, 172.7, 167.0, 155.4, 135.3, 128.9, 128.7, 128.4, 80.3, 67.6, 51.6, 48.1, 36.6, 31.0, 28.4, 19.0, 18.5 ppm; FTIR (neat film): 3307, 1683, 1522, 1159, 845 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{21}\text{H}_{29}\text{N}_3\text{O}_6\text{Na}$ [$M+\text{Na}$] $^+$: 442.1954; found 442.1956.

BocThrAzyAlaOBn (4b). *N,N*-Diisopropylethylamine (76 μL , 0.44 mmol, 2.0 equiv) was added dropwise to a solution of Boc-threonine (74 mg, 0.33 mmol, 1.5 equiv), HAzyAlaOBn (55 mg, 0.22 mmol, 1.0 equiv), and BOP (147 mg, 0.33 mmol, 1.5 equiv) in chloroform (5.1 mL) at 23 $^\circ\text{C}$. The resulting solution was stirred at this temperature for 1 h, diluted with dichloromethane (110 mL), and washed subsequently with 10 % aqueous citric acid (40 mL), saturated aqueous sodium bicarbonate (40 mL), and saturated aqueous sodium chloride (40 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica gel flash chromatography (50 % ethyl acetate in dichloromethane, followed by 66 % ethyl acetate in toluene) to afford the tripeptide **4b** (76 mg, 76 %) as a white solid. M. p. 47 $^\circ\text{C}$; R_f = 0.43 (ethyl acetate); ^1H NMR (500 MHz, CDCl_3): δ = 7.40-7.32 (m, 5 H), 7.10 (d, J = 7.7 Hz, 1H), 5.63 (d, J = 8.7 Hz, 1H), 5.19 (ABq, J_{AB} = 12.2 Hz, 2H), 4.64 (pentet, J = 7.2 Hz, 1H), 4.43 (m, 1H), 4.22 (dd, J = 8.9, 1.8 Hz, 1H), 3.18 (dd, J = 6.2, 3.2 Hz, 1H), 2.81 (d, J = 4.0 Hz, 1H), 2.71 (d, J = 6.5 Hz, 1H), 2.43 (d, J = 2.5 Hz, 1H), 1.43 (m, 12H), 1.27 ppm (d, J = 6.3 Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ = 183.2, 172.8, 167.1, 156.3, 135.3, 129.2, 128.9, 128.8, 80.4, 67.9, 67.6, 60.8, 48.2, 36.7, 30.7, 28.4, 19.9, 18.3 ppm; FTIR (neat film): 3351, 1694, 1530, 1164 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{22}\text{H}_{31}\text{N}_3\text{O}_7\text{Na}$ [$M+\text{Na}$] $^+$: 472.2060; found 472.2046.

BocValAzyAlaOBn (4c). *N,N*-Diisopropylethylamine (76 μL , 0.44 mmol, 2.0 equiv) was added dropwise to a solution of Boc-valine (73 mg, 0.33 mmol, 1.5 equiv), HAzyAlaOBn (54 mg, 0.22 mmol, 1.0 equiv), and BOP (145 mg, 0.33 mmol, 1.5 equiv) in chloroform (5.1 mL) at 23 $^\circ\text{C}$. The resulting solution was stirred at this temperature for 2 h, diluted with dichloromethane (110 mL), and washed subsequently with 10 % aqueous citric acid (40 mL), saturated aqueous sodium bicarbonate (40 mL), and saturated aqueous sodium chloride (40 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica gel flash chromatography (25 % ethyl acetate in toluene to 33 % ethyl acetate in toluene) to afford the tripeptide **4c** (80 mg, 81 %) as a colorless oil. R_f = 0.61 (ethyl acetate); ^1H NMR (500

MHz, CDCl₃): δ = 7.37-7.32 (m, 5 H), 6.97 (d, J = 7.7 Hz, 1H), 5.18 (m, 3H), 4.63 (pentet, J = 7.1 Hz, 1H), 4.13 (dd, J = 6.0, 2.5 Hz, 1H), 3.11 (dd, J = 6.5, 3.2 Hz, 1H), 2.71 (d, J = 6.5 Hz, 1H), 2.36 (d, J = 3.0 Hz, 1H), 2.25 (m, 1H), 1.40 (m, 12H), 1.01 (d, J = 6.7 Hz, 3H), 0.97 ppm (d, J = 6.9 Hz, 3H); ¹³C NMR (126 MHz, CDCl₃): δ = 184.1, 172.9, 167.1, 156.0, 135.3, 128.9, 128.4, 128.3, 80.2, 67.5, 61.1, 48.0, 36.6, 31.3, 31.0, 28.4, 19.6, 18.5, 17.7 ppm; FTIR (neat film): 3326, 1694, 1500, 1167 cm⁻¹; HRMS (FAB)⁺ : m/z calcd for C₂₃H₃₃N₃O₆Na [M+Na]⁺: 470.2267; found 470.2254.

BocPheAzyAlaOBn (4d). *N,N*-Diisopropylethylamine (60 μ L, 0.34 mmol, 2.6 equiv) was added dropwise to a solution of Boc-phenylalanine (69 mg, 0.26 mmol, 2.0 equiv), HAzyAlaOBn (32 mg, 0.13 mmol, 1.0 equiv), and BOP (116 mg, 0.26 mmol, 2.0 equiv) in chloroform (3.2 mL) at 23 °C. The resulting solution was stirred at this temperature for 1 h, diluted with dichloromethane (100 mL), and washed subsequently with 10 % aqueous citric acid (35 mL), saturated aqueous sodium bicarbonate (35 mL), and saturated aqueous sodium chloride (35 mL). The organic layer was dried (sodium sulfate), filtered, and concentrated. The residue was purified by silica gel flash chromatography (29 % ethyl acetate in toluene to 33 % ethyl acetate in toluene) to afford the tripeptide **4d** (57 mg, 89 %) as a white solid. M. p. 152 °C; R_f = 0.61 (ethyl acetate); ¹H NMR (500 MHz, CDCl₃): δ = 7.41-7.18 (m, 10 H), 6.80 (d, J = 7.8 Hz, 1H), 5.20 (m, 3H), 4.58 (pentet, J = 7.2 Hz, 1H), 4.46 (q, J = 7.4 Hz, 1H), 3.11 (dd, J = 13.4, 7.4 Hz, 1H), 3.07 (dd, J = 13.4, 6.7 Hz, 1H), 2.69 (dd, J = 6.6, 3.2 Hz, 1H), 2.56 (d, J = 6.4 Hz, 1H), 2.18 (d, J = 3.8 Hz, 1H), 1.40 ppm (m, 12H); ¹³C NMR (100 MHz, CDCl₃): δ = 183.0, 172.5, 167.0, 155.5, 136.3, 135.5, 129.6, 128.9, 128.9, 128.7, 128.4, 127.4, 80.5, 67.4, 57.3, 48.0, 38.8, 36.0, 31.1, 28.4, 18.2 ppm; FTIR (neat film): 3436, 1644, 1165 cm⁻¹; HRMS (FAB)⁺ : m/z calcd for C₂₇H₃₄N₃O₆ [M+H]⁺: 496.2448; found 496.2449.

