Supporting Information

Fucosylated Ubiquitin and Orthogonally Glycosylated Mutant A28C: Conceptually New Ligands for Burkholderia ambifaria Lectin (BambL)

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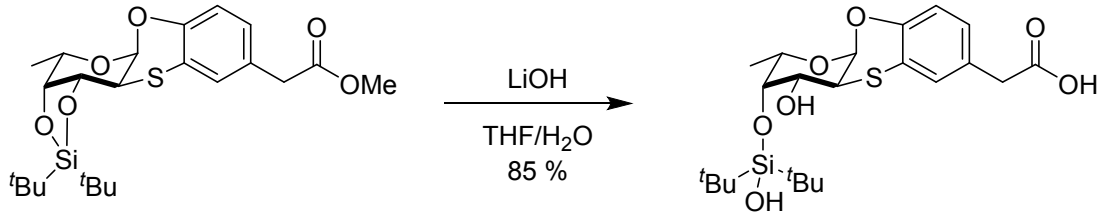
Table of content

Synthesis of compound 5:

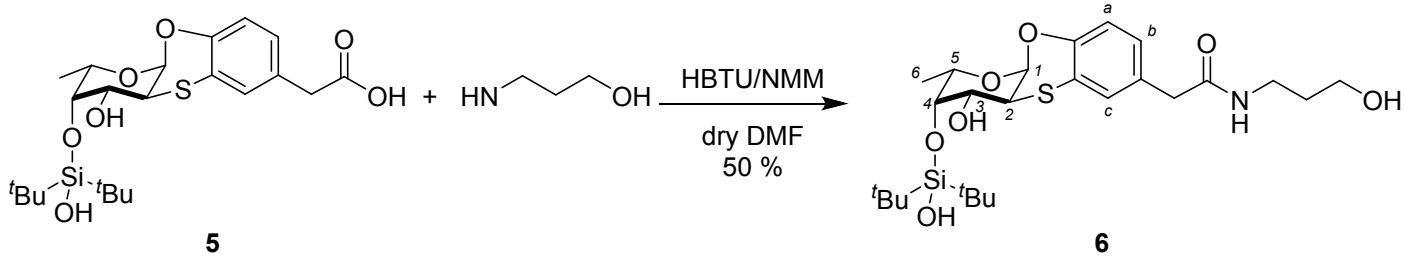


4 **5**

Aqueous solution of LiOH (1M, 3.9 eq, 10 mL) was added to a solution of **4** (1.26 g, 2.60 mmol) in THF (96 mL) and H_2O (38 mL). The mixture was stirred at room temperature for 2 hours then the solvents were removed *in vacuo* and the crude mixture was purified via flash chromatography on silica gel (DCM/MeOH/formic acid: 90/10/0.1) yielding **5** as a white solid (1.04 g, 85%).

MW: ($C_{22}H_{34}O_7SSi$) 470.65
m.p. 73.3-75.7 °C
ESI-MS: *m/z* (%) 493 (100) $[M + Na]^+$
$[\alpha]_D^{22}$ = - 65.10 (c = 0.001 in CH_3OH)
^{1}H NMR: (500 MHz, CD_3OD) δ 7.03 (d, $J_{c\text{-}b}$ = 2.1 Hz, 1H, C*H*-c), 6.95 (dd, $J_{b\text{-}a}$ = 8.3 Hz, $J_{c\text{-}b}$ = 2.1 Hz, 1H, C*H*-b), 6.78 (d, $J_{b\text{-}a}$ = 8.3 Hz, 1H, C*H*-a), 5.62 (d, $J_{1\text{-}2}$ = 2.8 Hz, 1H, C*H*-1), 4.30 (d, *J* = 1.9 Hz, 1H, C*H*-4), 4.28 (q, $J_{5\text{-}Me}$ = 6.5 Hz, 1H, C*H*-5), 3.73 (dd, $J_{2\text{-}3}$ = 10.9 Hz, *J* = 2.5 Hz, 1H, C*H*-3), 3.49-3.46 (m, 3H, CH_2 + C*H*-2), 1.33 (d, $J_{5\text{-}Me}$ = 6.5 Hz, 3H, CH_3-5), 1.07 (s, 18H, $C(CH_3)_3$).
^{13}C NMR: (125 MHz, CD_3OD) δ 175.56 (*C*O), 152.44 (C_q-f), 129.90 (C_q-d), 128.72 (*C*H-c), 128.28 (*C*H-b), 118.83 (*C*H-a), 115.81 (C_q-e), 96.95 (*C*H-1), 76.68 (*C*H-4), 70.95 (*C*H-5), 69.26 (*C*H-3), 41.70 (*C*H-2), 41.00 (CH_2), 28.54-28.15 ($C(CH_3)_3$), 22.28-21.65 ($C(CH_3)_3$), 17.60 (CH_3-5).

Synthesis of compound 6:



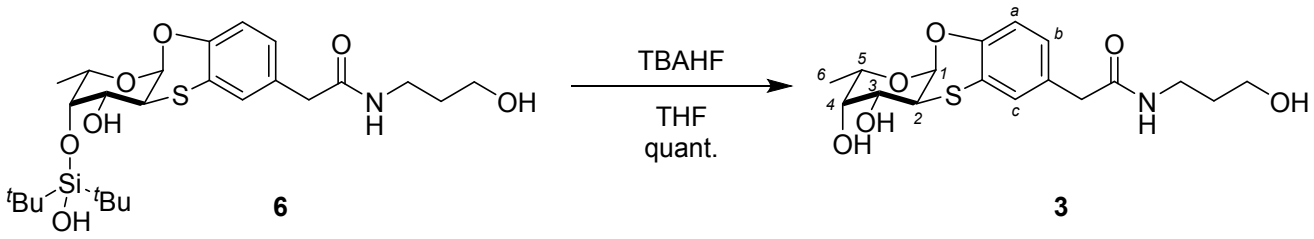
5 **6**

HBTU (174 mg, 0.46 mmol) and NMM (46.5 mg, 51 µL, 0.46 mmol) were dissolved in 2.3 mL dry DMF and stirred at room temperature for 20 minutes. This mixture was then added to a solution of **5** (100 mg, 0.23 mmol) in 1.15 mL DMF. The mixture was stirred at room temperature for 20 minutes after which 3-amino-1-propanol (35 mg, 0.46 mmol, 35 µL in 180 µL dry DMF) was

added. After 2 hours, solvents were removed *in vacuo*. The crude mixture was purified via Flash Chromatography (DCM/MeOH: 95/5) to yield 57 mg of the pure product as a white solid (50%)

MW: ($C_{25}H_{41}NO_7SSi$) 527.75 g/mol
ESI-MS: *m/z* (%) 550.50 (100) $[M+Na]^+$
$[\alpha]_D^{22}$ = - 58 (c = 0.001 in CH_3OH)
^{1}H NMR: (500 MHz, CDCl3) δ 6.99 (d, $J_{c\text{-}b}$ = 1.91 Hz, 1H, C*H*-c), 6.87 (dd, $J_{b\text{-}a}$ = 8.75 Hz, $J_{c\text{-}b}$ = 1.91 Hz, 1H, C*H*-b), 6.82 (d, $J_{b\text{-}a}$ = 8.75 Hz, 1H, C*H*-a), 6.58 (t, J = 5.77 Hz, 1H, NH), 5.68 (d, $J_{1\text{-}2}$ = 2.8 Hz, 1H, C*H*-1), 5.41 (bs, 1H), 5.11 (bs, 1H), 4.27 (m, 1H, C*H*-5, 1H, C*H*-4), 3.85 (bs, 1H), 3.72 (d, $J_{2\text{-}3}$ = 11.1 Hz, 1H, C*H*-3), 3.56 (bt, 2H, CH_2), 3.53 (dd, $J_{1\text{-}2}$ = 2.76 Hz, $J_{2\text{-}3}$ = 11.12 Hz, 1H, C-H_2), 3.39 (s, 2H), 3.28 (m, 2H), 1.62 (bt, 2H), 1.38 (d, $J_{6\text{-}5}$ = 6.55 Hz, 3H, C-H_6), 1.07 (s, 18H, $C(CH_3)_3$).
^{13}C NMR: (125 MHz, $CDCl_3$) δ 172.40, 151.33, 128.36, 128.01, 17.38, 118.35, 114.44, 95.50, 74.50, 69.89, 67.75, 59.92, 42.40, 40.04, 37.13, 31.60, 27.95, 27.67, 21.43, 20.70, 17.17

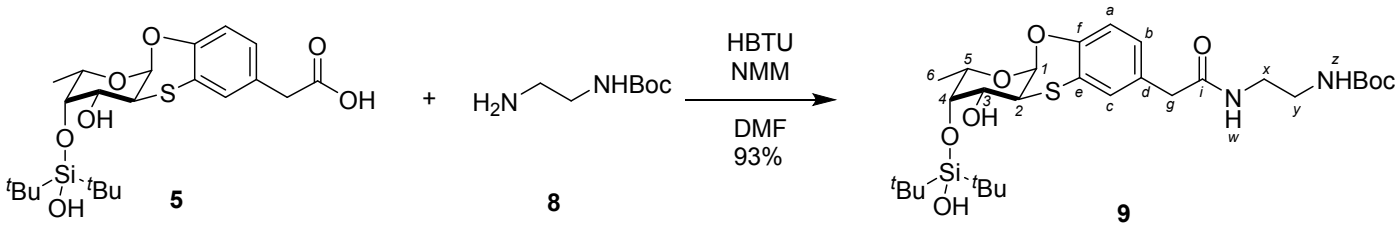
Synthesis of compound 3:



100 µL of a 2M solution of TBA.HF in THF (0.19 mmol) was added to a solution of **6** (50 mg, 0.095 mmol) in 1 mL THF. The solution was stirred at 40°C overnight, after which another equivalent of TBA.HF was added (50 µL of a 2M solution in THF) and the reaction mixture was stirred at 40°C for another night. The formed precipitate was filtered and further washed with THF to yield the pure product as a white solid (35 mg, quantitative).

MW: ($C_{17}H_{23}NO_6S$) 369.43
ESI-MS: *m/z* (%) 370.33 (100) $[M+H]^+$, 392.33 (50) $[M+Na]^+$
$[\alpha]_D^{22}$ = - 36 (c = 0.001 in CH_3OH)
^{1}H NMR: (500 MHz, MeOD) δ 7.05 (d, $J_{c\text{-}b}$ = 1.84 Hz, 1H, C*H*-c), 6.97 (dd, $J_{b\text{-}a}$ = 8.42 Hz, $J_{c\text{-}b}$ = 1.84 Hz, 1H, C*H*-b), 6.79 (d, $J_{b\text{-}a}$ = 8.42 Hz, 1H, C*H*-a), 5.61 (d, $J_{1\text{-}2}$ = 2.82 Hz, 1H, C*H*-1), 4.25 (q, C*H*-5, $J_{5\text{-}6}$=6.53 Hz, 1H), 3.71 (bd, 1H), 3.66 (dd, $J_{3\text{-}4}$ = 2.91 Hz, $J_{3\text{-}2}$ = 10.82 Hz, 1H, C-H_3), 3.57 (t, J=6.35, 2H, CH_2), 3.44 (dd, $J_{1\text{-}2}$ = 2.83 Hz, $J_{2\text{-}3}$ = 11.0 Hz, 1H, C-H_2), 3.38 (bs, 2H), 3.27 (t, 6.89, 2H, CH_2), 1.71 (q, 6.52, 2H, CH_2), 1.30 (d, $J_{6\text{-}5}$ = 6.54 Hz, 3H, C-H_6)
^{13}C NMR: (125 MHz, MeOD) δ 172.77, 151.13, 129.25, 127.11, 126.52, 117.48, 114.74, 95.56, 71.67, 68.67, 67.33, 59.01, 41.51, 39.85, 36.23, 31.72, 15.45

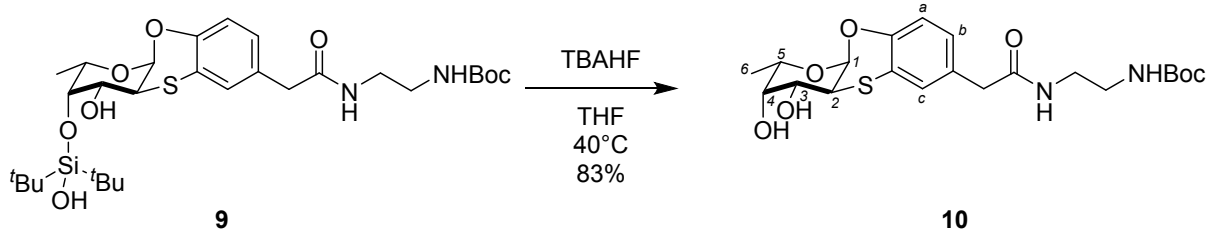
Synthesis of compound 9:



HBTU (302 mg, 0.80 mmol) and NMM (188 mg, 1.86 mmol) were added to a solution of **5** (250 mg, 0.53 mmol), in DMF (10 mL)). After 20 minutes, **8** (127 mg, 0.80 mmol) was added. The mixture was stirred at room temperature for 3 hours after which organic solvents were dried *in vacuo* and the crude mixture was purified via flash chromatography (DCM/EtOAc: 7/3 to EtOAc), yielding 306 mg of pure product as a white solid (93%).

MW: ($C_{29}H_{48}N_2O_8SSi$) 612.85
m.p.: 105-110 °C
ESI-MS: *m/z* (%) 635 (100) $[M + Na]^+$
$[\alpha]_D^{25}$ = -121.5 (c 0.001 in CH_3OH)
^{1}H NMR: (400 MHz, $CDCl_3$) δ 6.97 (d, $J_{c\text{-}b}$=1.9 Hz, 1H, *H*-c), 6.88 (dd, $J_{b\text{-}a}$= 8.4 Hz, $J_{c\text{-}b}$=1.9 Hz, 1H, *H*-b), 6.80 (d, $J_{b\text{-}a}$= 8.4 Hz, 1H, *H*-a), 6.48 (bs, 1H, N*H*-w), 5.68 (d, $J_{1\text{-}2}$ = 2.6 Hz, 1H, C*H*-1), 5.07 (bs, 1H, N*H*-z), 4.87 (bs, 1H, O*H* -3), 4.78 (s, 1H, O*H* -Si), 4.31-4.23 (m, 2H, C*H*-4 + C*H*-5), 3.75 (d, $J_{2\text{-}3}$ = 11.1 Hz, 1H, C*H*-3), 3.53 (dd, $J_{2\text{-}3}$ = 11.1 Hz, $J_{1\text{-}2}$ = 2.6 Hz, 1H, C*H*-2), 3.36 (s, 2H, CH_2-g), 3.31-3.09 (m, 4H, CH_2-x + CH_2-y), 1.42 (s, 9H, OC(CH_3)$_3$), 1.38 (d, $J_{5\text{-}Me}$ = 6.5 Hz, 3H, CH_3-5), 1.06 (s, 18H, SiC(CH_3)$_3$).
^{13}C NMR: (100 MHz, $CDCl_3$) δ 171.88 (*C*O-i), 151.25 (*C*O + *C*q-f), 128.43 (*C*q-d), 128.06 (*C*H-c), 127.49 (*C*H-b), 118.49 (*C*H- a), 110.49 (*C*q-e), 95.31 (*C*H-1), 79.93 (*C*(CH$_3$)$_3$), 74.00 (*C*H-4), 69.86 (*C*H-5), 67.80 (*C*H-3), 42.42 (*C*H$_2$-g), 40.73 (*C*H$_2$-x), 39.96 (*C*H$_2$-y), 39.89 (*C*H-2), 28.37 (OC(*C*H$_3$)$_3$), 27.68 (C(*C*H$_3$)$_3$), 27.9 (C(*C*H$_3$)$_3$), 21.43 (*C*(CH$_3$)$_3$), 20.71 (*C*(CH$_3$)$_3$), 17.20 (*C*H$_3$-5).

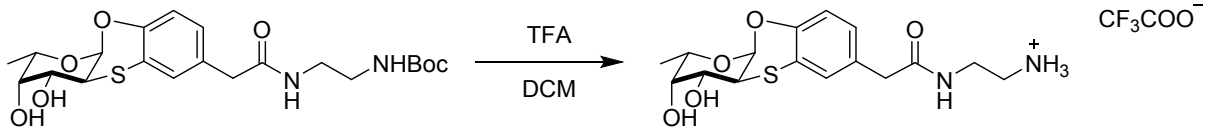
Synthesis of compound 10:



A 2M solution (1.42 mL) of TBAHF was added to a solution of **9** (582 mg, 0.95 mmol) in THF (6.5 mL) and the mixture is stirred at 40°C for 48 hours. The white suspension was filtered on #3 frit and the solid was washed with THF to yield 291 mg of pure product. The filtrate was evaporated and resuspended in THF, filtered, then the precipitate was washed with THF yielding another 66 mg of pure product as a white solid (total yield 83%).