General procedure for ligations with Azy-tripeptides. Reaction of BocAlaAzyAlaOBn with EtSH. DBU (1.0 μ L, 0.006 mmol, 0.1 equiv) was added to a solution of BocAlaAzyAlaOBn **4a** (27 mg, 0.06 mmol, 1 equiv), and ethanethiol (6 μ L, 0.08 mmol, 1.20 equiv) in acetonitrile (315 μ L) at 23 °C (*Note*: Ligations with thio 2-*N*-acetylamino-3,4,6-tri-*O*-acetyl-1,2-dideoxy- α -D-galactopiranoside **7**¹ as a nucleophile were performed in chloroform). Formation of a white precipitate was observed after 12 min. The resulting solution was stirred at this temperature for 3.5 h, and then concentrated in vacuo. The residue was purified by silica-gel flash chromatography (25 % ethyl acetate in dichloromethane) to afford cysteine derivative **5a** (27 mg, 85 %), as a white solid and β^2 -amino acid adduct **6a** (0.4 mg, 1 %), as a white solid (crude ¹H NMR ratio of **5a** : **6a** ~ 20:1).

BocAlaCys(Et)AlaOBn (5a, R = Et): M. p. = 53 °C; R_f = 0.30 (33 % ethyl acetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.39-7.33 (m, 5 H), 7.26 (br s, 1H), 7.06 (d, J = 6.4 Hz, 1H), 5.18 (ABq, J_{AB} = 12.3 Hz, 2H), 4.96 (br s, 1H), 4.59 (pentet, J = 7.2 Hz, 1H), 4.54 (m, 1H), 4.13 (m, 1H), 3.08 (dd, J = 14.8, 2.5 Hz, 1H), 2.77 (dd, J = 14.0, 7.1 Hz, 1H), 2.59 (m, 2H), 1.46 (s, 9H), 1.44 (d, J = 7.1 Hz, 3H), 1.39 (d, J = 7.3 Hz, 3H), 1.26 ppm (t, J = 7.5 Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ = 172.9, 172.4, 170.1, 156.0, 135.5, 128.8, 128.6, 128.4, 80.7, 67.4, 52.4, 50.9, 48.7, 33.5, 28.5, 26.5, 18.2, 18.0, 14.9 ppm; FTIR (neat film): 3317, 1737, 1680, 1645, 1532, 1166, 850 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{23}\text{H}_{36}\text{N}_3\text{O}_6\text{S}$ [$M+\text{H}$] $^+$: 482.2325; found 482.2318.

BocAla β^2 EtS-HGlyAlaOBn (6a, R = Et): R_f = 0.17 (33 % ethyl acetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.50 (d, J = 6.98 Hz, 1H), 7.40-7.34 (m, 5H), 7.18 (br s, 1H), 5.22 (ABq, J_{AB} = 12.2 Hz, 2H), 5.04 (d, J = 4.3 Hz, 1H), 4.66 (pentet, J = 7.7 Hz, 1H), 4.03 (pentet, J = 7.7 Hz, 1H), 3.93 (m, 1H), 3.55 (m, 2H), 2.56 (q, J = 7.4, 2H), 1.52 (d, J = 7.2 Hz, 3H), 1.44 (s, 9H), 1.32 (d, J = 7.0 Hz, 3H), 1.23 ppm (t, J = 7.5 Hz, 3H); FTIR (neat film): 3291, 1651, 1546, 1037 cm^{-1} ; HRMS (ESI): m/z calcd for $\text{C}_{23}\text{H}_{36}\text{N}_3\text{O}_6\text{S}$ [$M+\text{H}$] $^+$: 482.2325; found 482.2325.

BocAlaCys($t\text{Bu}$)AlaOBn (5a, R = $t\text{Bu}$): Colorless oil; R_f = 0.24 (25 % ethyl acetate in dichloromethane); ^1H NMR (500 MHz, CDCl_3): δ = 7.38-7.33 (m, 5 H), 7.19 (br s, 1H), 7.00 (d, J = 6.4 Hz, 1H), 5.18 (ABq, J_{AB} = 12.3 Hz, 2H), 4.98 (br s, 1H), 4.59 (pentet, J = 7.2 Hz, 1H), 4.53 (m, 1H), 4.14 (m, 1H), 3.09 (dd, J = 13.0, 4.1 Hz, 1H), 2.78 (dd, J = 13.0, 6.9 Hz, 1H), 1.46 (s, 9H), 1.44 (d, J = 7.2 Hz, 3H), 1.39 (d, J = 7.2 Hz, 3H), 1.34 ppm (s, 9H); ^{13}C NMR (126 MHz, CDCl_3): δ = 172.7, 172.4, 169.8, 155.9, 135.6, 128.8, 128.6, 128.4, 80.7, 67.3, 53.1, 50.9, 48.7, 43.4, 31.1, 30.4, 28.5, 28.5, 18.4, 18.2 ppm; FTIR (neat film): 3326, 1646, 1527, 1167 cm^{-1} ; HRMS (FAB) $^+$: m/z calcd for $\text{C}_{25}\text{H}_{40}\text{N}_3\text{O}_6\text{S}$ [$M+\text{H}$] $^+$: 510.2638; found 510.2644.