MW: ($C_{21}H_{30}N_2O_7S$) 454.54 g/mol
m.p.: 217-219 °C
ESI-MS *m/z* (%) 477 (100) $[M+Na]^+$
$[\alpha]_D^{22}$ = -58.1 (c = 0.001 in CH_3OH)
^{1}H NMR: (500 MHz, DMSO-d6) δ 7.99 (bs, 1H, N*H*-x), 6.98 (d, $J_{b\text{-}c}$ = 2.0 Hz, 1H, C*H*-c), 6.89 (dd, $J_{a\text{-}b}$ = 8.4 Hz, $J_{b\text{-}c}$ = 2.0 Hz, 1H,C*H*-b), 6.80 (bs, 1H, N*H*-y), 6.77 (d, $J_{a\text{-}b}$ = 8.4 Hz, 1H, C*H*-a), 5.56 (d, $J_{1\text{-}2}$ = 2.7 Hz, 1H, C*H*-1), 5.24 (d, $J_{OH\text{-}3}$ = 6.9 Hz, 1H, O*H*-3), 4.87 (d, $J_{OH\text{-}4}$ = 4.9 Hz, 1H, O*H*-4), 4.11 (q, $J_{5\text{-}Me}$ = 6.6 Hz, 1H, C*H*-5), 3.58-3.53 (m, 1H, C*H*-4), 3.52-3.47 (m, 1H, C*H*-3), 3.39 (dd, $J_{2\text{-}3}$ = 11.0 Hz, $J_{1\text{-}2}$ = 2.7 Hz, 1H, C*H*-2), 3.27 (s, 2H, CH_2-g), 3.09-3.02 (m, 2H, CH_2-x), 3.00-2.93 (m, 2H, CH_2-y), 1.38 (s, 9H, $OC(CH_3)_3$), 1.17 (d, $J_{5\text{-}Me}$ = 6.6 Hz, 3H, CH_3-5).
^{13}C NMR: (125 MHz, DMSO-d6) δ 170.73 (*C*O-i), 156.08 (*C*O-l), 150.82 (C_q-e), 130.30 (C_q-f), 127.41 (*C*H-c), 127.01 (*C*H-b), 117.92 (*C*H-a), 115.12 (C_q-d), 95.59 (*C*H-1), 78.14 ($C(CH_3)_3$), 71.57 (*C*H-4), 69.16 (*C*H-5), 67.33 (*C*H-3), 41.80 (CH_2-g), 40.58 (CH_2-g), 39.30 (CH_2 x+y), 28.70 ($C(CH_3)_3$), 17.02 (CH_3-5).

Synthesis of compound 11:



10 **11**

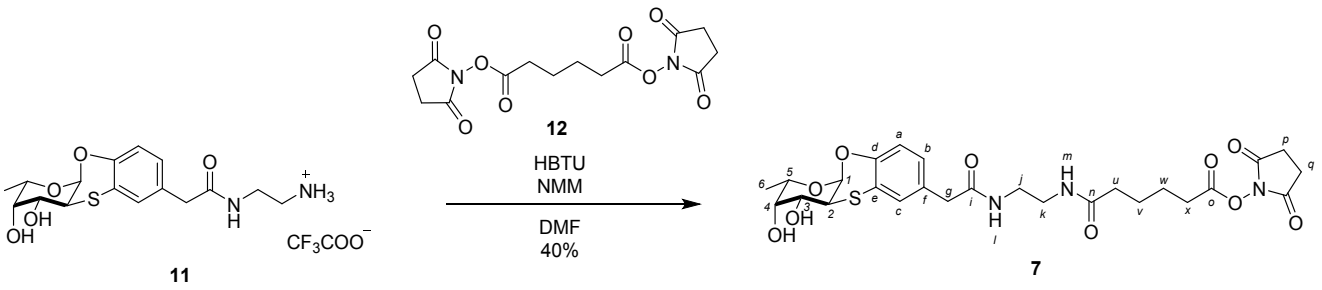
TFA (0.9 mL, 15 eq) was added dropwise to a suspension of **10** (134 mg, 0.30 mmol) in DCM (3 mL) at 0°C. The solution was stirred at room temperature for 2 hours after which TFA and DCM were removed *in vacuo*. The crude mixture was used without further purification.

M.W.: ($C_{18}H_{23}F_3N_2O_7S$) 468.44 g/mol
ESI-MS *m/z* (%) 355.5 (100) $[M]^+$

^{1}H NMR: (400 MHz, D_2O) δ 7.13 (d, *J* = 1.9 Hz, 1H), 7.05 (dd, *J* = 8.5 Hz, *J* = 1.9 Hz, 1H), 6.91 (d, *J* = 8.5 Hz, 1H), 5.79 (d, *J* = 3.0 Hz, 1H), 4.34 (q, *J* = 6.5 Hz, 1H), 3.85 (d, *J* = 3.0 Hz, 1H),

3.76 (dd, J = 11.1 Hz, J = 2.9 Hz, 1H), 3.55 (s, 2H), 3.53-3.43 (m, 3H), 3.17-3.10 (m, 2H), 1.29 (d, J = 6.5 Hz, 3H).

Synthesis of compound 7:

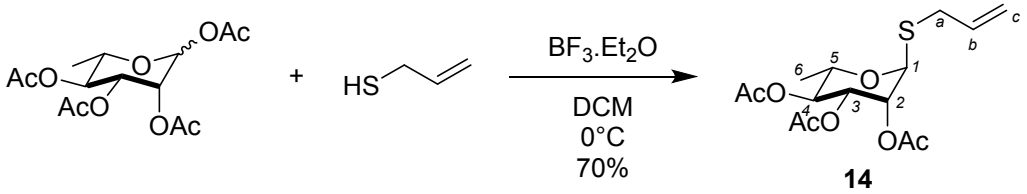


NMM (0.20 mL, 1.84 mmol) was added to a solution of **11** (239 mg, 0.51 mmol) in dry DMF (5 mL). After 20 mns, **12** (695 mg, 2 mmol) was added. The mixture was stirred at room temperature for 18 hours after which the suspension was filtered on #3 frit and washed with DMF. The filtrate was evaporated to dryness and purified via Flash Chromatography (DCM/Acetone: 65/35 to Acetone) to yield 110 mf the pure product as a white solid (40% yield over two steps)

M.W.: ($C_{26}H_{33}N_3O_{10}S$) 579.62 g/mol
m.p.: 156-159 °C
ESI-MS *m/z* (%) 602 (100) $[M + Na]^+$, 637 (55) $[M + K]^+$
$[\alpha]_D^{25}$ = -55.7 (c = 0.001 in CH_3OH)

^{1}H NMR: (500 MHz, DMSO -d6) δ 8.01 (bs, 1H, N*H*-l), 7.85 (bs, 1H, N*H*-m), 6.98 (d, $J_{c\text{-}b}$ = 2.0 Hz, 1H, C*H*-c), 6.89 (dd, $J_{a\text{-}b}$ = 8.4 Hz, $J_{b\text{-}c}$ = 2.0 Hz, H, C*H*-b), 6.78 (d, $J_{a\text{-}b}$ = 8.4 Hz, 1H, C*H*-a), 5.56 (d, $J_{1\text{-}2}$ = 2.7 Hz, 1H, C*H*-1), 5.25 (bs, 1H, O*H*-3), 4.88 (bs, 1H, O*H*-4), 4.10 (q, $J_{5\text{-}Me}$ = 6.4 Hz, 1H, C*H*-5), 3.55 (bs, 1H, C*H*-4), 3.49 (d, $J_{2\text{-}3}$ = 10.7 Hz, 1H, C*H*-3), 3.39 (dd, $J_{2\text{-}3}$ = 10.7 Hz, $J_{1\text{-}2}$ = 2.7 Hz, 1H, C*H*-2), 3.27 (s, 2H, CH_2-g), 3.08 (bs, 4H, CH_2-j + CH_2-k), 2.81 (s, 4H, CH_2-p + CH_2-q), 2.70-2.65 (m ,2H, CH_2-x), 2.12-2.07 (m, 2H, CH_2-u), 1.59 (bs, 4H, CH_2-w + CH_2-v), 1.17 (d, $J_{5\text{-}Me}$ = 6.4 Hz, 3H, CH_3-5).
^{13}C NMR: (125 MHz, DMSO -d6) δ 172.37 (*C*O-n), 170.77 (*C*O-o), 170.73 (*C*O-i), 169.37 (*C*O NHS), 150.84 (C_q -e), 130.27 (C_q-f), 127.41 (C_q-c), 127.03 (C_q-b), 117.92 (*C*H-a), 115.11 (C_q-d), 95.59 (*C*H-1), 71.56 (*C*H-4), 69.16 (*C*H-5), 67.33 (*C*H-3), 41.81 (*C*H$_2$-g), 40.40 (*C*H-2), 38.98 + 38.71 (*C*H$_2$-j + *C*H$_2$-k), 35.22 (*C*H$_2$-u), 30.40 (*C*H$_2$-x), 25.90 (*C*H$_2$-p + *C*H$_2$-q), 24.72 (*C*H$_2$-v), 24.31 (*C*H$_2$-w), 17.02 (*C*H$_3$-5).

Synthesis of compound 14:



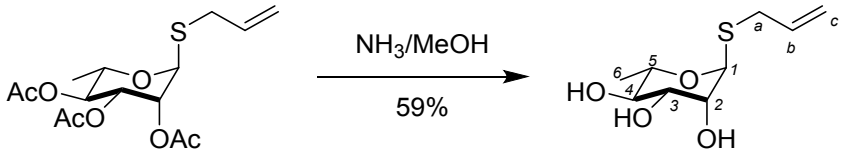
14

Allyl mercaptan (1.01 mL, 1.25 mmol) and $BF_3.Et_2O$ (1.65 mL, 13.37 mmol) were added to a solution of peracetylated rhamnose (3.7 g, 11.14 mmol) in DCM (110 mL) at 0°C. Reaction mixture was stirred at room temperature for 4 hours, after which it was diluted with DCM and washed with sat. $NaHCO_3$ (4 times) and brine (1 time). The organic layer was dried over $NaSO_4$ and solvents were removed *in vacuo*. The crude mixture was purified via Flash Chromatography (DCM/MeOH: 85/15) yielding 2.7 g of pure **14** as a colorless oil (70%).

M.W.: ($C_{15}H_{22}O_7S$) 346.39 g/mol
ESI-MS: *m/z* (%) 369 (100) $[M + Na]^+$
$[\alpha]_D^{22}$ = -152 (c = 0.001 in CH_3OH)

^{1}H NMR: (500 MHz, $CDCl_3$) δ 5.85-5.75 (m, 1H, C*H*-a), 5.35 (dd, *J* =1.5 Hz, $J_{1\text{-}2}$ = 3.4 Hz, 1H, C*H*-1), 5.24 (dd, $J_{2\text{-}3}$ = 10.1 Hz, $J_{1\text{-}2}$ = 3.4 Hz, 1H, C*H*-2), 5.21-5.08 (m, 4H, *C*H-3, C*H*-4, C*H*-b, C*H*-c), 4.26-4.19 (dq, $J_{\text{Me-5}}$ = 6.2 Hz, *J* = 9.7 Hz, 1H, C*H*-5), 3.27-3.12 (m, 2H, CH_2), 2.16 (s, 3H, CH_3 Ac), 2.07 (s, 3H, CH_3 Ac), 2.00 (s, 3H, CH_3 Ac), 1.25 (d, $J_{\text{Me-5}}$ = 6.2 Hz, 3H, CH_3-5).
^{13}C NMR: (125 MHz, $CDCl_3$) δ 169.98 (C_q Ac), 169.958 (C_q Ac), 169.84 (C_q Ac), 132.82 (*C*H-a), 118.35 (*C*H_2-b+c), 80.67 (*C*H-3), 71.35 (*C*H-1), 71.28 (*C*H-4), 69.69 (*C*H-2), 67.13 (*C*H-5), 33.31 (*C*H_2), 20.94 (*C*H_3 Ac), 20.80 (*C*H_3 Ac), 20.66 (*C*H_3 Ac), 17.40 (*C*H_3-5).

Synthesis of compound 13:



14 **13**

Compound **14** (2 g, 5.77 mol) was dissolved in 5 mL of a 4M solution of NH_3 in MeOH. The mixture was stirred at room temperature for 24 hours after which the reaction mixture was

evaporated to dryness and purified via flash chromatography (DCM/MeOH: 9/1) to yield 750 mg of product as a colourless oil (59%).

M.W.: ($C_9H_{16}O_4S$) 220.28 g/mol
ESI-MS: *m/z* (%) 243 (100) $[M + Na]^+$ 463 (80) $[2M + Na]^+$
$[\alpha]_D^{22}$ = -182 (c = 0.001 in CH_3OH)

^{1}H NMR: (500 MHz, CD_3OD) δ 5.89-5.79 (m, 1H, C*H*-a), 5.17 (dd, J_{trans} = 17.0 Hz, J_{gem} = 1.0 Hz, 1H, C*H*-c), 5.12 (d, J_{cis} = 7.7 Hz, 1H, C*H*-b), 5.11 (bs, 1H, C*H*-1), 3.97-3.91 (m, 1H, C*H*-5), 3.90 (dd, J_{2-3} = 3.2 Hz, J_{1-2} = 1.3 Hz, 1H, C*H*-2), 3.61 (dd, J_{3-4} = 9.5 Hz, J_{2-3} = 3.2 Hz, 1H, C*H*-3), 3.43 (t, J_{3-4} = 9.5 Hz, 1H, C*H*-4), 3.25 (dd, J_{gem} = 13.8 Hz, J_{CH2-a} = 8.4 Hz, 1H, CH_2) 3.17 (dd, J_{gem} =13.8 Hz, J_{CH2-a} = 5.7 Hz, 1H, CH_2), 1.29 (d, J_{Me-5} = 6.2 Hz, 3H, CH_3-5).
^{13}C NMR: (125 MHz, CD_3OD) δ 133.78 (*C*H-a), 116.34 (*C*H_2-b+c), 83.46 (*C*H-1), 72.84 (*C*H-4), 72.41 (*C*H-2), 71.80 (*C*H-3), 68.91 (*C*H-5), 32.54 (*C*H_2), 16.54 (*C*H_3-5).

Procedure for lysine functionalization:

Compound **7** was dissolved in a minimal amount of 150 mM sodium phosphate (NaPi) pH 7.5 buffer and the solution (70 equivalents, 10 equivalents per lysine) was added to a solution of ubiquitin (450 μM in sodium phosphate 150 mM, pH 7.5). The mixture was stirred overnight at 4 °C and then washed with 20 mM sodium phosphate, 1 mM TCEP, pH 7.5 buffer by cyclical dilution/concentration steps using 3 kDa MWCO Amicon Ultra Centrifugal Filters. The occurrence of the reaction was controlled by SDS-PAGE, 1D ^{1}H and 2D ^{1}H-^{15}N-HSQC NMR experiments. The number of functionalized lysine was confirmed with MALDI/TOF-TOF analysis.

Procedure for cysteine functionalization of Ubi-A28C with 13:

Five equivalents of **13** (in solution, 1 mg/10 μL in water) and 5 equivalents of DPAP (in solution, 1 mg/10 μL in DMSO) were added to a solution of ubiquitin mutant A28C (450 μM in 150 mM NaPi, pH 7.5). Argon was gently bubbled in the solution for 5 minutes and the reaction mixture was irradiated by UV light at 365 nm (UVGL-55 Mineralight 26W). Cysteine-functionalization was complete after 20 minutes, as revealed by NMR experiments (1D ^{1}H and 2D ^{1}H-^{15}N-HSQC). The obtained glycoconjugates were washed from the excess of **13** by cyclical

dilution/concentration steps using 3 kDa MWCO Amicon Ultra Centrifugal Filters with 20 mM NaPi, 1 mM TCEP, pH 7.5 buffer, and NMR experiments were recorded again.

Expression and Purification of Ubiquitin WT and A28C:

E. coli BL21(DE3) Gold cells were transformed with pET-21a(+) plasmid encoding for ubiquitin (WT or A28C). The cells were cultured in a minimal medium (M9) supplied with 1.2 g/L of ^{15}N-labelled ammonium sulfate, 3 g/L of glucose, 0.2 mM $CaCl_2$, 2.0 mM $MgSO_4$ and 0.1 mg/mL of ampicillin at 37 °C until OD_{600} reached the value 0.6, then induced with 1 mM IPTG for 16 hours at 37 °C. Cells were harvested by centrifugation and resuspended in a 20 mM Tris-HCl pH 7.2 buffer (20 mL per liter of culture) supplied with 12.5 µg/mL DNAse and 20 mM $MgSO_4$. The disruption of the cells was performed by sonication. After centrifugation, the lysate was treated with a concentrated $HClO_4$ solution until reaching pH 4.5. The precipitate formed during acidic treatment was removed via centrifugation and the protein contained in the solution was purified by cationic exchange chromatography (5 mL HiTrap SP FF column). Fractions of purified protein were identified by SDS-PAGE. A second purification step was performed by gel filtration chromatography (HiPrep Superdex 75 PG E26/60 Column). The protein was finally eluted in 50 mM NaPi, pH 7.5 buffer (supplied with 1 mM TCEP in the case of A28C mutant)

X-ray crystallography

Molecular cloning, expression and purification of BambL were performed as previously described.[1,2] The protein was lyophilized and stored at -20 °C prior to crystallization experiment. The lyophilized protein was dissolved in a buffer pH 7.5 containing 20 mM Tris-HCl, 100 mM NaCl, and 100 µM $CaCl_2$. 1 µL of **3** (100 mM in 100% DMSO) was aliquot onto a siliconized glass circle cover slides (22 mm, Hampton research) and left to complete dryness at room temperature. 2 µL of the protein solution was mixed with 2 µL of a reservoir solution (200 mM trisodium citrate, 100 mM sodium acetate pH 5.0 and 24% PEG 8000, 1% DMSO) and the mixture was deposited on the dried compound **3**. Crystallization was performed by the hanging drop vapor diffusion method on a 24-well plate with sealant (Hampton Research) at 19 °C, and the protein crystals were observed after 2-3 days. The protein crystals were cryo-protected in 30% PEG8000, 1% DMSO, 100 mM sodium acetate pH 5, 200 mM trisodium citrate and flashed cooled in liquid nitrogen. X-ray diffraction data were collected at SOLEIL-PROXIMA1 (Saint Aubin, France) using a Pilatus

6M hybrid photon counting detector (Dectris). The recorded data were indexed, integrated and scaled using XDS [3] and merged using AIMLESS.[4] The structures were solved by molecular replacement using 3ZW0 as a searching template in PHASER,[5] followed by further iterations of manual rebuilding in COOT [6] and restrained refinement in REFMAC5.[7] **3** model was manually built in ACEDRG in CCP4i2 suite.[8] The final models were validated with MOLPROBITY,[9] PDB-redo (https://pdb-redo.eu/) and wwPDB validation service (http://validate-rcsb-1.wwpdb.org/). All structural figures were prepared using CCP4MG.[10]

Table S1. X-ray data collection and processing of BambL structure in complex with compound **3**

Data set	**BambL-3 (PDB code:6ZFC)**
Data Collection	
Beamline	PROXIMA1 (SOLEIL)
Wavelength (Å)	0.9786
Detector	Pilatus 6M
Resolution (Å)[a]	45.21-1.65 (1.68-1.65)
Space Group	I 1 2 1
a, b, c (Å)	109.09, 49.16, 115.10
α, β, γ (°)	90.0, 91.4, 90.0
Total observations	502,219
Unique reflections	72,307
Multiplicity[a]	6.9 (7.1)
Mean $I/\sigma(I)$[a]	11.8 (1.5)
Completeness (%)[a]	98.1 (96.5)
R_{merge}[a,b]	0.088 (1.260)
$CC_{½}$[a,c]	1.0 (0.6)
Refinement	
Reflections: working/free[d]	72,306/3,534
R_{work}/ R_{free}[e]	0.177/0.209
Ramachandran plot: allowed/favoured/outliers (%)[f]	3/97/0
R.m.s. bond deviations (Å)	0.0115
R.m.s. angle deviations (°)	1.687
Mean *B*-factors: protein/ligand[f]**/ /water (Å²)**	25/26/31

[a] Values for the outer resolution shell are given in parentheses.
[b] $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

[c] $CC_{½}$ is the correlation coefficient between symmetry-related intensities taken from random halves of the dataset.
[d] The data set was split into "working" and "free" sets consisting of 95 and 5% of the data, respectively. The free set was not used for refinement.
[e] The R-factors R_{work} and R_{free} are calculated as follows: $R = \sum(|F_{obs} - F_{calc}|)/\sum|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factor amplitudes, respectively
[f] refers to ligands bound in the active site and potential surface binding sites

Isothermal Titration Calorimetry:

Purified and lyophilized BambL and AFL were dissolved in buffer (20 mM Tris-HCl, 150 mM NaCl, 100 µM $CaCl_2$ buffer containing 2.5% DMSO) and degassed. Compound **3** and αMeFuc were dissolved directly into the same buffer degassed, and placed in the injection syringe (concentration from 2 to 6 mM). Isothermal titration calorimetry was performed with an ITC-200 (MicroCal Inc - Malvern). BambL and AFL were placed in the 200 µM sample cell, at 25 °C, at concentrations of 50 to 150 µM. Glyco-compounds were injected by 10 µL steps every 300 s. Data were fitted with MicroCal Origin 7 software, according to standard procedures. Fitted data yielded the stoichiometry (n), the association constant (Ka) and the enthalpy of binding (ΔH). Other thermodynamic parameters (i.e. changes in free energy, ΔG, and entropy, ΔS) were calculated from the equation $\Delta G = \Delta H - T\Delta S = -RT \ln K_a$ where T is temperature and R = 8.314 J mol^{-1} K^{-1}. Two or three independent titrations were performed for each ligand tested.

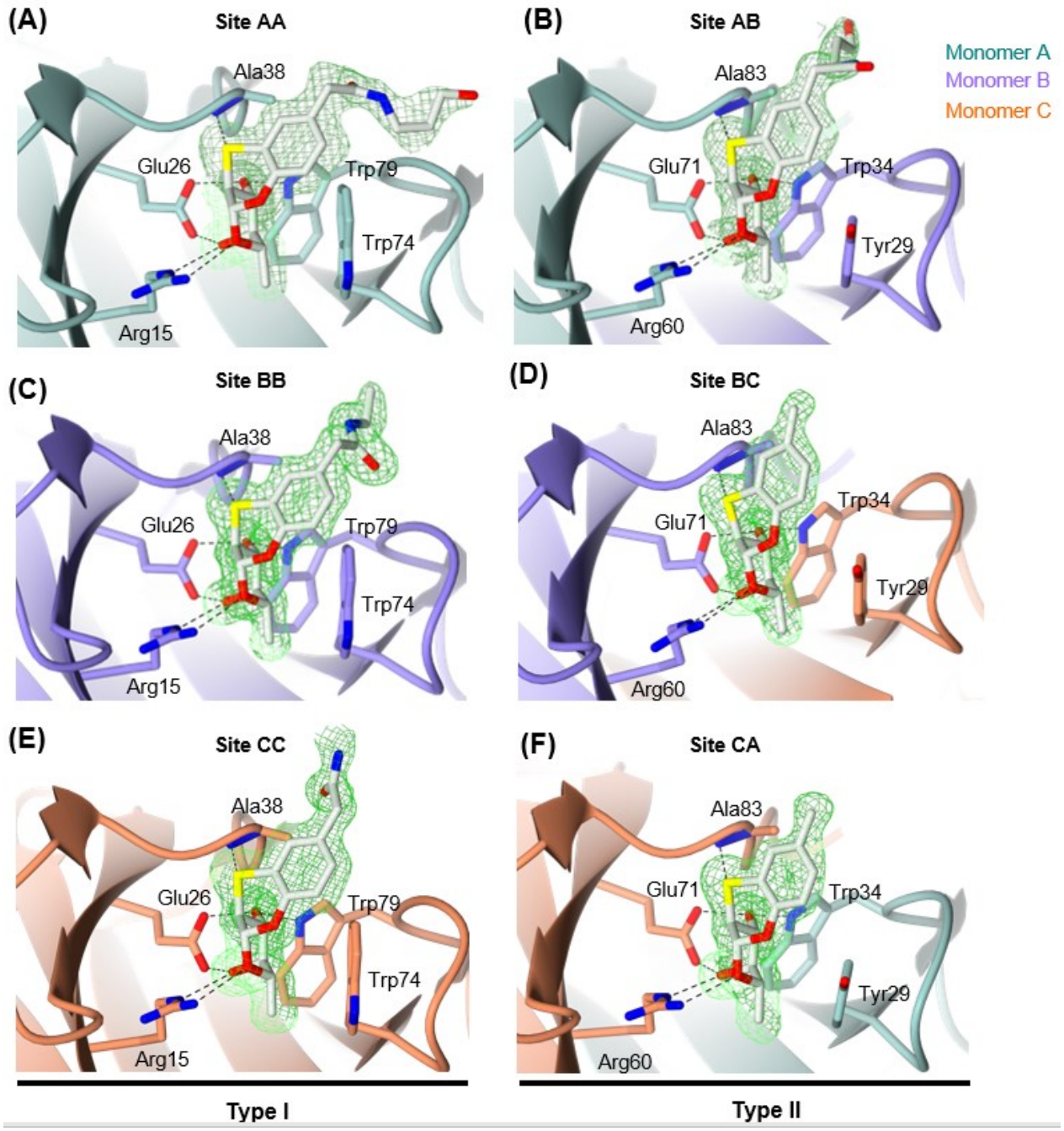


Figure S1. The binding sites of compound **3** in BamBL. The names of the binding sites are indicated in the figure. Green mesh represents the 2m*Fo* -D*Fc* electron density maps for the ligand contoured at 1σ.

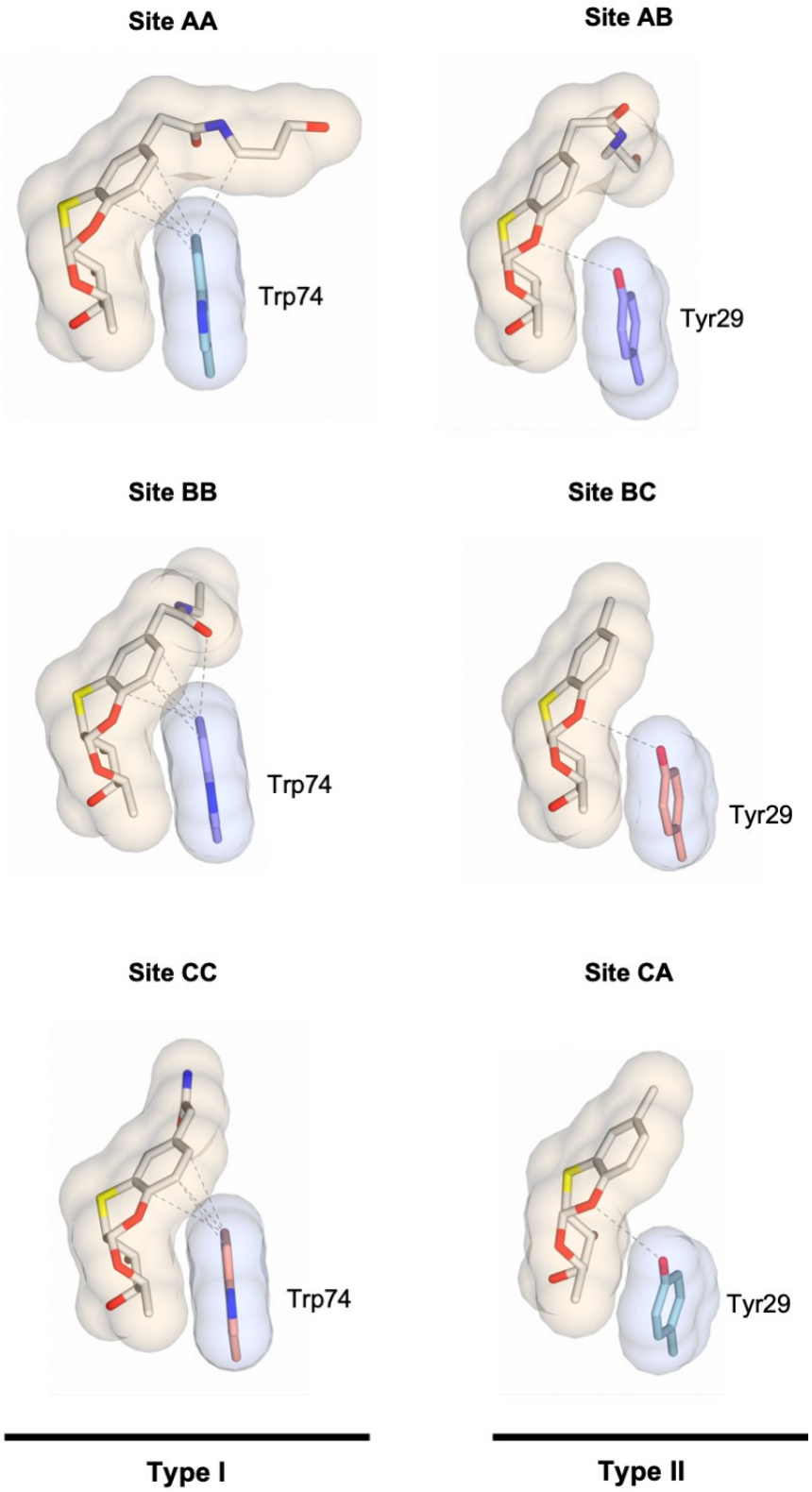


Figure S2. Surface and contact analysis of compound **3** with intra and inter binding sites. The surfaces are shown in blobs and contacts within 4 Å are shown as grey dashes.

MALDI-TOF of Fuc-Ub:

MALDI-TOF measurement were acquired on Bruker Daltonics Ultraflex III TOF/TOF.

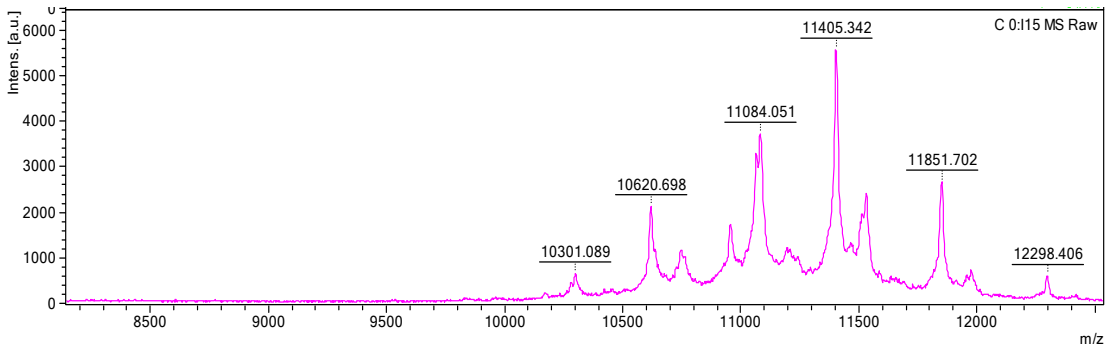


Figure S3: MALDI-TOF analysis of Fuc-Ub conjugates reveals a distribution of 3 to 8 fucose residues conjugated to the protein.

Surface Plasmon Resonance (SPR):

SPR experiments were performed using a Biacore X100 biosensor instrument (GE Healthcare) at 25°C. BambL was immobilized by amine coupling on CM5 chips (GE Healthcare) that were coated previously with streptavidin on flow cell 1 using standard manufacturer's procedures. BambL was diluted to 2 µg·ml^{-1}in HBS-T (HEPES-buffered saline, pH 7.4, with 0.05% Tween 20) before being injected into flow cell 2 of the chip until high immobilization levels of 3385 RU is attained. A reference surface was always present inflow cell 1, thus allowing for the subtraction of bulk effects and non-specific interactions with streptavidin. The running buffer consisted of the same HBS-T. **Fuc-Ub** was injected over the flow cell surface at 30 µl.min^{-1}in a series of 2-fold dilutions. The dissociation of this analyte was achieved by passing running buffer for 4–6 min. Surfaces were regenerated with four consecutive 80 s injections of 1M fucose, also at 30 µl·min^{-1}. The information on the affinity was determined by steady state analyses, using the BIACORE evaluation software.

NMR measurements

The NMR spectra for the analysis of Ub conjugates and NMR titration with BambL were acquired on a Bruker AVANCE NEO NMR spectrometer operating at 700 MHz, ^{1}H Larmor frequency, and equipped with a cryogenically cooled probe. All the spectra were acquired at 298 K. The assignment of the spectra of free Ub was based on the data reported in the Biological Magnetic Resonance Data Bank under the accession code 6457.[11] The assignment of the spectra of the functionalized Ub-A28C was helped by the analysis of 3D ^{1}H-^{15}N NOESY-HSQC acquired on a Bruker AVANCE MHD NMR spectrometer operating at 950 MHz, ^{1}H Larmor frequency, and equipped with a cryogenically cooled probe. The titration with BambL was performed using ^{15}N-isotopically enriched **Fuc-Ub** or **Fuc-Rha-A28C** or **Rha-A28C** at the concentration of 100 μM in 20 mM Tris buffer at pH 7.5 and 100 mM NaCl. Two different aliquots of BambL solution (0.6 mM of monomer in 20 mM Tris buffer at pH 7.5, 100 mM NaCl), to reach the final concentrations in solution of 50 and 100 μM, were added to **Fuc-Ub** or **Fuc-Rha-A28C** or **Rha-A28C** and 2D ^{1}H-^{15}N HSQC acquired at 700 MHz after each addition. In the titrations involving **Fuc-Ub** or **Fuc-Rha-A28C,** the signal intensity was decreased by more than 50% after the first addition, and the signals completely disappeared after the second.

The spectra were processed with the program Topspin 4.0 and analyzed with CARA .[12]

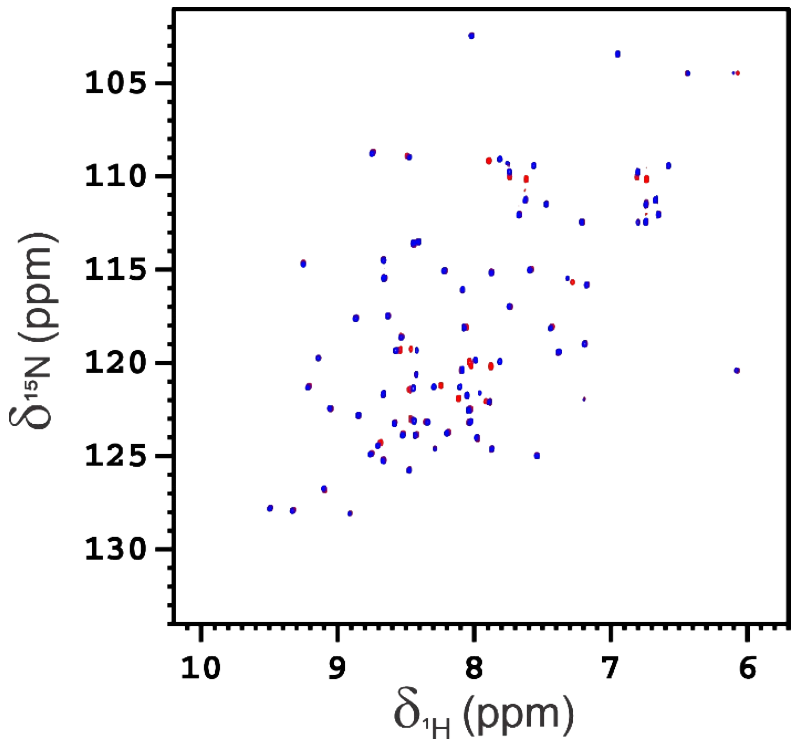


Figure S4. 2D ^{1}H-^{15}N HSQC spectra of Ub-A28C (red) and Ub-A28C functionalized with the rhamnose derivative on the cysteine residue (**Rha-A28C,** blue). The protein was 350 µM in 20 mM NaPi buffered solution at pH 7.5.