BocAlaCys($O\text{-Ac-}\alpha\text{-GalNAc}$)AlaOBn (5a, R = $O\text{-Ac-}\alpha\text{-GalNAc-SH}$): Colorless oil; R_f = 0.32 (ethyl acetate); ^1H NMR (500 MHz, CDCl_3): δ = 7.36 (m, 5H), 7.03 (d, J = 7.1 Hz, 1H), 6.99 (d, J = 7.3 Hz, 1H), 5.78 (d, J = 8.7 Hz, 1H), 5.56 (d, J = 5.4 Hz, 1H), 5.38 (d, J = 2.2 Hz, 1H), 5.18 (ABq, J = 12.3 Hz, 2H), 5.01 (dd, J = 11.9, 3.2 Hz, 1H), 4.79 (ddd, J = 11.6, 8.5, 5.4 Hz, 1H), 4.65 (dt, J = 7.7, 5.2 Hz, 1H), 4.57

(pentet, $J = 7.1$ Hz, 1H), 4.53 (t, $J = 6.4$ Hz, 1H), 4.16 (m, 3H), 3.10 (dd, $J = 13.7$, 7.2 Hz, 1H), 2.99 (dd, $J = 13.6$, 5.0 Hz, 1H), 2.17 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H), 1.46 (s, 9H), 1.43 (d, $J = 7.2$ Hz, 3H), 1.38 ppm (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): $\delta = 173.7$, 172.4, 171.2, 171.0, 170.8, 170.5, 170.4, 169.6, 155.9, 135.5, 128.8, 128.6, 128.4, 85.8, 68.5, 67.7, 67.4, 67.3, 62.0, 53.3, 50.1, 48.5, 48.1, 33.1, 28.5, 23.4, 23.3, 20.9, 20.9, 20.9, 20.9, 18.5, 18.0 ppm; FTIR (neat film): 3307, 1748, 1660, 1534, 1369, 1236 cm^{-1} ; HRMS (ESI) : m/z calcd for $\text{C}_{35}\text{H}_{51}\text{N}_4\text{O}_{14}\text{S}$ $[M+H]^+$: 783.3122; found 783.3119.

BocAla- β^2 (O-Ac- α -GalNAc)-HGlyAlaOBn (6a, O-Ac- α -GalNAc-SH): $R_f = 0.32$ (ethyl acetate); ^1H NMR (500 MHz, C_6D_6): $\delta = 7.32$ (br s, 1H), 7.12 (m, 5H), 7.00 (br s, 1H), 6.16 (d, $J = 4.9$ Hz, 1H), 5.82 (d, $J = 6.6$ Hz, 1H), 5.45 (br s, 1H), 5.22 (dd, $J = 11.6$, 2.3 Hz, 1H), 5.16 (dd, $J = 7.5$, 5.0 Hz, 1H), 5.09 (d, $J = 6.8$ Hz, 1H), 4.98 (ABq, $J_{AB} = 12.1$ Hz, 2H), 4.64 (pentet, $J = 7.3$ Hz, 1H), 4.44 (t, $J = 5.3$ Hz, 1H), 4.32 (dd, $J = 11.2$, 7.3 Hz, 1H), 4.20 (m, 2H), 3.88 (m, 1H), 3.72 (m, 2H), 1.84 (s, 3H), 1.70 (s, 3H), 1.61 (s, 6H), 1.45 (s, 9H), 1.26 (d, $J = 7.3$ Hz, 3H), 1.12 ppm (d, $J = 6.9$ Hz, 3H); FTIR (neat film): 3307, 1756, 1652, 1520, 1238, 1051 cm^{-1} ; HRMS (ESI): m/z calcd for $\text{C}_{35}\text{H}_{51}\text{N}_4\text{O}_{14}\text{S}$ $[M+H]^+$: 783.3122; found 783.3120.

BocThrCys(O-Ac- α -GalNAc)AlaOBn (5b): White solid. M.p. = 96 $^{\circ}\text{C}$; $R_f = 0.19$ (ethyl acetate); ^1H NMR (500 MHz, CDCl_3): $\delta = 7.37 - 7.33$ (m, 5H), 7.27 (m, 2H), 6.12 (d, $J = 7.9$ Hz, 1H), 5.63 (m, 2H), 5.39 (d, $J = 2.7$ Hz, 1H), 5.17 (ABq, $J = 12.2$ Hz, 2H), 5.03 (dd, $J = 11.8$, 3.3 Hz, 1H), 4.75 (m, 2H), 4.56 (pentet, $J = 7.4$ Hz, 1H), 4.52 (t, $J = 6.8$ Hz, 1H), 4.41 (m, 1H), 4.17 (m, 3H), 3.52 (s, 1H), 3.07 (dd, $J = 14.3$, 8.1 Hz, 1H), 3.00 (dd, $J = 14.0$, 4.4 Hz, 1H), 2.17 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.46 (s, 9H), 1.41 (d, $J = 7.2$ Hz, 3H), 1.20 ppm (d, $J = 6.3$ Hz, 3H); ^{13}C NMR (126 MHz, C_6D_6): $\delta = 172.5$, 172.4, 171.3, 170.7, 170.6, 170.3, 170.2, 156.6, 136.0, 128.7, 128.4, 128.3, 85.4, 79.9, 68.9, 68.0, 67.9, 67.6, 67.0, 62.1, 60.1, 53.4, 48.7, 28.2, 22.9, 20.4, 20.4, 20.0, 19.3, 17.4 ppm; FTIR (neat film): 3307, 1748, 1668, 1652, 1538, 1371, 1235, 1161 cm^{-1} ; HRMS (ESI): m/z calcd for $\text{C}_{36}\text{H}_{53}\text{N}_4\text{O}_{15}\text{S}$ $[M+H]^+$: 813.3228; found 813.3226.

BocThr-β²(O-Ac-α-GalNAc)-HGlyAlaOBn (6b): R_f = 0.19 (ethyl acetate); ¹H NMR (500 MHz, CDCl₃): δ = 7.39-7.35 (m, 5H), 7.19 (d, J = 6.7 Hz, 1H), 5.78 (d, J = 5.4 Hz, 1H), 5.67 (d, J = 6.8 Hz, 1H), 5.54 (d, J = 6.6 Hz, 1H), 5.40 (d, J = 2.0 Hz, 1H), 5.20 (ABq, J_{AB} = 12.2 Hz, 2H), 5.07 (dd, J = 11.0, 2.9 Hz, 1H), 4.72 (m, 1H), 4.54 (m, 2H), 4.35 (m, 1H), 4.15 (m, 2H), 4.02 (dd, J = 8.1, 2.0 Hz, 1H), 3.93 (m, 1H), 3.64 (m, 2H), 2.17 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.47 (m, 12H), 1.20 ppm (d, J = 6.4 Hz, 3H); FTIR (neat film): 3293, 1744, 1656, 1529, 1245 cm⁻¹; HRMS (ESI): m/z calcd for C₃₆H₅₃N₄O₁₅S [$M+H$]⁺: 813.3228; found 813.3222.

BocValCys(O-Ac-α-GalNAc)AlaOBn (5c): White solid. M.p. = 109 °C; R_f = 0.37 (ethyl acetate); ¹H NMR (500 MHz, CDCl₃): δ = 7.39 – 7.33 (m, 5H), 7.26 (br s, 1H), 5.41 (m, 2H), 5.17 (ABq, J = 12.3 Hz, 2H), 5.06 (dd, J = 11.7, 2.9 Hz, 1H), 4.86 (m, 1H), 4.65 (dt, J = 8.2, 5.5 Hz, 1H), 4.57 (m, 2H), 4.23 (br s, 1H), 4.19 (dd, J = 11.5, 5.8 Hz, 1H), 4.13 (m, 2H), 3.04 (dd, J = 13.1, 8.7 Hz, 1H), 2.97 (dd, J = 13.04, 5.6 Hz, 1H), 2.19 (m, 1H), 2.17 (s, 3H), 2.04 (s, 6H), 2.00 (s, 3H), 1.46 (s, 9H), 1.40 (d, J = 7.2 Hz, 3H), 0.97 (d, J = 6.7 Hz, 3H), 0.88 ppm (d, J = 6.9 Hz, 3H); ¹³C NMR (126 MHz, CDCl₃): δ = 173.2, 172.3, 171.5, 171.1, 170.9, 170.6, 169.9, 156.4, 135.5, 128.8, 128.6, 128.4, 85.4, 80.5, 68.6, 67.6, 67.2, 62.3, 52.9, 48.5, 47.8, 32.3, 31.5, 28.6, 23.2, 20.9, 20.9, 20.9, 19.4, 18.0, 17.0 ppm; FTIR (neat film): 3280, 1748, 1682, 1660, 1645, 1557, 1538, 1371, 1242, 1085 cm⁻¹; HRMS (ESI): m/z calcd for C₃₇H₅₄N₄O₁₄NaS [$M+Na$]⁺: 833.3255; found 833.3282.

BocPheCys(O-Ac-α-GalNAc)AlaOBn (5d): White solid. M.p. = 99 °C; R_f = 0.45 (ethyl acetate); ¹H NMR (500 MHz, CDCl₃): δ = 7.38 – 7.19 (m, 10H), 7.02 (br s, 2H), 5.96 (br s, 1H), 5.42 (d, J = 4.9 Hz, 1H), 5.38 (dd, J = 2.8, 0.8 Hz, 1H), 5.17 (ABq, J = 12.3 Hz, 2H), 5.12 (br s, 1H), 5.01 (dd, J = 12.1, 3.2 Hz, 1H), 4.79 (ddd, J = 11.7, 9.0, 5.2 Hz, 1H), 4.62 (dt, J = 7.6, 5.2 Hz, 1H), 4.56 (pentet, J = 7.23 Hz, 1H), 4.52 (t, J = 6.4 Hz, 1H), 4.47 (br s, 1H), 4.19 (dd, J = 11.3, 6.1 Hz, 1H), 4.08 (dd, J = 11.3, 6.4 Hz, 1H), 3.14 (dd, J = 13.9, 5.6 Hz, 1H), 3.04 (dd, J = 13.7, 7.8 Hz, 1H), 2.97 (dd, J = 13.9, 7.7 Hz, 1H), 2.93 (dd, J = 13.7, 4.8 Hz, 1H), 2.17 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.98 (s, 3H), 1.41 ppm (m, 12H); ¹³C NMR (126 MHz, CDCl₃): δ = 172.5, 172.4, 171.3, 171.0,

170.7, 170.5, 169.5, 155.9, 136.6, 135.5, 129.5, 128.8, 128.7, 128.4, 127.1, 85.8, 80.7, 68.6, 67.7, 67.5, 67.3, 62.1, 55.5, 53.3, 48.5, 48.0, 38.6, 33.0, 28.5, 23.3, 20.9, 20.9, 20.9, 18.0 ppm; FTIR (neat film): 3301, 1748, 1660, 1652, 1538, 1236, 1052 cm^{-1} ; HRMS (ESI): m/z calcd for $\text{C}_{41}\text{H}_{55}\text{N}_4\text{O}_{14}\text{S}$ $[M+H]^+$: 859.3435; found 859.3438.

Fmoc-aziridine-2-carboxylic acid: A solution of FmocOSu (1.79g, 5.31 mmol, 1 equiv) in acetonitrile (20 mL) was added dropwise to a solution of the lithium salt of aziridine-2-carboxylic acid² (494 mg, 5.31 mmol, 1.0 equiv) and triethylamine (740 μL , 5.31 mmol, 1.0 equiv) in water (20 mL) at 0 °C. Formation of a white precipitate, which disappeared after 12 min, was noted. The resulting solution was stirred at this temperature for 50 min, then acidified to pH~1.5 with 1 M aqueous HCl, and partitioned between ethyl acetate (300 mL) and 1 M aqueous HCl (100 mL). The organic layer was washed with saturated aqueous sodium chloride (100 mL), and immediately (without concentration) loaded on and eluted off a pre-packed silica gel flash column (50 % ethylacetate in dichloromethane, followed by 20 % methanol in dichloromethane). Fractions containing product were combined, and concentrated to ~ 70 mL, and then coevaporated with benzene (6 x 70 mL), each time to ~ 70 mL residual volume. The remaining benzene was removed by lyophilization to afford the Fmoc-aziridine (1.40 g, 85%) as a white solid. (The purified product should not be concentrated to dryness by rotary evaporation, as that causes partial decomposition. After lyophilization, the white solid is stable to prolonged storage.) M.p. > 300 °C; R_f = 0.32 (20 % methanol in dichloromethane); ^1H NMR (500 MHz, DMF-d_7): δ = 7.93 (d, J = 7.7 Hz, 2H), 7.72 (t, J = 7.4 Hz, 2H), 7.43 (m, 2H), 7.36 (m, 2H), 4.39-4.27 (m, 3H), 2.97 (dd, J = 5.0, 3.5 Hz, 1H), 2.39 (dd, J = 3.3, 2.2 Hz, 1H), 2.23 ppm (dd, J = 5.1, 2.2 Hz, 1H); ^{13}C NMR (126 MHz, DMF-d_7): δ = 173.9, 162.3, 144.8, 144.4, 141.5, 141.4, 128.7, 128.1, 128.0, 127.5, 125.9, 125.8, 120.4, 120.4, 67.9, 47.2, 38.3, 30.6 ppm; FTIR (neat film): 3436, 1715, 1594, 1450, 1202, 738; 3306, 1699, 1655, 2365, 1182 cm^{-1} ; HRMS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{16}\text{NO}_4$ $[M+H]^+$: 310.1079; found 310.1077.