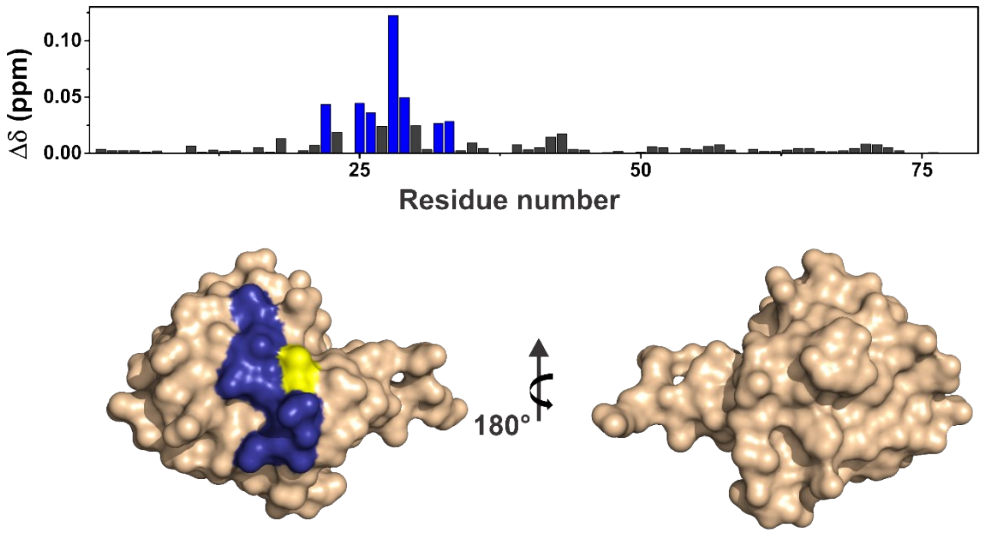


Figure S5. Graphical representation of the per residue chemical shift perturbation (CSP) of Ub-A28C functionalized on the cysteine side chain with rhamnose derivative with respect to the free protein. The CSP has been evaluated according to the formula $\Delta\delta = {}^{1}/_{2}\sqrt{(\Delta\delta_H)^2 + \left({}^{\Delta\delta_N}/_{5}\right)^2}$. The residue exhibiting the

highest CSP have been highlighted in blue in the plot (A) and on the surface representation of the protein (PDB: 1UBQ); the cysteine residue is in yellow.

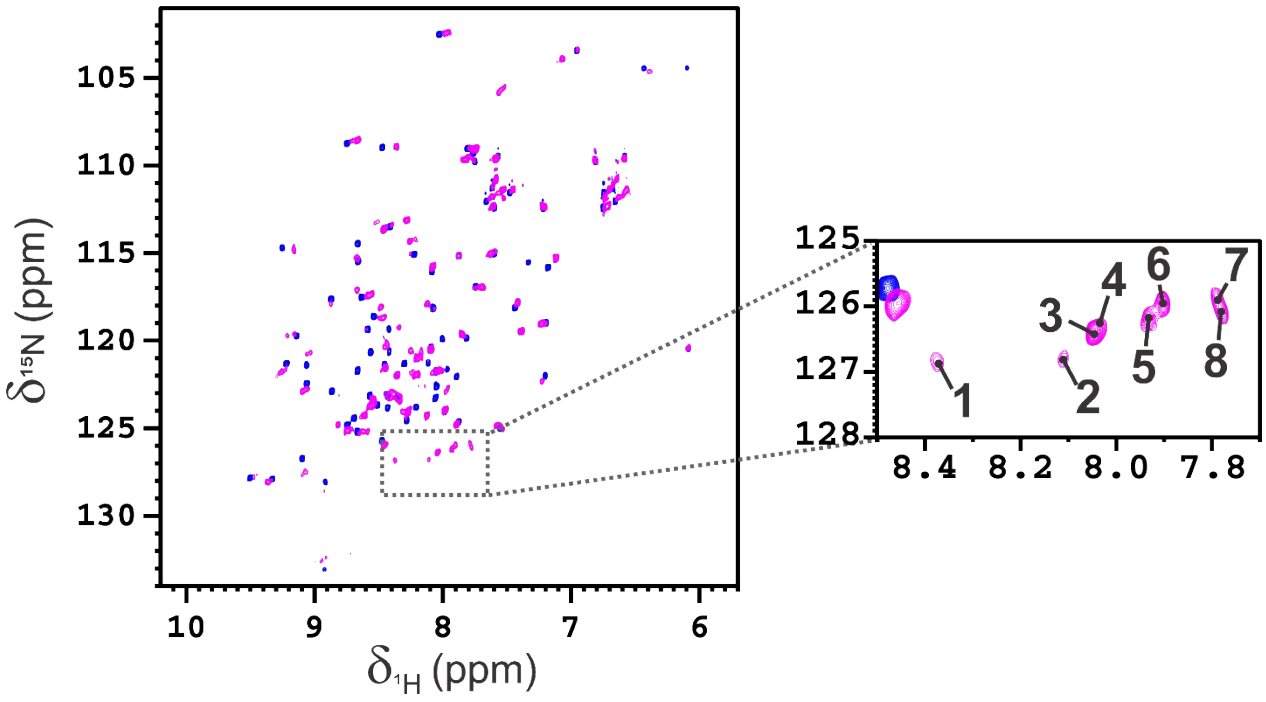


Figure S6. 2D ^{1}H-^{15}N HSQC spectra of **Rha-A28C** (blue) and **Fuc-Rha-A28C** (magenta) acquired at 298 K and 700 MHz. The signals in the area, selected by the dashed lines (enlarged on the right), correspond to the new amide groups obtained by the conjugation of the ε-NH_2 group of lysine sidechains and the N-terminus with fucoside **7**.

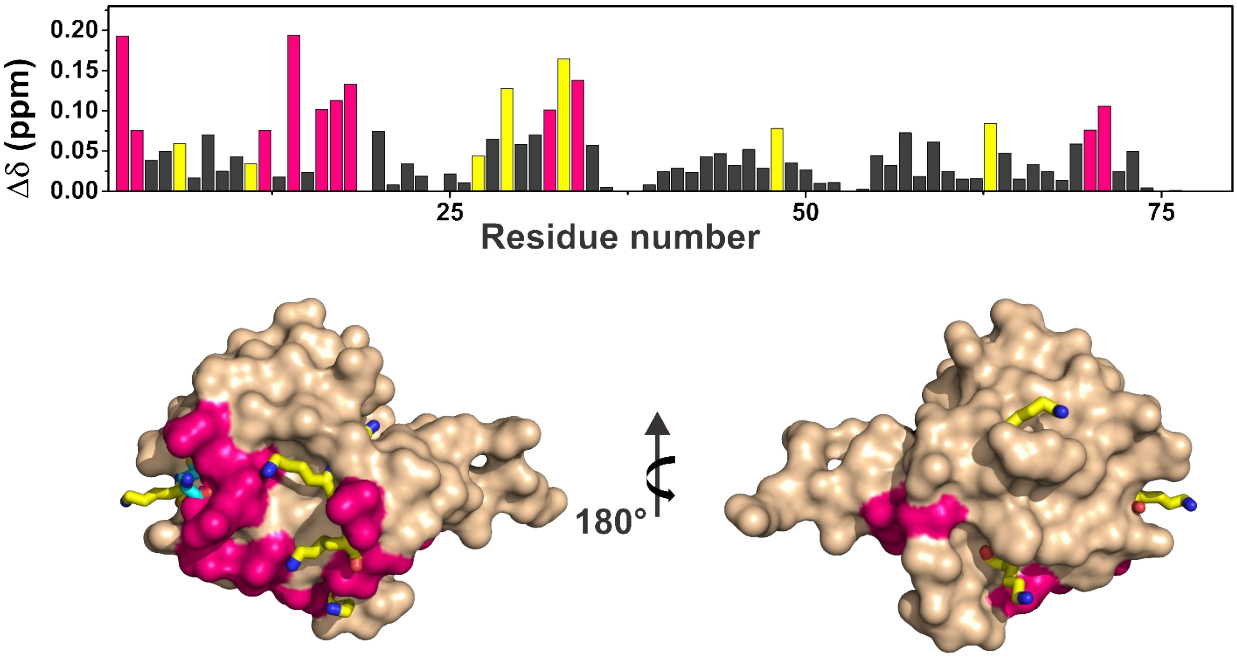


Figure S7. (A) Graphical representation of the per residue chemical shift perturbation (CSP) of **Rha-A28C** with respect to **Fuc-Rha-A28C**. The CSP has been evaluated according to the formula $\Delta\delta = {}^{1}/_{2}\sqrt{(\Delta\delta_H)^2 + \left({}^{\Delta\delta_N}/_{5}\right)^2}$. The residues exhibiting the highest CSPs have been highlighted in pink, while the lysine residues are in yellow. (B) Surface representation of Ub (PDB: 1UBQ) with highlighted in pink the residues exhibiting the highest CSP; the lysine residues are represented as yellow sticks, and the N-terminus as cyan sticks.

SEC-MALS-QELS analysis

SEC-MALS-QELS analysis was performed using a coupled method consisting of a size-exclusion chromatography (SEC) and multiangle light scattering and quasi-elastic light scattering detectors (MALS-QELS). The system was composed of several modules:

- an HPLC system (Knauer) equipped with a Superdex 200 HR 10/30 column (GE Healthcare) and an Optilab rEX interferometric refractometer detector (Wyatt Technology Corporation) for monitoring dRI elution profiles,
- a quasi-elastic light scattering (QELS) module for measuring RH values,
- a DAWN EOS MALS module set at 690 nm for scattering evaluation.

150 µL of the NMR sample of the complex formed using a 1:1 ratio of monomeric BambL:Fuc-Ub were injected onto the column, previously equilibrated with 20 mM Tris-HCl, pH 7.5, 100 mM NaCl buffer. The sample was eluted at 0.6 mL min^{-1}. Data were acquired and processed using the ASTRA 5.4.3 software (Wyatt Technology Corporation) which recorded dRI and scattering profiles and estimated molar mass values. Two main dRI peaks were revealed. The first one (13.5-14.5 mL) corresponded to an average molar mass of 153900 g mol^{-1}, whereas the second one (around 17.5 mL) to a molar mass range of 47570-71460 g mol^{-1}. Since the molar mass of BambL is 28143 g mol^{-1} and average molar mass of Fuc-Ub is 11169 g mol^{-1}, the latter range is compatible with 2 to 4 Fuc-Ub units bound to each trimeric BambL. The presence in solution of a complex where a Fuc-Ub unit bridges two trimeric BambL could be also possible.

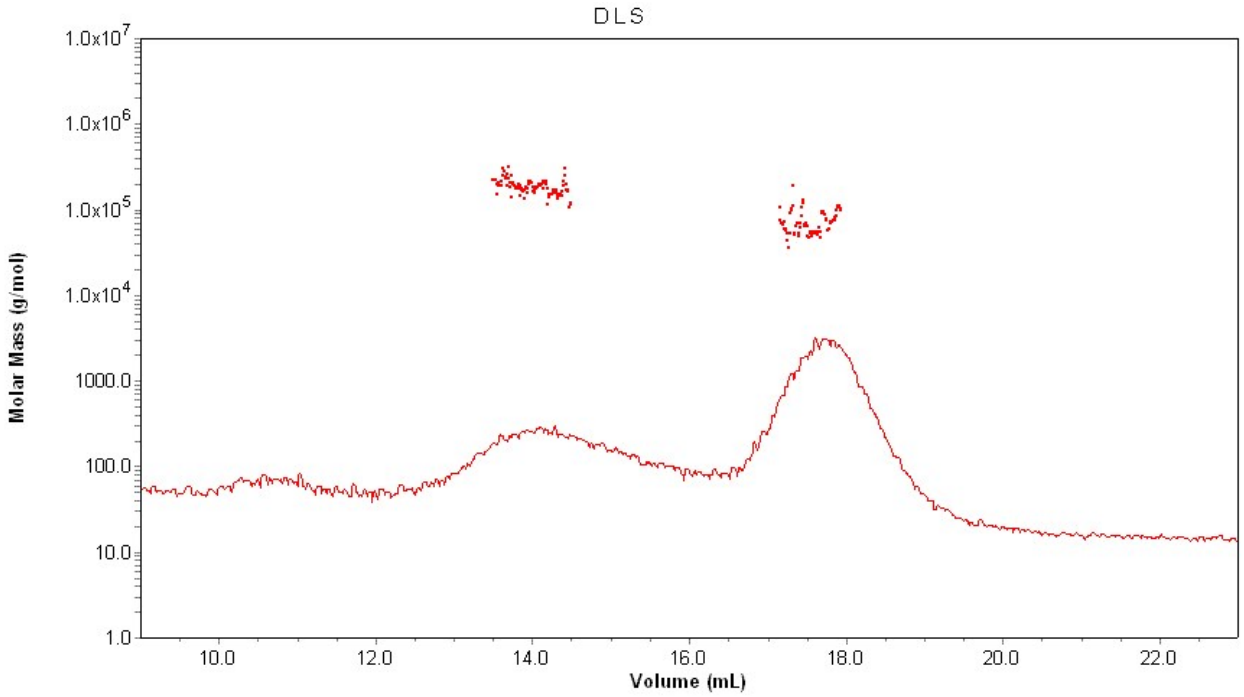


Figure S8. dRi profile (curve) and molar mass values (dots) of the NMR sample of the complex formed using a 1:1 ratio of monomeric BambL:Fuc-Ub.

Cell culture

The mouse monocyte/macrophage cell line RAW 264.7 was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in a 37 °C incubator with 5% CO_2. Cells were grown in 75 cm^2 flasks and sub-cultured by scraping when they reached 90% confluence with a 1:5 or 1:10 ratio in fresh medium. Before each experiment, viable cells were assessed by

trypan blue staining, then, cells were seeded in a complete fresh medium 24 h prior to compound treatments.

Cell viability assay

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide (MTT) assay, as previously described. RAW 264.7 cells were seeded (0.5×10^5cells/ well) in 24-well plates and treated with increasing concentrations (0.01-1 μM) of test compounds for 24 h at 37 °C in a 5% CO_2 humidified incubator. The percentage of cell viability was calculated as [100 (x−y)/ (z−y)], where x, y, and z were the absorbance read in compound-treated, resting, and compound-untreated cells, respectively.

TNF-α evaluation

Compounds were tested for their effects on the production of pro-inflammatory mediators, such as tumor necrosis factor (TNF)-α, as previously described. RAW 264.7 cells were seeded at a density of 0.6×10^5 cells/well in 24-well plates. The day of experiment, cells were pre-treated with increasing concentrations (0.01-1 μM) of each test compound for 1 h and then unstimulated/stimulated with LPS (0.1 μg/ml) for 24 h. At the end of each experiment, supernatants were collected and stored at −20 °C until assays. The amounts of TNF-α in cell culture medium were assayed using enzyme-linked immunosorbent assay (ELISA) kits (Biolegend), according to the manufacturer's instructions. The concentrations of TNF-α in the samples were determined by extrapolation from specific reference standard curves.

Statistical analysis

Results are expressed as means ± SEM of at least four different experiments run in triplicate. Statistical significance was evaluated by Student's t-test for unpaired varieties. Differences were considered statistically significant when $p < 0.05$.