Solid phase peptide synthesis (SPPS). All peptides prepared on the solid phase were synthesized manually. 2-Chlorotrityl resins pre-loaded with C-terminal amino acids were purchased from Novabiochem. Extent of couplings, as well as the efficiency of Fmoc deprotection was determined by Kaiser test.³ (Note: Deprotected Azy residue, similar to Pro, stains red by this test).

General SPPS procedure. AcGlyGlyPheAzyAla-2-Chlorotrityl Resin (10). Alanine pre-loaded 2-chlorotrityl resin **8** (228 mg, 0.15 mmol, 1 equiv; loading 0.64 mmol/g) was swollen in $\text{CH}_2\text{Cl}_2/\text{DMF}$ (30 %, v/v) in a solid phase peptide synthesis reaction vessel (see Ace Glass) for 1 h. After swelling, resin was rinsed with DMF (10 x 2 mL). FmocAzyOH (190 mg, 0.6 mmol, 4 equiv), *O*-benzotriazole-*N,N,N',N'*-

tetramethyluronium hexafluorophosphate (HBTU, 227 mg, 0.6 mmol, 4 equiv), and 0.4 M *N*-methylmorpholine (NMM) in DMF (6 mL, 2.4 mmol, 16 equiv) were premixed in a vial and added to the resin. Nitrogen was bubbled through the reaction vessel for 2.5 h, after which the resin was rinsed with DMF (10 x 2 mL). To ensure complete reaction, coupling was repeated (FmocAzyOH: 93 mg, 0.3 mmol, 2 equiv; HBTU: 114 mg, 0.3 mmol, 2 equiv; 0.4 M NMM/DMF: 3 mL, 1.2 mmol, 8 equiv; 1 h). The resin was subsequently rinsed with DMF (10 x 2 mL), Fmoc was removed with 1% DBU/DMF (6 mL, 0.4 mmol, 2.7 equiv) for 1 min,⁴ and then rinsed with DMF (10 x 2 mL). Fmoc deprotection was repeated twice, in the same manner. FmocPheOH (236 mg, 0.6 mmol, 4 equiv), HBTU (228 mg, 0.6 mmol, 4 equiv) and 0.4 M NMM/DMF (6 mL, 2.4 mmol, 16 equiv) were premixed in a vial, and added to the reaction vessel containing resin. Nitrogen was bubbled through for 2.5 h, and resin was rinsed with DMF (10 x 2 mL), and the coupling was repeated to ensure complete acylation of the secondary amine within the Azy residue (FmocPheOH: 234 mg, 0.6 mmol, 4 equiv; HBTU: 227 mg, 0.6 mmol, 4 equiv; 0.4 M NMM/DMF: 6 mL, 2.4 mmol, 16 equiv; 1h). The resin was subsequently rinsed with DMF (10 x 2 mL), Fmoc was removed with 1 % DBU/DMF (6 mL, 0.4 mmol, 2.7 equiv) for 1 min, and then rinsed with DMF (10 x 2 mL). Fmoc deprotection was repeated twice, in the same manner. FmocGlyGlyOH (219 mg, 0.6 mmol, 4 equiv), HBTU (230 mg, 0.6 mmol, 4 equiv) and 0.4 M NMM/DMF (6 mL, 2.4 mmol, 16 equiv) were premixed in a vial, and added to the reaction vessel containing resin. Nitrogen was bubbled through for 2 h, and the resin was rinsed with DMF (10 x 2 mL). Fmoc was removed with 1% DBU/DMF (6 mL, 0.4 mmol, 2.7 equiv) for 1 min, and the resin was rinsed with DMF (10 x 2 mL). Fmoc deprotection was repeated twice, in the same manner. A solution of Ac₂O in pyridine (1:3, v/v; 15.5 mL, 37.5 mmol, 250 equiv) was then added to the SPPS reaction vessel with the resin, through which nitrogen was bubbled for 15 min. Following acetylation, the resin was washed with DMF and CH₂Cl₂, and stored at -10 °C under Ar until used for ligations. A portion of the product was cleaved from the resin with 20 % hexafluoroisopropanol (HFIPA) in dichloromethane (v/v). ¹H NMR analysis of a crude product showed ca. 85-90 % purity for the desired peptide. ¹H NMR (500 MHz, DMF-d₇): δ=8.64 (d, *J* = 6.9 Hz, 1H), 8.49 (br.s, 1H), 8.33 (t, *J* = 5.5 Hz, 1H), 8.21 (t, *J* = 6.0 Hz, 1H), 8.18 (d, *J* = 7.2 Hz, 1H), 7.33-7.21 (m, 5H), 4.68 (m, 1H), 4.39 (pentet, *J* = 7.1 Hz, 1H), 3.89-3.76 (m, 4H), 3.30 (dd, *J* = 5.6, 3.2 Hz, 1H), 3.25 (dd, *J* = 14.3, 4.5 Hz, 1H), 3.01 (dd, *J* = 14.0, 9.3 Hz, 1H), 2.65 (dd, *J* = 5.6, 2.0 Hz, 1H), 2.45 (dd, *J* = 3.2, 1.9 Hz, 1H), 1.96 (s, 3H), 1.41 ppm (d, *J* = 7.2 Hz, 3H). HRMS (ESI): *m/z* calcd for C₂₁H₂₈N₅O₇ [*M*+H]⁺: 462.1989; found 462.1992.

AcGlyGlyPheAzyAsp(^tBu)-2-Chlorotrityl Resin (11): ¹H NMR analysis of an analytical sample of the crude product cleaved from the resin showed ca. 80-85 % purity for the desired peptide. ¹H NMR (500 MHz, DMF-d₇): δ = 8.56 (d, *J* = 8.1 Hz, 1H), 8.47 (br.s, 1H), 8.30 (t, *J* = 5.4 Hz, 1H), 8.20 (t, *J* = 6.1 Hz, 1H), 8.15 (d, *J* = 7.8 Hz, 1H), 7.34-7.21 (m, 5H), 4.76 (m, 1H), 4.69 (ddd, *J* = 9.4, 7.8, 4.3 Hz, 1H), 3.90-3.76 (m, 4H), 3.31 (dd, *J* = 5.6, 3.0 Hz, 1H), 3.26 (dd, *J* = 14.0, 4.4 Hz, 1H), 3.01 (dd, *J* = 14.0, 9.4 Hz,

1H), 2.86 (dd, $J = 16.2, 6.0$ Hz, 1H), 2.78 (dd, $J = 16.2, 6.9$ Hz, 1H), 2.69 (dd, $J = 5.7, 1.8$ Hz, 1H), 2.43 (dd, $J = 3.0, 1.8$ Hz, 1H), 1.96 (s, 3H), 1.44 ppm (s, 9H). HRMS (ESI): m/z calcd for $C_{26}H_{36}N_5O_9$ $[M+H]^+$: 562.2513; found 562.2506.

AcLys(Boc)Ser(^tBu)Glu(^tBu)GlyPheAzyAla-2-Chlorotrityl Resin (12): 1H NMR analysis of an analytical sample of the crude product cleaved from the resin showed ca. 80-85 % purity for the desired peptide. HRMS (ESI): m/z calcd for $C_{46}H_{73}N_8O_{14}$ $[M+H]^+$: 961.5246; found 961.5233.

AcGlyGlyPheCys(Et)AlaOH (13a). DBU (2.4 μ L, 0.016 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAla-2-chlorotrityl resin **10** (32 mg, 0.016 mmol, 1 equiv) and ethanethiol (12 μ L, 0.16 mmol, 10 equiv) in DMF (650 μ L) at 23 $^{\circ}$ C. The resulting suspension was stirred at this temperature for 16 h, then the resin was washed with CH_2Cl_2 (25 x 1.5 mL) and the crude product mixture was cleaved from the resin with 20 % HFIPA/ CH_2Cl_2 (v/v; 9 mL, 15 min, 23 $^{\circ}$ C). Concentration in vacuo, and purification of the residue by preparative RP-HPLC (10 % CH_3CN in H_2O to 70 % CH_3CN in H_2O + 0.1 % TFA, in 50 min) provided conjugate **13a** (4 mg, 52 % from HAla-preloaded resin **8**; 9 steps, 93 % /step), as a white solid. R_t (10 % CH_3CN in H_2O to 70 % CH_3CN in H_2O + 0.1 % TFA, in 50 min, analytical column) = 14.60 min. 1H NMR (500 MHz, CD_3OD): δ = 8.35 (t, $J = 5.4$ Hz, 1H), 8.20 (d, $J = 7.3$ Hz, 1H), 8.16 (d, $J = 8.0$ Hz, 1H), 8.13 (d, $J = 7.6$ Hz, 1H), 7.27-7.19 (m, 5 H), 4.63 (ddd, $J = 9.6, 7.9, 5.1$ Hz, 1H), 4.52 (m, 1H), 4.36 (pentet, $J = 7.5$ Hz, 1H), 3.80 (m, 4H), 3.21 (dd, $J = 14.0, 5.1$ Hz, 1H), 3.04 (dd, $J = 13.9, 5.2$ Hz, 1H), 2.97 (dd, $J = 14.0, 9.6$ Hz, 1H), 2.80 (dd, $J = 14.0, 9.0$ Hz, 1H), 2.59 (qd, $J = 7.5, 1.7$ Hz, 2H), 2.02 (s, 3H), 1.41 (d, $J = 7.4$ Hz, 3H), 1.24 ppm (t, $J = 7.3$ Hz, 3H); HRMS (ESI): m/z calcd for $C_{23}H_{34}N_5O_7S$ $[M+H]^+$: 524.2179; found 524.2186.

AcGlyGlyPheCys(^tBu)AlaOH (13b). DBU (3.1 μ L, 0.021 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAla-2-chlorotrityl resin **10** (40 mg, 0.021 mmol, 1 equiv) and ethanethiol (24 μ L, 0.21 mmol, 10 equiv) in DMF (1.5 mL) at 23 $^{\circ}$ C. The resulting suspension was stirred at this temperature for 28 h, then the resin was washed with CH_2Cl_2 (25 x 1.5 mL) and the crude reaction mixture was cleaved from the resin with 20 % HFIPA/ CH_2Cl_2 (v/v; 9 mL, 16 min, 23 $^{\circ}$ C). Concentration in vacuo, and purification of residue by preparative RP-HPLC (10 % CH_3CN in H_2O to 70 % CH_3CN

in H₂O + 0.1 % TFA, in 50 min) provided conjugate **13b** (5 mg, 40 % from HAla-preloaded resin **8**; 9 steps, 90 % /step), as a white solid. *R*_t (10 % CH₃CN in H₂O to 70 % CH₃CN in H₂O + 0.1 % TFA, in 50 min, analytical column) = 19.95 min. ¹H NMR (500 MHz, CD₃OD): δ = 8.34 (t, *J* = 5.7 Hz, 1H), 8.18 (d, *J* = 7.8 Hz, 1H), 8.13 (d, *J* = 7.2 Hz, 1H), 8.12 (d, *J* = 6.2 Hz, 1H), 7.28-7.18 (m, 5 H), 4.62 (ddd, *J* = 9.3, 7.6, 5.0 Hz, 1H), 4.49 (m, 1H), 4.36 (pentet, *J* = 7.2 Hz, 1H), 3.80 (m, 4H), 3.20 (dd, *J* = 14.0, 5.0 Hz, 1H), 3.05 (dd, *J* = 13.2, 5.4 Hz, 1H), 2.96 (dd, *J* = 14.1, 9.5 Hz, 1H), 2.87 (dd, *J* = 13.0, 8.9 Hz, 1H), 2.03 (s, 3H), 1.41 (d, *J* = 7.4 Hz, 3H), 1.33 ppm (s, 9H); HRMS (ESI) : *m/z* calcd for C₂₅H₃₈N₅O₇S [*M*+H]⁺: 552.2492; found 552.2489.