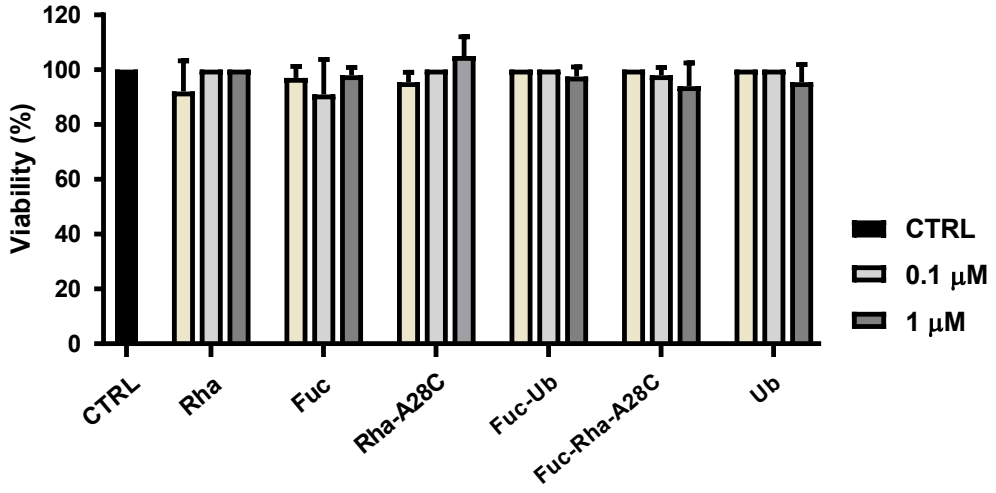
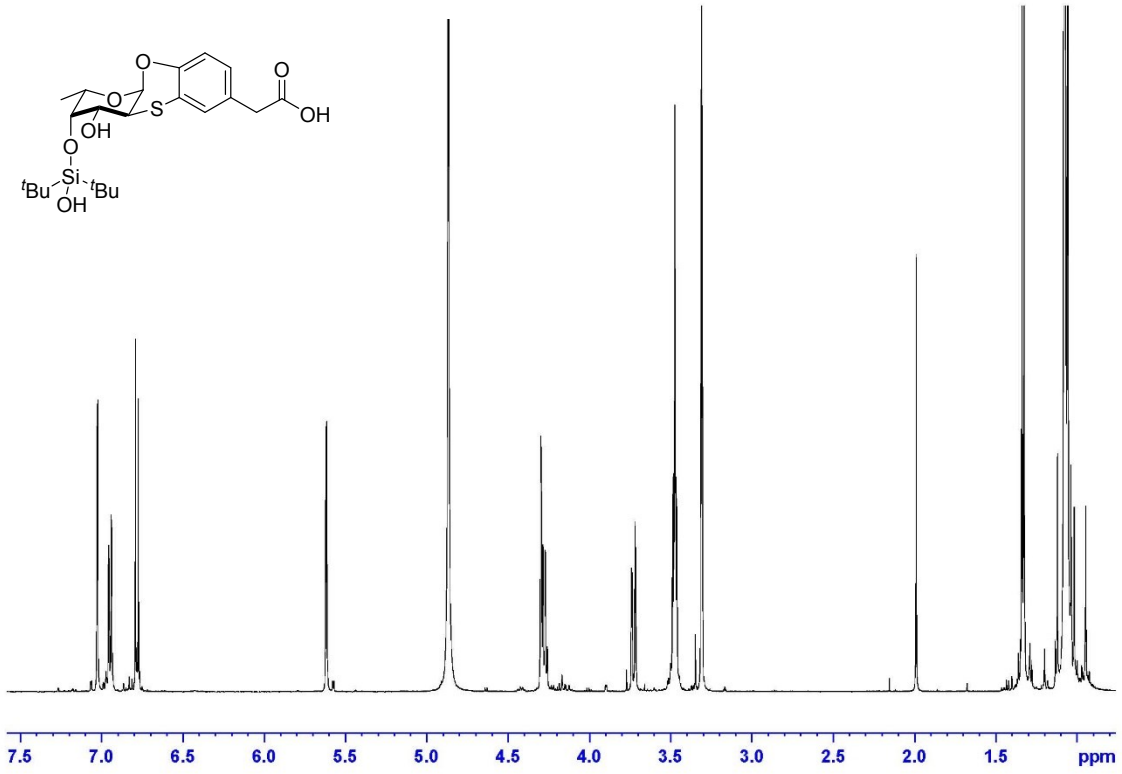


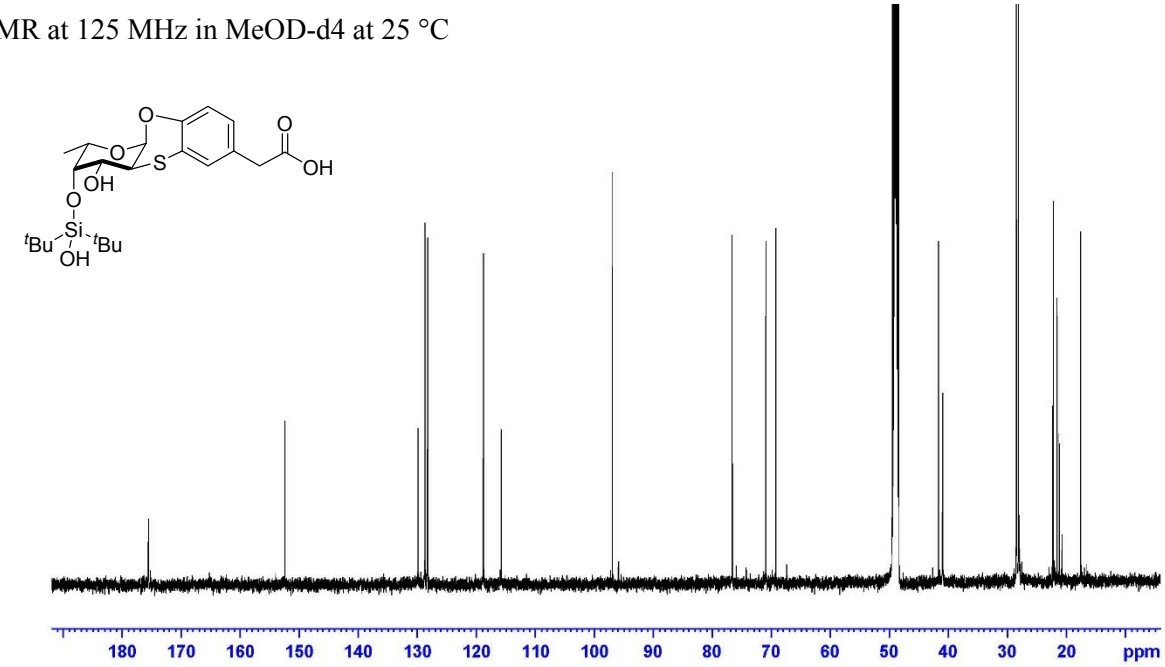
Figure S9. Effects of compounds on cell viability. RAW 264.7 cells were treated with increasing concentrations (0.01-1 μM) of each compound for 24 h and cell viability was assessed by MTT. The data represent mean ±SEM of at least three independent experiments run in triplicate.

NMR and ESI-MS Spectra

^{1}H NMR at 500 MHz in MeOD-d4 at 25 °C

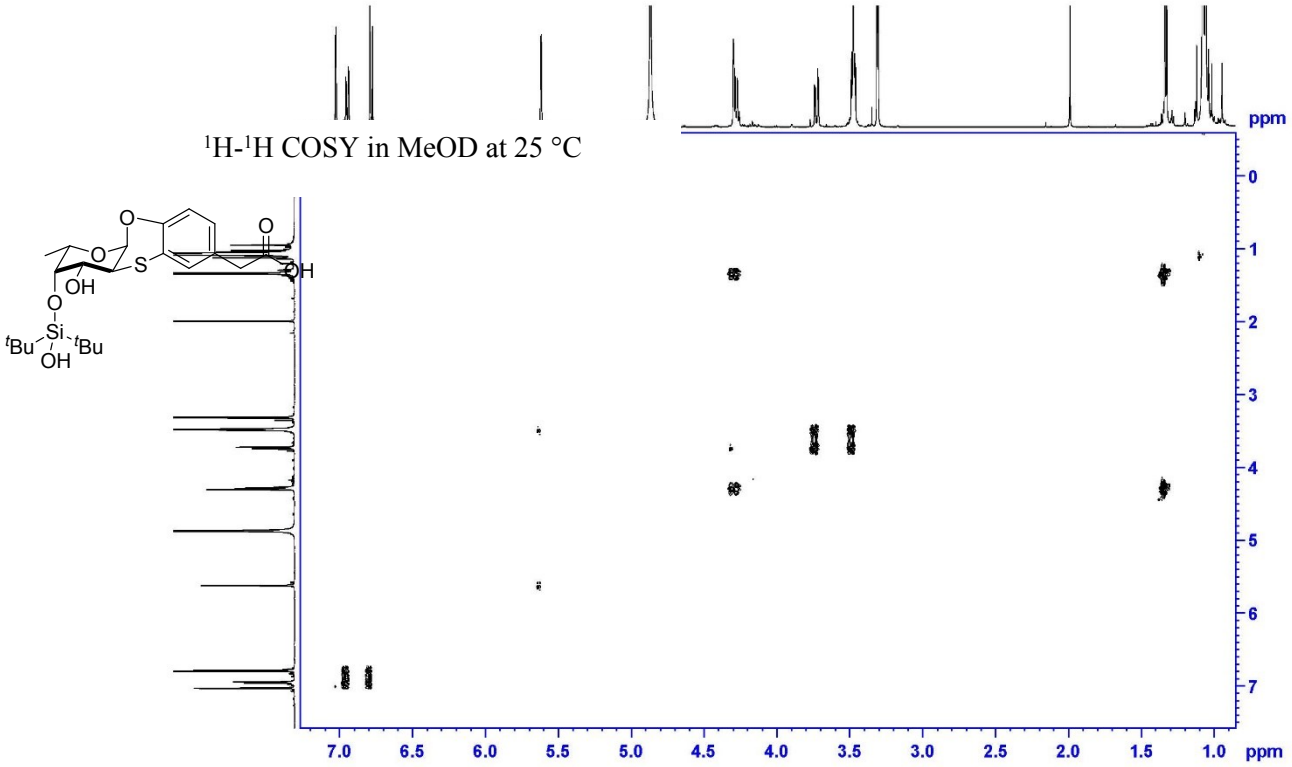


^{13}C NMR at 125 MHz in MeOD-d4 at 25 °C

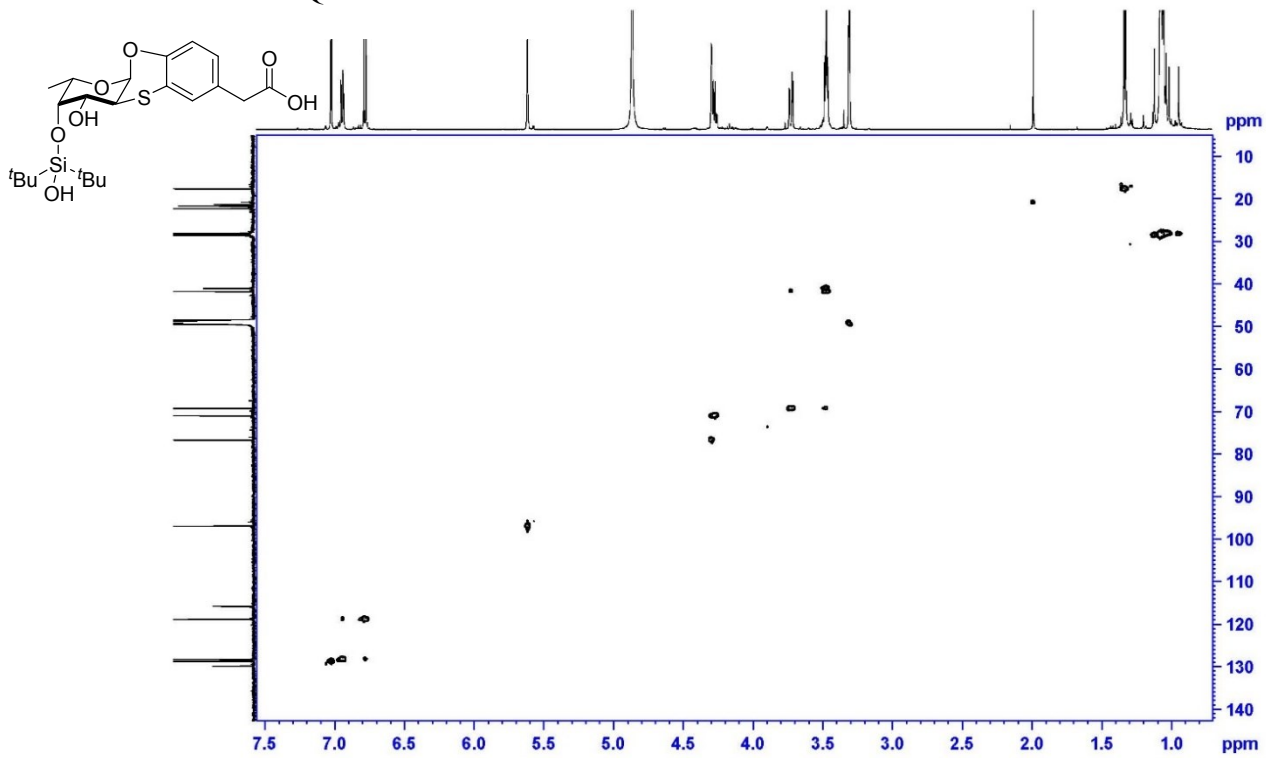


NMR Spectra of compound 5:

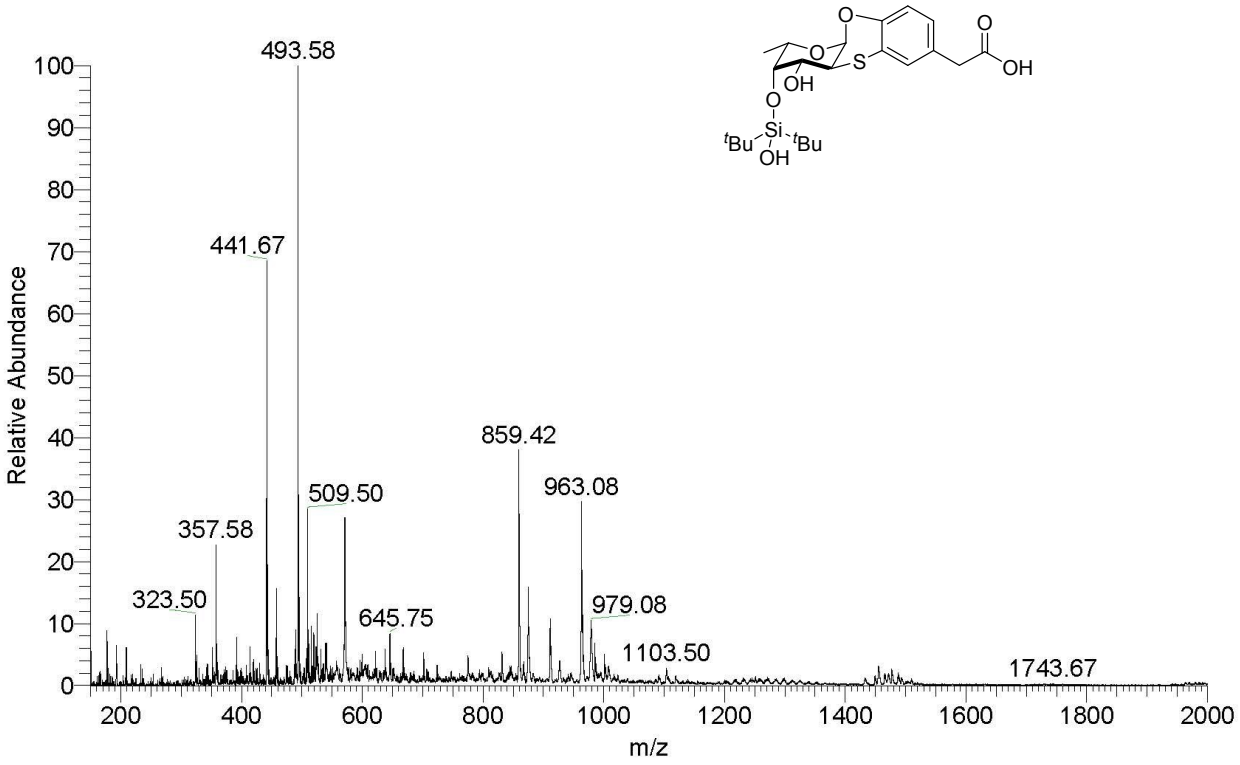
^{1}H-^{1}H COSY in MeOD at 25 °C



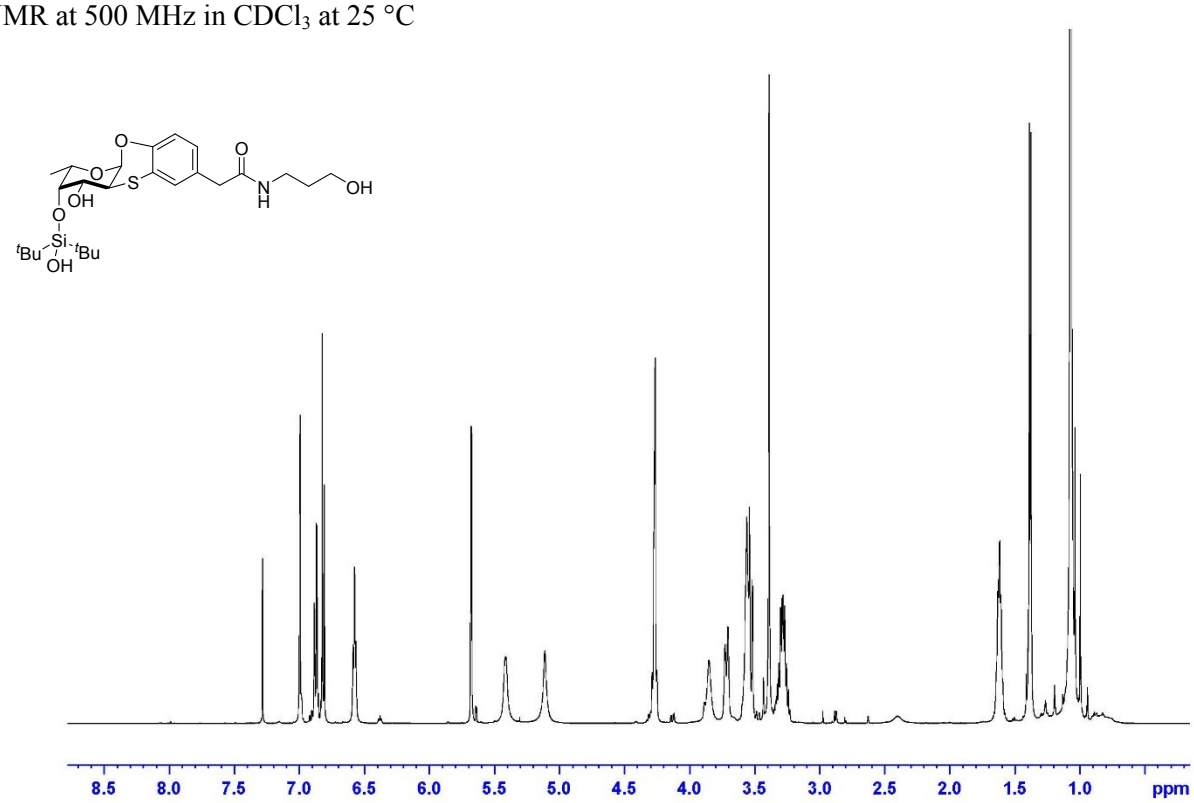
^{1}H-^{13}C 2D 2D-HSQC in MeOD at 25 °C