AcGlyGlyPheCys(Et)AspOH (14a). DBU (4.4 μL, 0.03 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAsp(^tBu)-2-chlorotrityl resin **11** (52 mg, 0.03 mmol, 1 equiv) and ethanethiol (22 μL, 0.29 mmol, 10 equiv) in DMF (1.2 mL) at 23 °C. The resulting suspension was stirred at this temperature for 22 h, and then the resin was filtered and washed with dichloromethane (25 x 1.5 mL). The ligation product was cleaved from the resin by treatment with TFA: CH₂Cl₂: ¹Pr₃SiH: HS(CH₂)₂SH = 10:10: 1: 1 (10 mL) for 1.5 h at 23 °C. The resin was filtered off, and the filtrate coevaporated sequentially with toluene (2 x 4mL), followed by cold Et₂O (5 mL). The resulting white precipitate was washed with hexanes (3 x 2 mL), and cold Et₂O (3 x 1 mL). Purification of the residue by preparative RP-HPLC (10 % CH₃CN in H₂O to 70 % CH₃CN in H₂O + 0.1 % TFA, in 50 min) provided conjugate **14a** (6 mg, 35 % from HAsp(^tBu)-preloaded resin **9**; 9 steps, 89 % /step). *R*_t (10 % CH₃CN in H₂O to 70 % CH₃CN in H₂O + 0.1 % TFA, in 50 min, analytical column) = 13.80 min. ¹H NMR (500 MHz, CD₃OD): δ = 8.22 (m, 2 H), 8.11 (d, *J* = 7.6 Hz, 1H), 7.26 (m, 5H), 4.72 (m, 1H), 4.64 (m, 1H), 4.54 (m, 1H), 3.82 (m, 4H), 3.21 (dd, *J* = 14.0, 4.7 Hz, 1H), 3.03 (dd, *J* = 14.0, 5.4 Hz, 1H), 2.97 (dd, *J* = 14.0, 9.6 Hz, 1H), 2.85 (m, 3H), 2.58 (qd, *J* = 7.4, 1.0 Hz, 2H), 2.03 (s, 3H), 1.24 ppm (t, *J* = 7.6 Hz, 3H); HRMS (ESI): *m/z* calcd for C₂₄H₃₄N₅O₉S [*M*+H]⁺: 568.2077; found 568.2073.

AcGlyGlyPheCys(α-GalNAc)AlaOH (13c). DBU (7.2 μL, 0.05 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAla-2-chlorotrityl resin **10** (99 mg, 0.05 mmol, 1 equiv) and thio 2-*N*-acetylamino-3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-*D*-galactopyranose **7** (91 mg, 0.24 mmol, 5 equiv) in DMF (3.4 mL) at 60 °C. The resulting suspension was stirred at this temperature for 22 h, and then the resin was filtered and washed with dichloromethane (25 x 1.5 mL). The ligation product was cleaved from the resin with 20 % HFIPA/CH₂Cl₂ (v/v; 15 mL, 15 min, 23 °C). The residue was concentration in vacuo, and dissolved in methanol (30 mL). A solution of NaOMe in MeOH (0.1 M) was added until the pH reached 8.5,⁵ and the resulting solution was stirred

for 1 h 20 min at 23 °C. Subsequently, a 1 M solution of AcOH in MeOH was added until the pH reached 6. Concentration in vacuo, and purification of the residue by preparative RP-HPLC (7 % CH₃CN in H₂O to 26 % CH₃CN in H₂O + 0.1 % TFA, in 40 min) provided conjugate **13c** (7 mg, 21 % from HAla-preloaded resin **8**; 10 steps, 86 % /step). *R*_t (7 % CH₃CN in H₂O to 30 % CH₃CN in H₂O + 0.1 % TFA, in 40 min, analytical column) = 15.01 min. ¹H NMR (500 MHz, CD₃OD): δ = 8.36 (d, *J* = 8.6 Hz, 1H), 8.27 (t, *J* = 6.2 Hz, 1H), 8.22 (d, *J* = 6.8 Hz, 1H), 8.14 (d, *J* = 7.6 Hz, 1H), 8.05 (d, *J* = 7.6 Hz, 1H), 7.29-7.19 (m, 5 H), 5.59 (d, *J* = 5.5 Hz, 1H), 4.67 (m, 1H), 4.61 (m, 1H), 4.43 (m, 1H), 4.38 (pentet, *J* = 7.2 Hz, 1H), 4.17 (dd, *J* = 7.8, 3.9 Hz, 1H), 3.87 (m, 6H), 3.72 (dd, *J* = 11.4, 4.3 Hz, 1H), 3.65 (dd, *J* = 11.4, 3.2 Hz, 1H), 3.23 (dd, *J* = 13.8, 4.8 Hz, 1H), 3.11 (dd, *J* = 13.8, 4.4 Hz, 1H), 3.00 (dd, *J* = 14.6, 4.4 Hz, 1H), 2.95 (dd, *J* = 14.0, 9.6 Hz, 1H), 2.02 (s, 3H), 1.98 (s, 3H), 1.41 ppm (d, *J* = 7.4 Hz, 3H); HRMS (ESI) : *m/z* calcd for C₂₉H₄₃N₆O₁₂S [*M*+H]⁺: 699.2660; found 699.2656.

AcGlyGlyPheCys(α-GalNAc)AspOH (14b). DBU (7.0 μL, 0.05 mmol, 1 equiv) was added to a suspension of AcGlyGlyPheAzyAsp(^tBu)-2-chlorotrityl resin **11** (92 mg, 0.05 mmol, 1 equiv) and thio 2-*N*-acetylamino-3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-galactopyranose **7** (88 mg, 0.23 mmol, 5 equiv) in DMF (3.3 mL) at 60 °C. The resulting suspension was stirred at this temperature for 22 h, and then the resin was filtered and washed with dichloromethane (25 x 1.5 mL). The ligation product was cleaved from the resin by the treatment with TFA: CH₂Cl₂: ¹Pr₃SiH: HS(CH₂)₂SH = 10:10: 1: 1 (8 mL) for 1.5 h at 23 °C. Resin was filtered off, and the filtrate sequentially coevaporated with toluene (2 x 4 mL), followed by cold Et₂O (4 mL). The resulting white precipitate was washed with hexanes (3 x 2 mL), and cold Et₂O (3 x 3 mL), and then dissolved in methanol (40 mL). A solution of NaOMe in MeOH (0.1 M) was added until the pH reached 8.5 and the resulting solution was stirred for 1 h 20 min at 23 °C. Subsequently, a 1 M solution of AcOH in MeOH was added until the pH reached 6. Concentration in vacuo, and purification of the residue by preparative RP-HPLC (5 % CH₃CN in H₂O to 28 % CH₃CN in H₂O + 0.1 % TFA, in 40 min) provided conjugate **14b** (6 mg, 17 % from HAsp(^tBu)-preloaded resin **9**; 10 steps, 84 % /step). *R*_t (5 % CH₃CN in H₂O to 28 % CH₃CN in H₂O + 0.1 % TFA, in 40 min, analytical column) = 14.88 min. ¹H NMR (500 MHz, D₂O): δ = 7.35-7.24 (m, 5 H), 5.51 (d, *J* = 5.5 Hz, 1H), 4.61 (m, 2H), 4.53 (t, *J* = 6.1 Hz, 1H), 4.32 (dd, *J* = 11.6, 5.6 Hz, 1H), 4.13 (dd, *J* = 7.6, 4.6 Hz, 1H), 3.93 (d, *J* = 2.8 Hz, 1H), 3.88 (s, 2H), 3.86 (s, 2H), 3.74 (m, 3H), 3.13 (dd, *J* = 14.1, 6.7 Hz, 1H), 3.00 (m, 3H), 2.85 (m, 2H), 2.03 (s, 3H), 1.98 ppm (s, 3H); HRMS (ESI) : *m/z* calcd for C₃₀H₄₃N₆O₁₄S [*M*+H]⁺: 743.2558; found 743.2559.