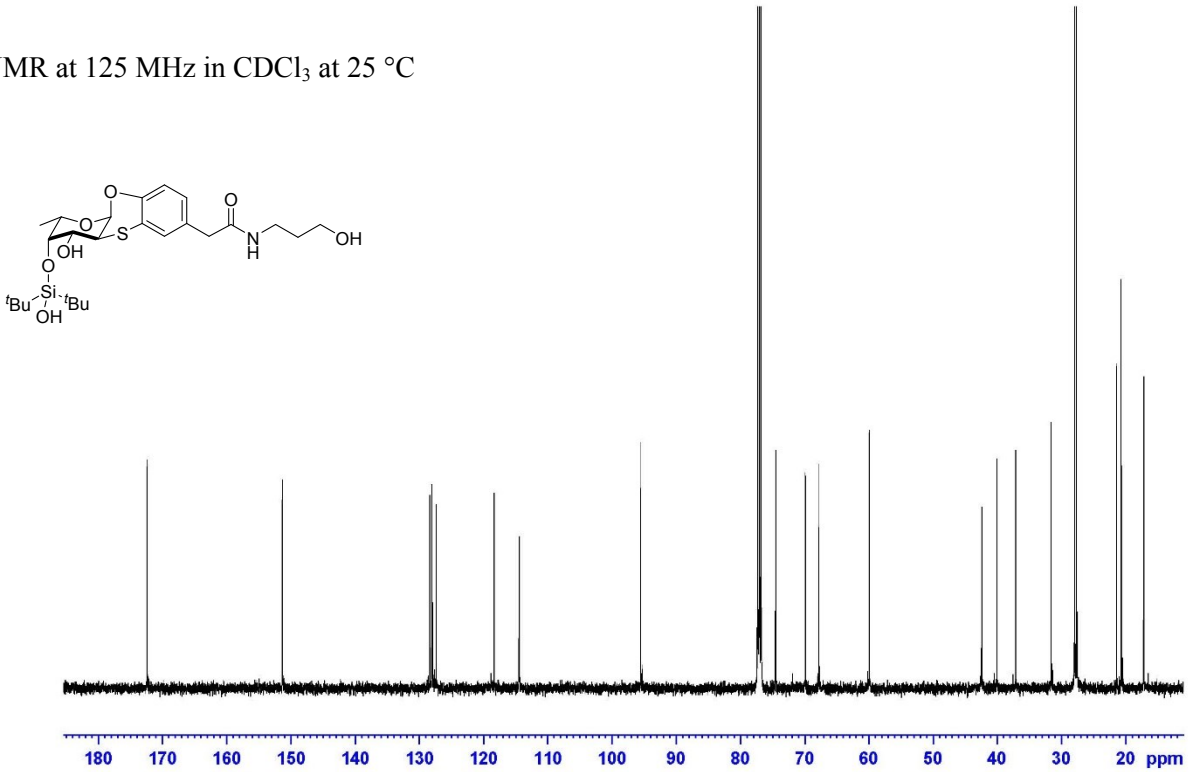
ESI-MS of compound 5:



^{1}H NMR at 500 MHz in $CDCl_3$ at 25 °C

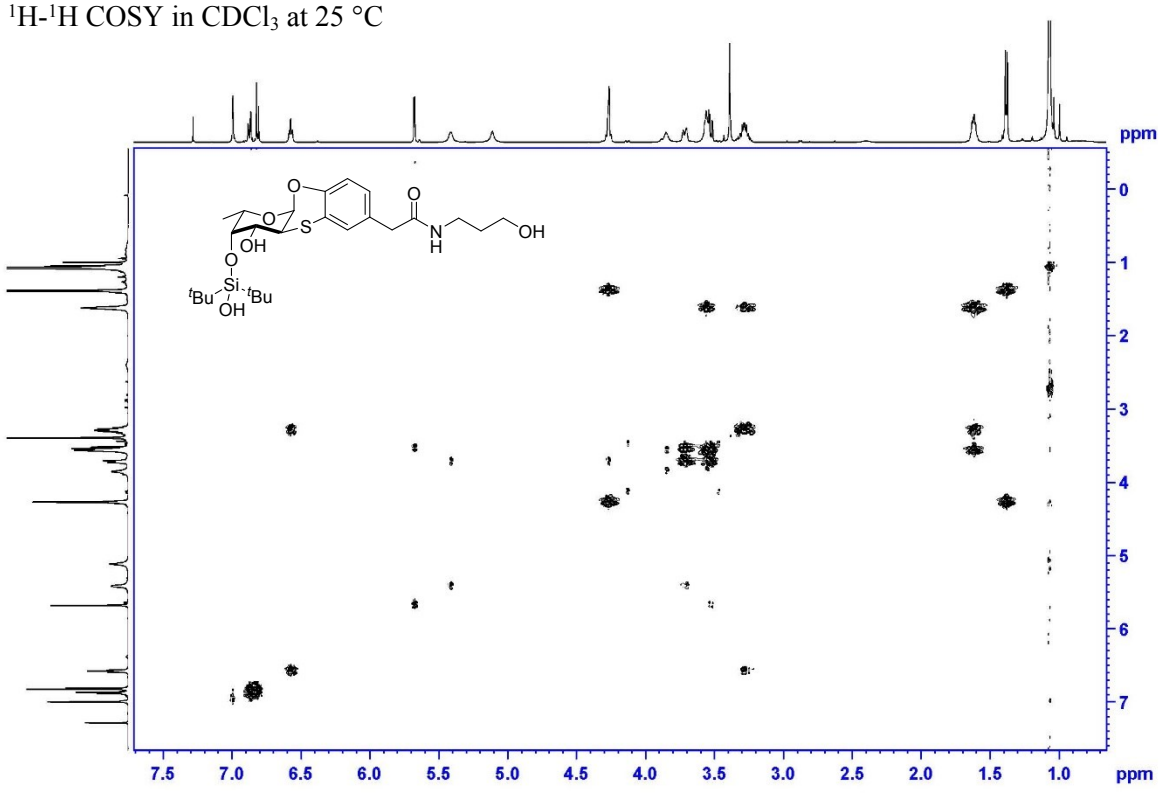


^{13}C NMR at 125 MHz in $CDCl_3$ at 25 °C

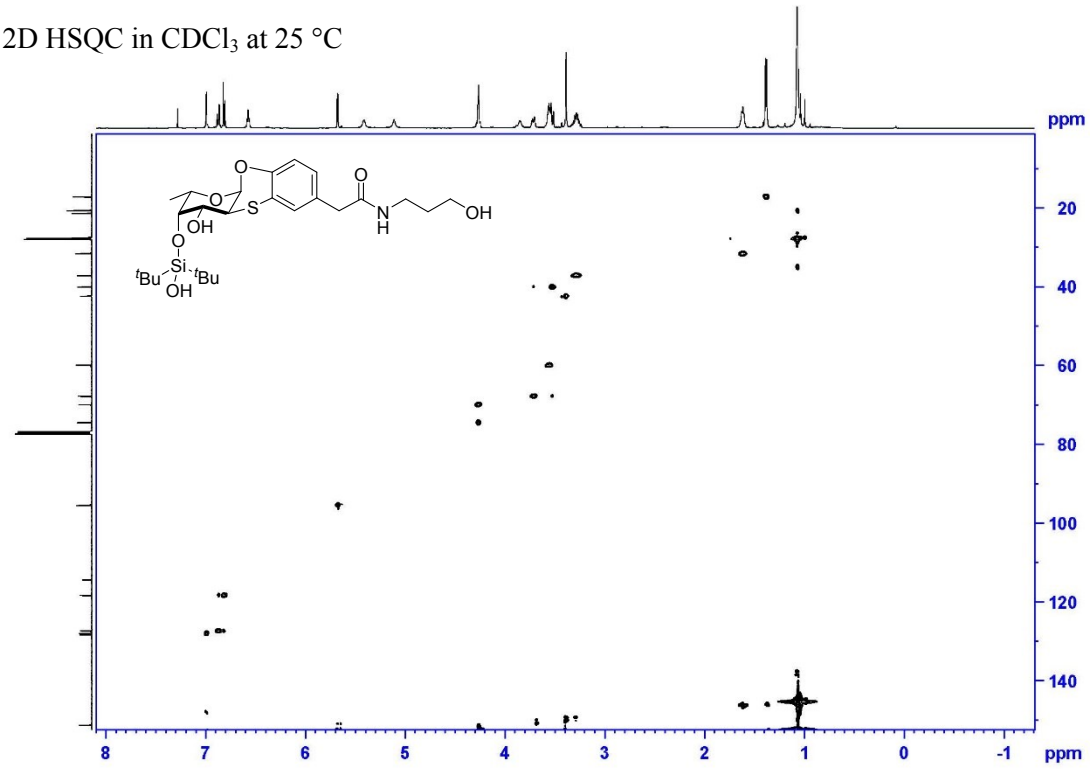


NMR of compound 6:

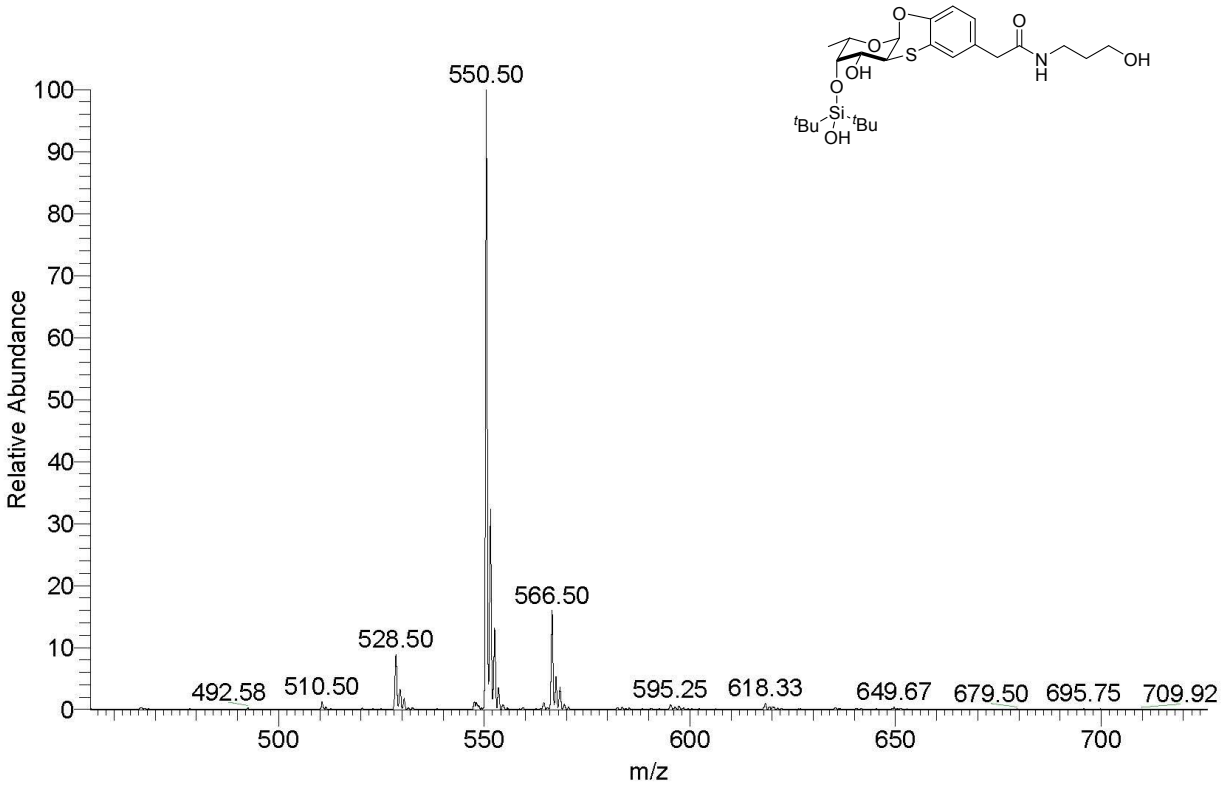
^{1}H-^{1}H COSY in $CDCl_3$ at 25 °C



^{1}H-^{13}C 2D HSQC in $CDCl_3$ at 25 °C

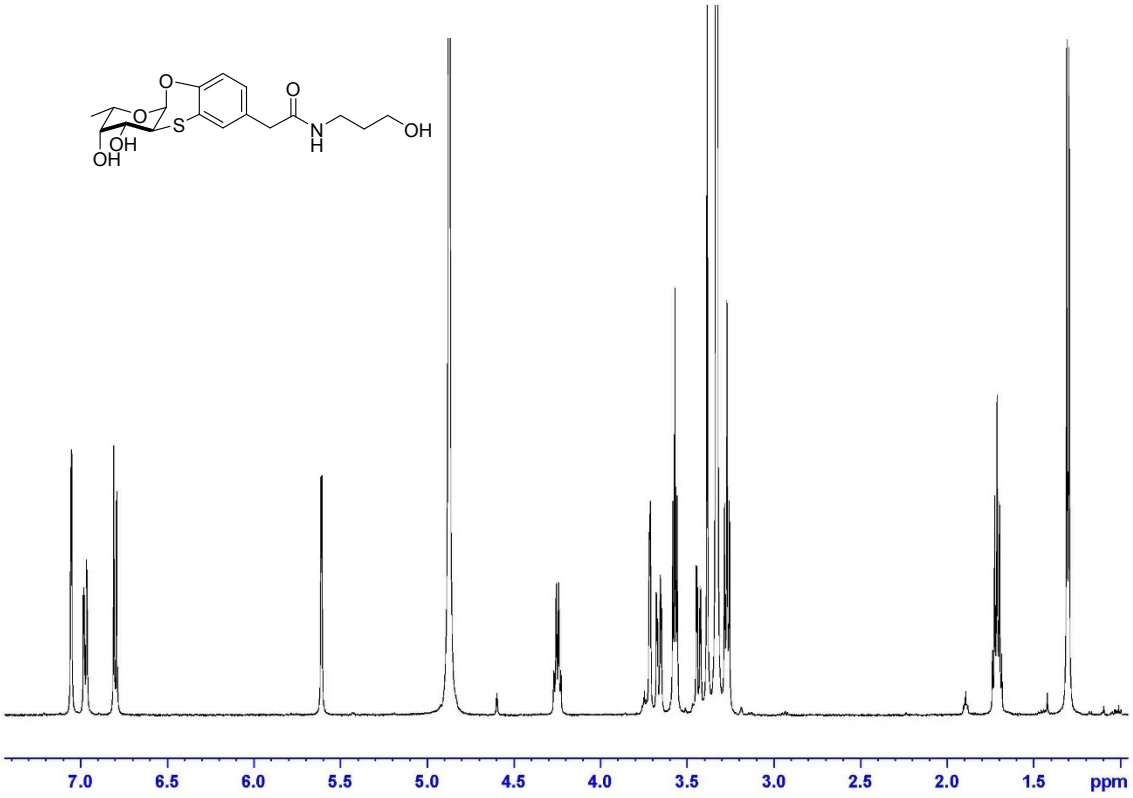


ESI-MS of compound 6:

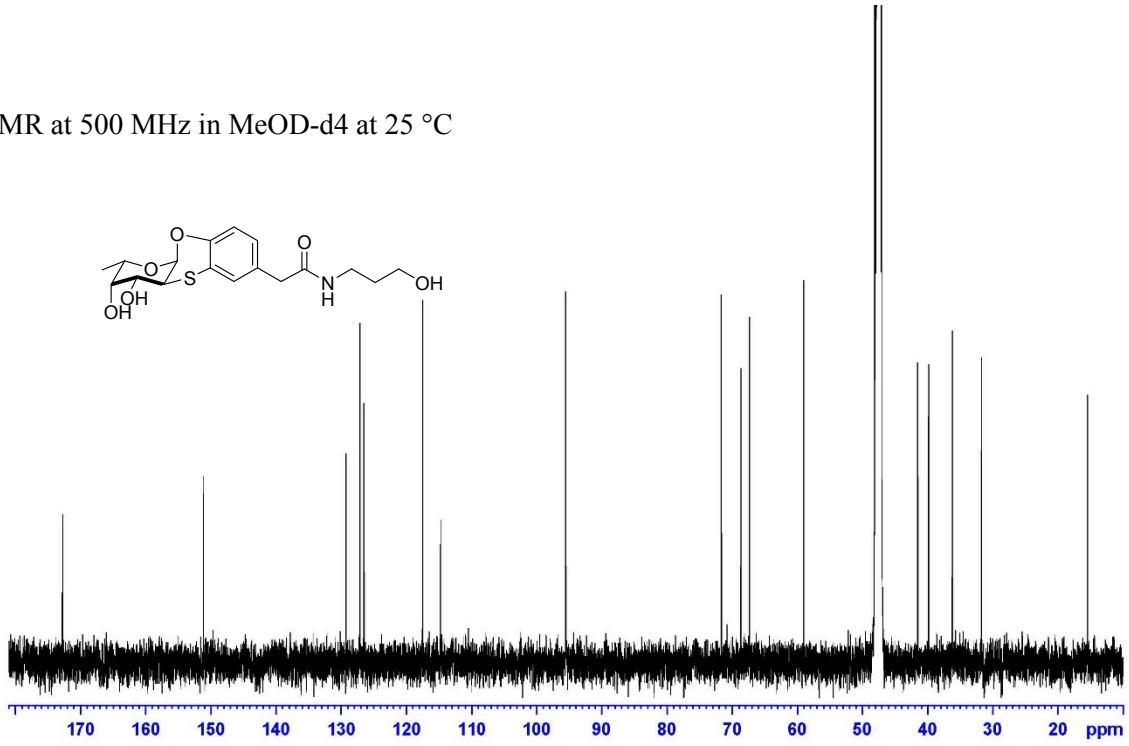


NMR Spectra of compound 3:

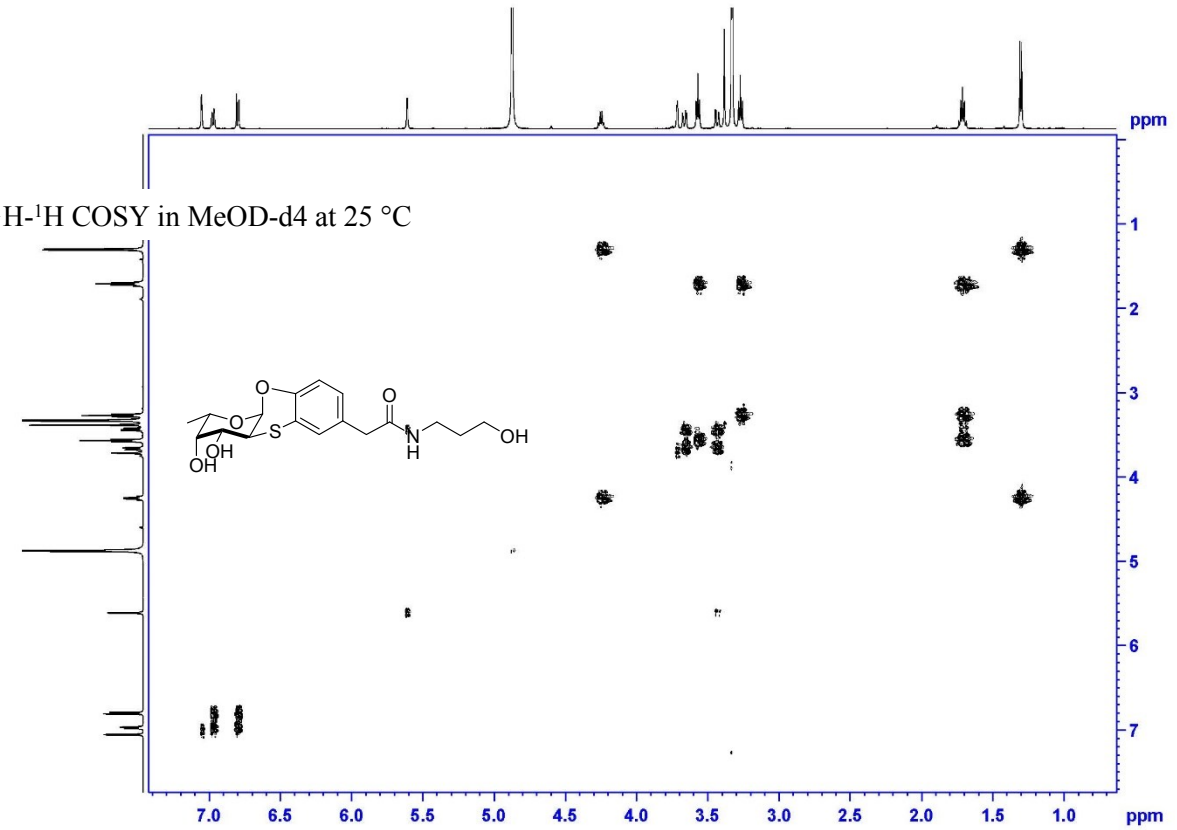
^{1}H NMR at 500 MHz in MeOD-d4 at 25 °C



^{13}C NMR at 500 MHz in MeOD-d4 at 25 °C

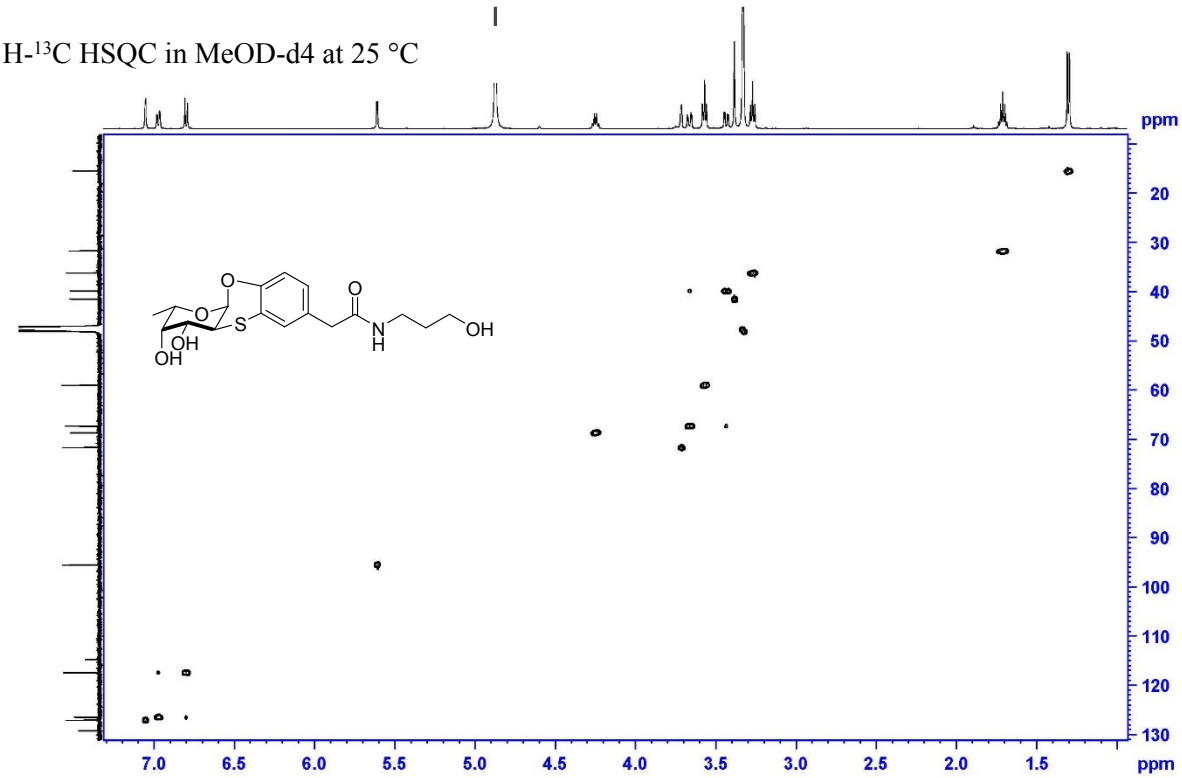


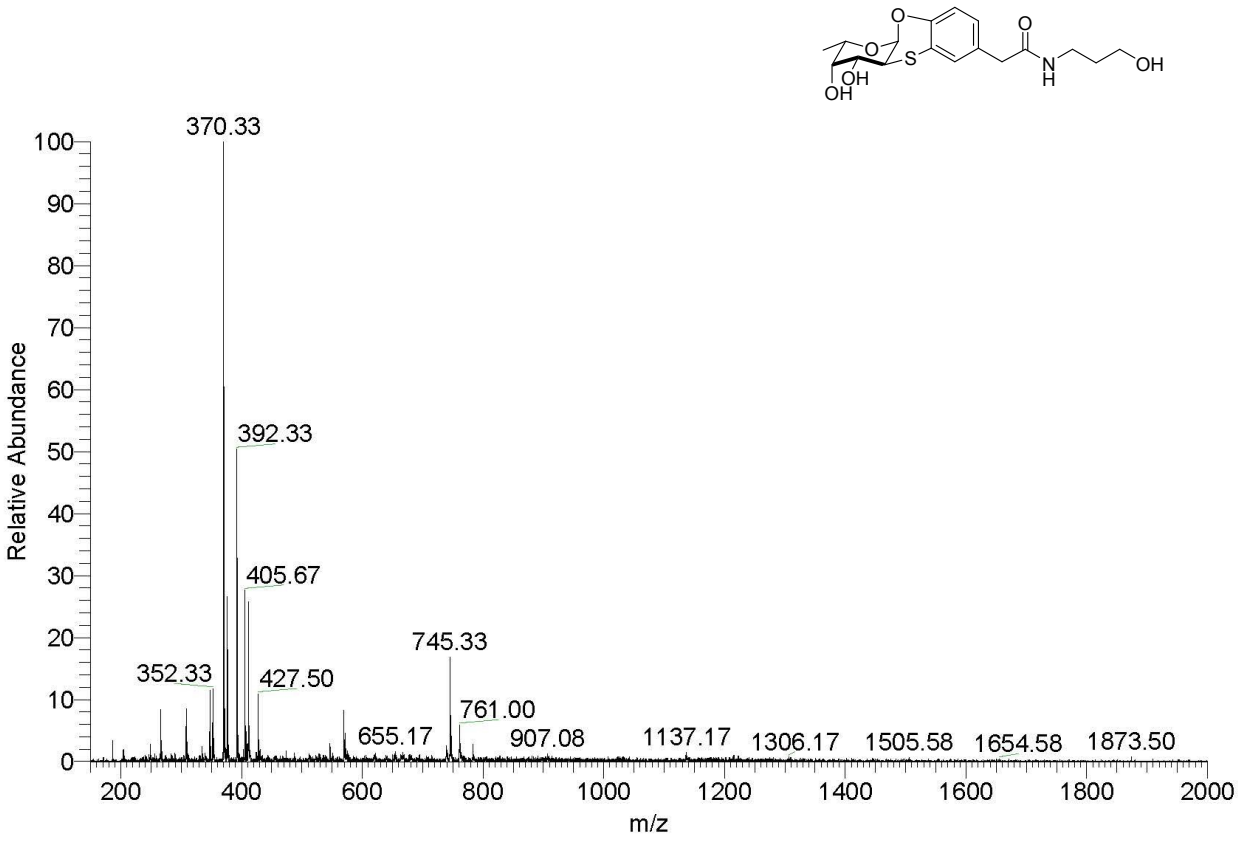
^{1}H-^{1}H COSY in MeOD-d4 at 25 °C



ESI-MS of compound 3:

^{1}H-^{13}C HSQC in MeOD-d4 at 25 °C

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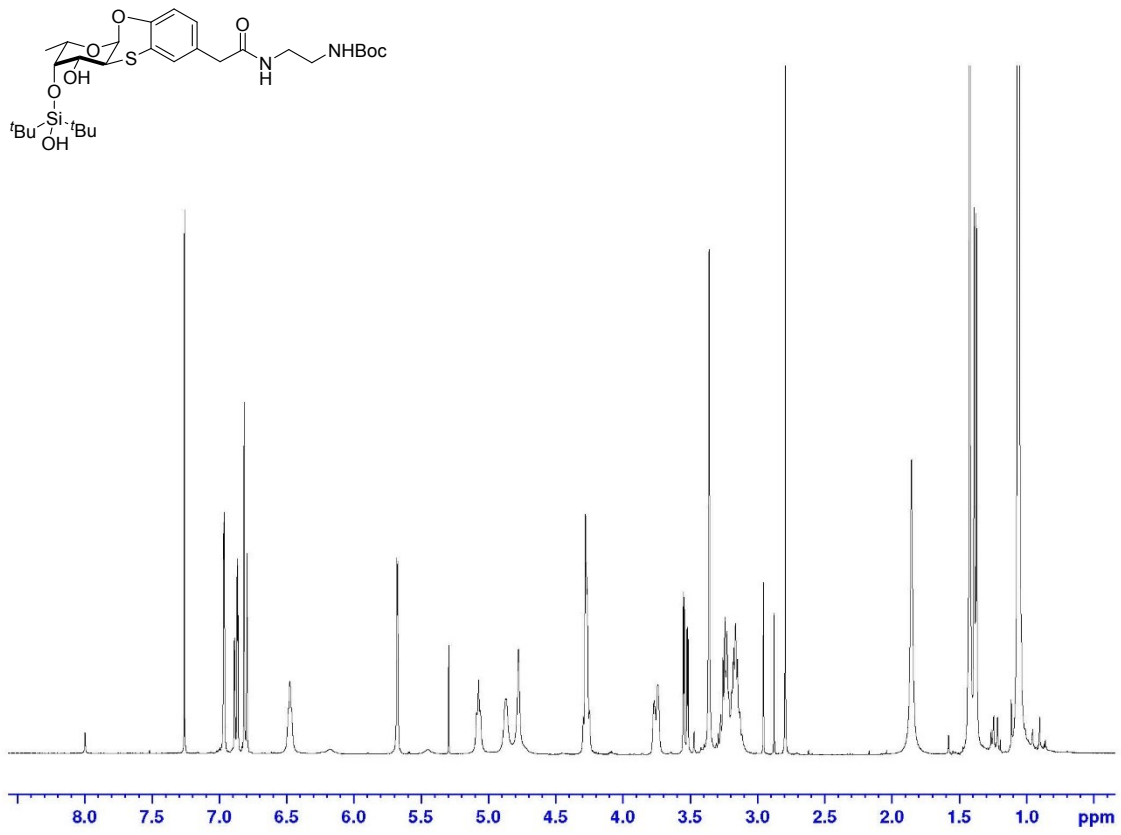
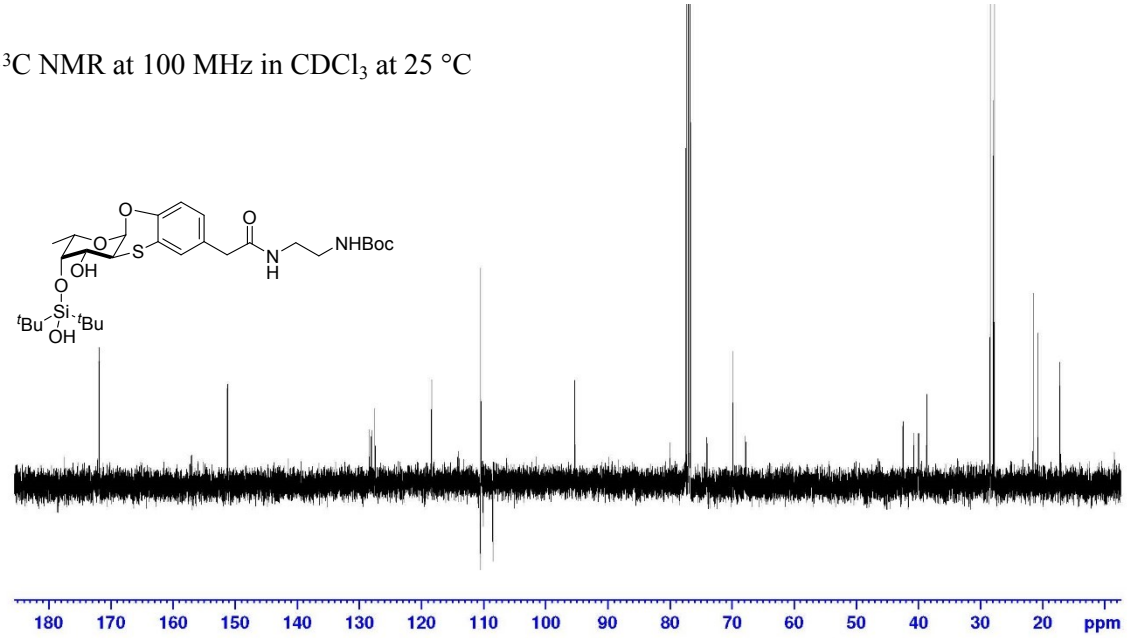
1505.58
1654.58
1873.50
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
200
400
600
800
1000
1200
1400
1600
1800
2000

NMR of compound 9:

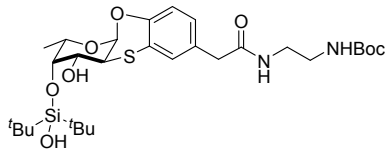
^{1}H NMR at 400 MHz in $CDCl_3$ at 25 °C