AcLysSerGluGlyPheCys(α -GalNAc)AlaOH (15). DBU (3.1 μ L, 0.02 mmol, 1 equiv) was added to a suspension of AcLys(Boc)Ser(^tBu)Glu(^tBu)GlyPheAzyAla-2-chlorotrityl resin **12** (47 mg, 0.02 mmol, 1 equiv) and thio 2-*N*-acetylamino-3,4,6-tri-*O*-acetyl-1,2-dideoxy- α -D-galactopyranose **7** (38 mg, 0.10 mmol, 5 equiv) in DMF (2.3 mL) at 60 °C. The resulting suspension was stirred at this temperature for 22 h, and then the resin was filtered and washed with dichloromethane (25 x 1.5 mL). The ligation product was cleaved from the resin by treatment with TFA: CH₂Cl₂: ⁱPr₃SiH: HS(CH₂)₂SH = 10:10: 1: 1 (15 mL) for 1.5 h at 23 °C. The resin was filtered off, and the filtrate sequentially coevaporated with toluene (2 x 4 mL), followed by cold Et₂O (3 mL). The resulting white precipitate was washed with hexanes (3 x 2 mL), and cold Et₂O (3 x 1 mL), and then dissolved in methanol (30 mL). A solution of NaOMe in MeOH (0.1 M) was added until the pH reached 8.5, and the resulting solution was stirred for 1 h 20 min at 23 °C. Subsequently, a 1 M solution of AcOH in MeOH was added until the pH reached 6. Concentration in vacuo, and purification of the residue by preparative RP-HPLC (5 % CH₃CN in H₂O to 30 % CH₃CN in H₂O + 0.1 % TFA, in 40 min) provided conjugate **15** (4 mg, 19 % from HAla-preloaded resin **8**; 14 steps, 88.5 % /step). R_t (5 % CH₃CN in H₂O to 30 % CH₃CN in H₂O + 0.1 % TFA, in 40 min, analytical column) = 18.20 min. ¹H NMR (500 MHz, 1 mL DMF-d₇ + 40 μ L, D₂O, after H to D exchange): δ =7.35-7.19 (m, 5 H), 5.62 (d, *J* = 5.1 Hz, 1H), 4.66 (dd, *J* = 8.0, 4.8 Hz, 1H), 4.64 (dd, *J* = 9.7, 4.6 Hz, 1H), 4.46-4.35 (m, 5H), 4.14 (t, *J* = 6.2 Hz, 1H), 3.93 (d, *J* = 3.5 Hz, 1H), 3.88 (m, 2H), 3.78 (m, 4H), 3.66 (dd, *J* = 11.5, 3.0 Hz, 1H), 3.23 (dd, *J* = 14.0, 4.5 Hz, 1H), 3.06 (m, 4H), 2.95 (m, 1H), 2.45 (m, 2H), 2.18 (m, 1H), 1.99 (m, 1H), 1.97 (s, 3H), 1.94 (s, 3H), 1.85 (m, 1H), 1.76 (t, *J* = 7.6 Hz, 2H), 1.72 (m, 1H), 1.53 (m, 2H), 1.39 ppm (d, *J* = 7.3 Hz, 3H); HRMS (ESI) : *m/z* calcd for C₄₁H₆₄N₉O₁₇S [*M*+H]⁺: 986.4141; found 986.4131.

AcGlyGlyPheCys(NeuNAc α 2-6GalNAc α 1)AlaOH (17). A solution of DBU (9.6 μ L, 0.06 mmol, 4 equiv) in DMF (690 μ L) was added to a suspension of AcGlyGlyPheAzyAla-2-chlorotrityl resin **10** (34 mg, 0.016 mmol, 1 equiv) and thio 4,7,8,9-tetra-*O*-acetyl-5-*N*-acetylamino- α -D-sialosyl-(2-6)-2-*N*-acetylamino-3,4,6-tri-*O*-acetyl-1,2-dideoxy- α -D-galactopyranose **16** (39 mg, 0.054 mmol, 3 equiv).¹ The resulting suspension was stirred at 23 °C for 4 d, and then the resin was filtered and washed with dichloromethane (25 x 1.5 mL). The ligation product was cleaved from the resin with 20

% HFIPA/CH₂Cl₂ (v/v; 15 mL, 15 min, 23 °C). The residue was concentration in vacuo, and dissolved in methanol (11 mL). A solution of NaOMe in MeOH (0.1 M) was added until the pH reached 8.5,⁵ and the resulting solution was stirred for 32 h at 23 °C. Subsequently, a 1 M solution of AcOH in MeOH was added until the pH reached 5.5. Concentration in vacuo, and purification of the residue by preparative RP-HPLC (5 % CH₃CN in H₂O to 28 % CH₃CN in H₂O + 0.1 % TFA, in 40 min) provided 12 mg of conjugate **17** containing minor quantity of inseparable DBU-TFA (molar ratio **17**: DBU: TFA = 1: 1.6: 4.2; ¹H and ¹⁹F NMR). The other examples in this manuscript did not co-purify with DBU and so were not analyzed for the presence of TFA. Calculated yield of **17** = 7 mg, 43 % from HAla-preloaded resin **8**; 10 steps, 92 % /step. R_t (5 % CH₃CN in H₂O to 28 % CH₃CN in H₂O + 0.1 % TFA, in 40 min, analytical column) = 16.37 min. ¹H NMR (500 MHz, CD₃OD): δ = 8.16 (m, 2H), 7.28 (m, 5H), 7.20 (m, 2H), 5.61 (d, *J* = 5.5 Hz, 1H), 4.59 (m, 2H), 4.39 (m, 2H), 4.22 (t, *J* = 6.3 Hz, 1H), 3.98-3.51 (m, 15H), 3.24 (dd, *J* = 13.8, 5.0 Hz, 1H), 2.98 (m, 3H), 2.71 (dd, *J* = 12.8, 4.8 Hz, 1H), 2.03 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H), 1.78 (t, *J* = 12.3 Hz, 1H), 1.41 ppm (d, *J* = 7.4 Hz, 3H); HRMS (ESI) : *m/z* calcd for C₄₀H₆₀N₇O₂₀S [*M*+H]⁺: 990.3614; found 990.3630.

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