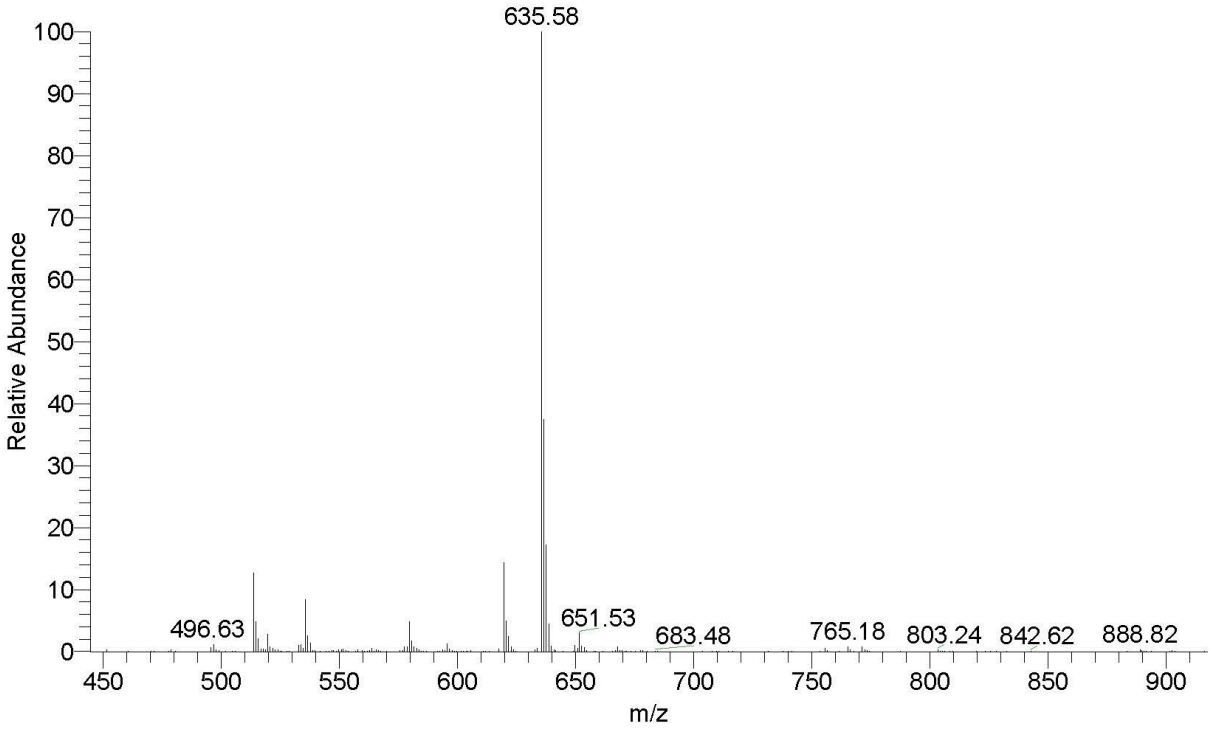


^{13}C NMR at 100 MHz in $CDCl_3$ at 25 °C

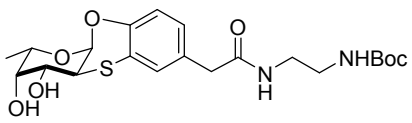


ESI-MS of compound 9:

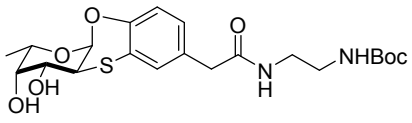




^{1}H NMR at 500 MHz in DMSO-d6 at 25 °C

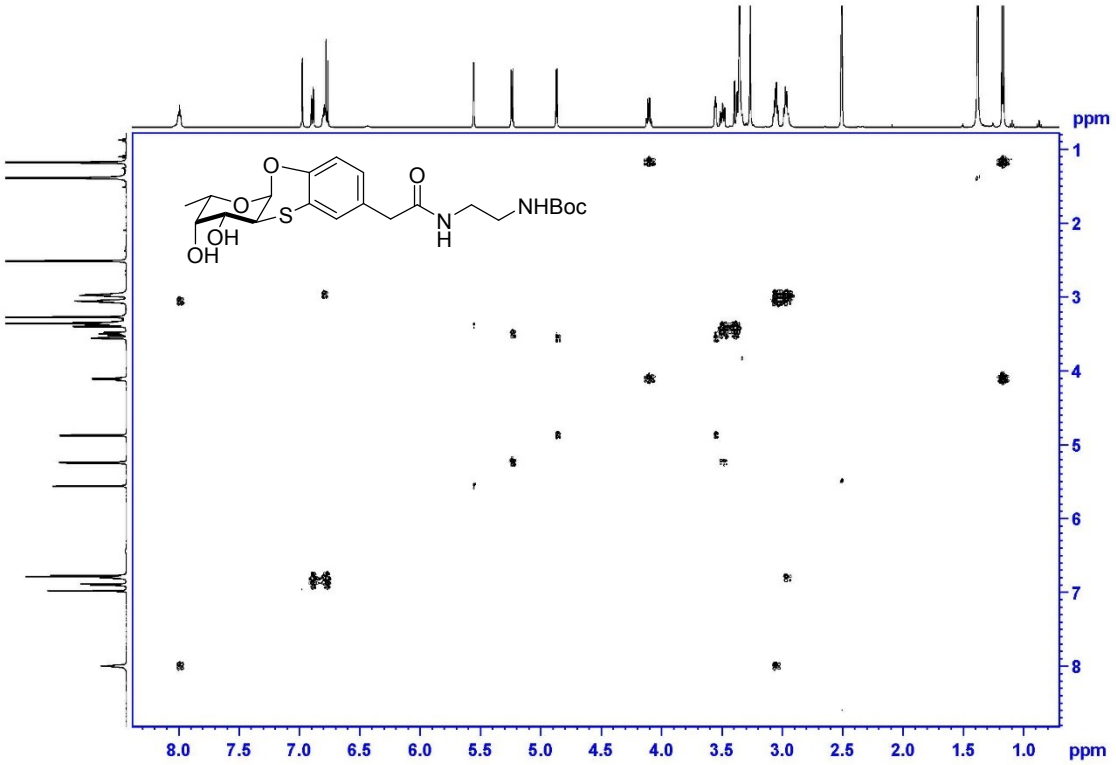


^{13}C NMR at 125 MHz in DMSO-d6 at 25 °C

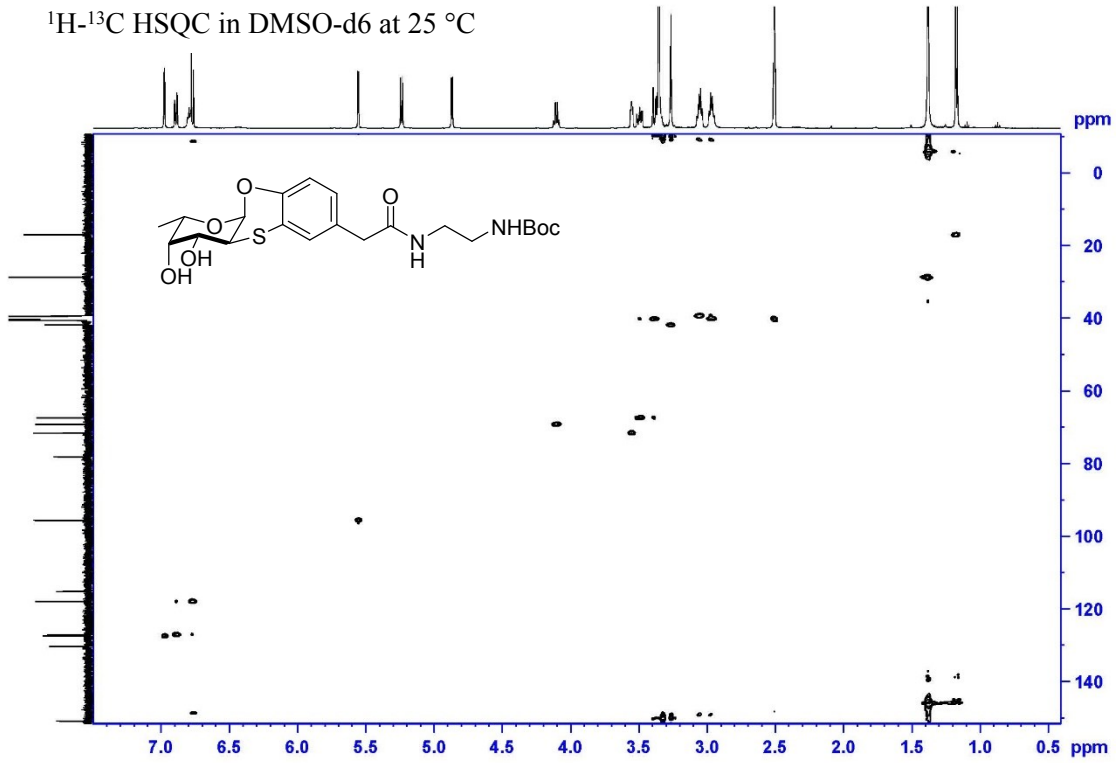


NMR of compound 10:

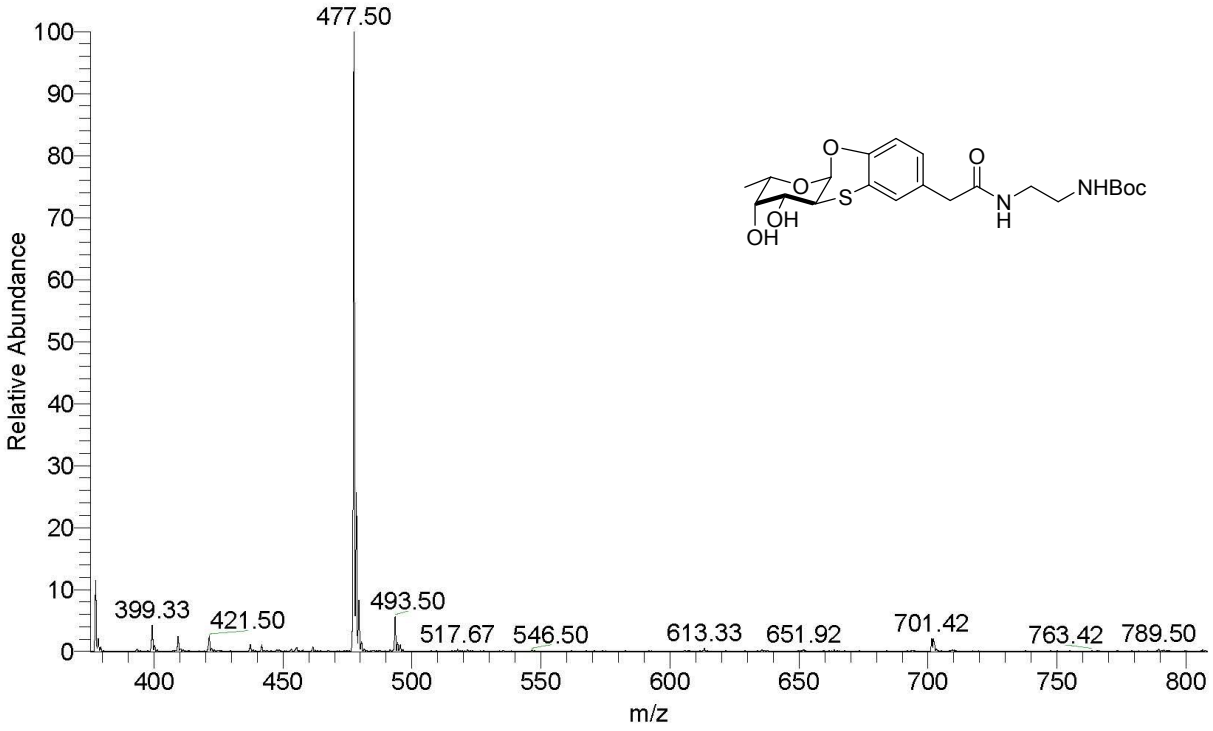
^{1}H-^{1}H COSY in DMSO-d6 at 25 °C



^{1}H-^{13}C HSQC in DMSO-d6 at 25 °C

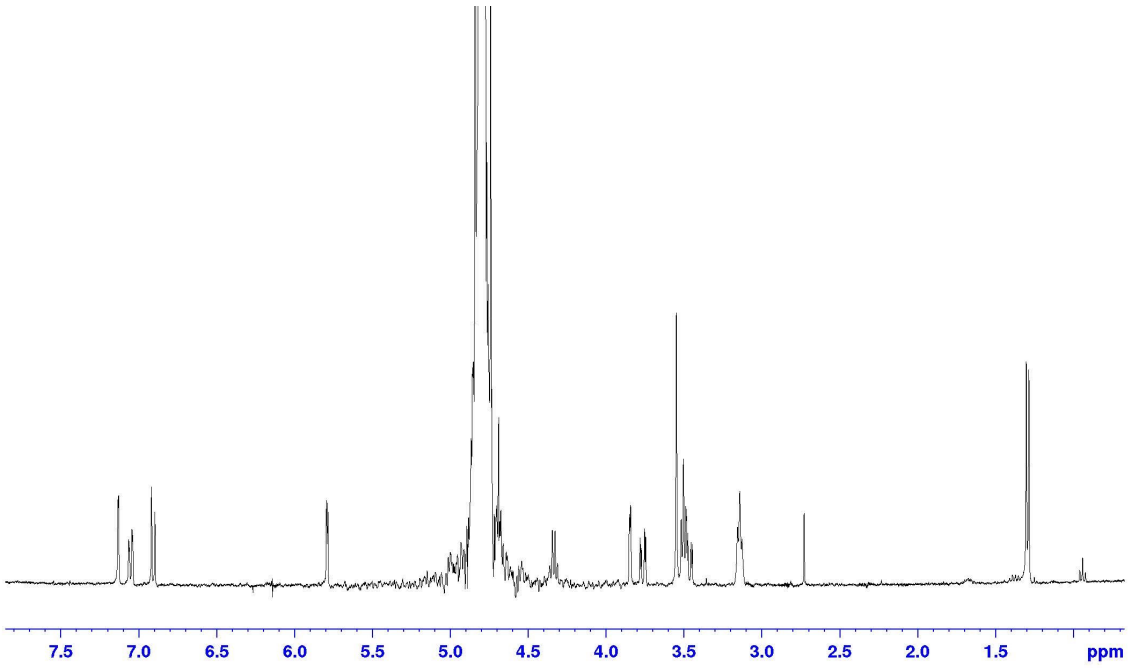


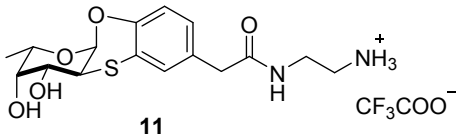
ESI-MS of compound 10:



NMR of compound 11:

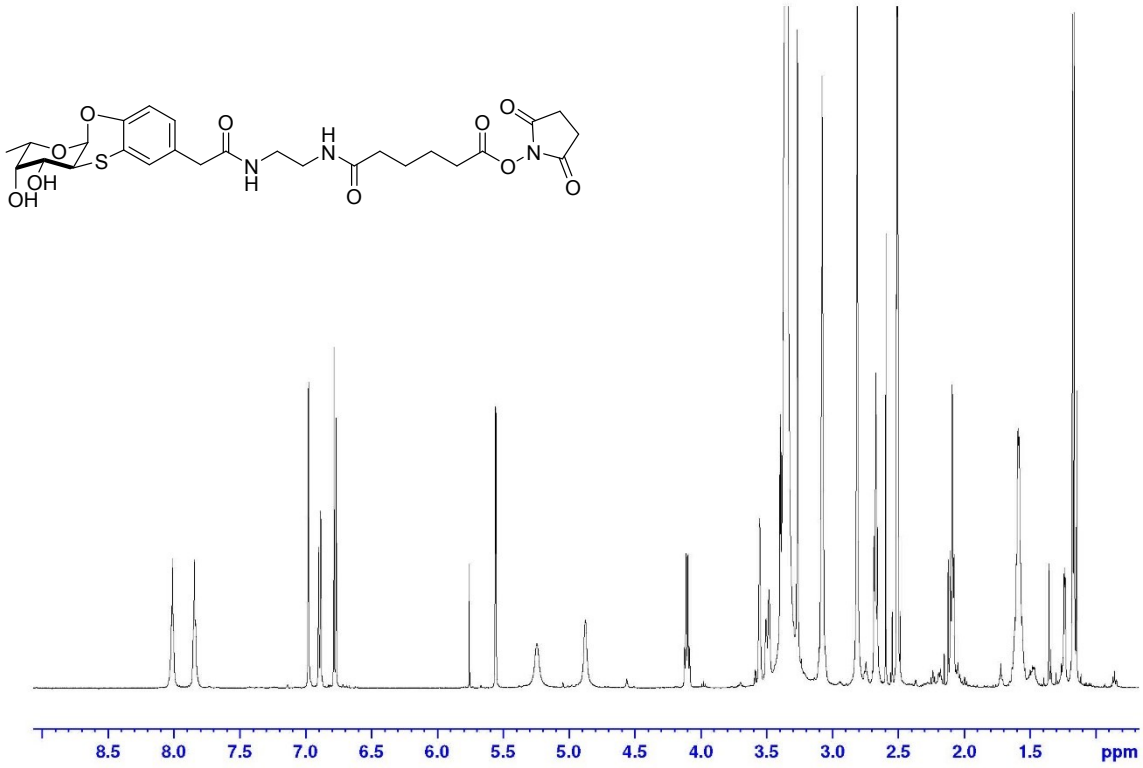
^{1}H NMR at 500 MHz in D_2O at 25 °C



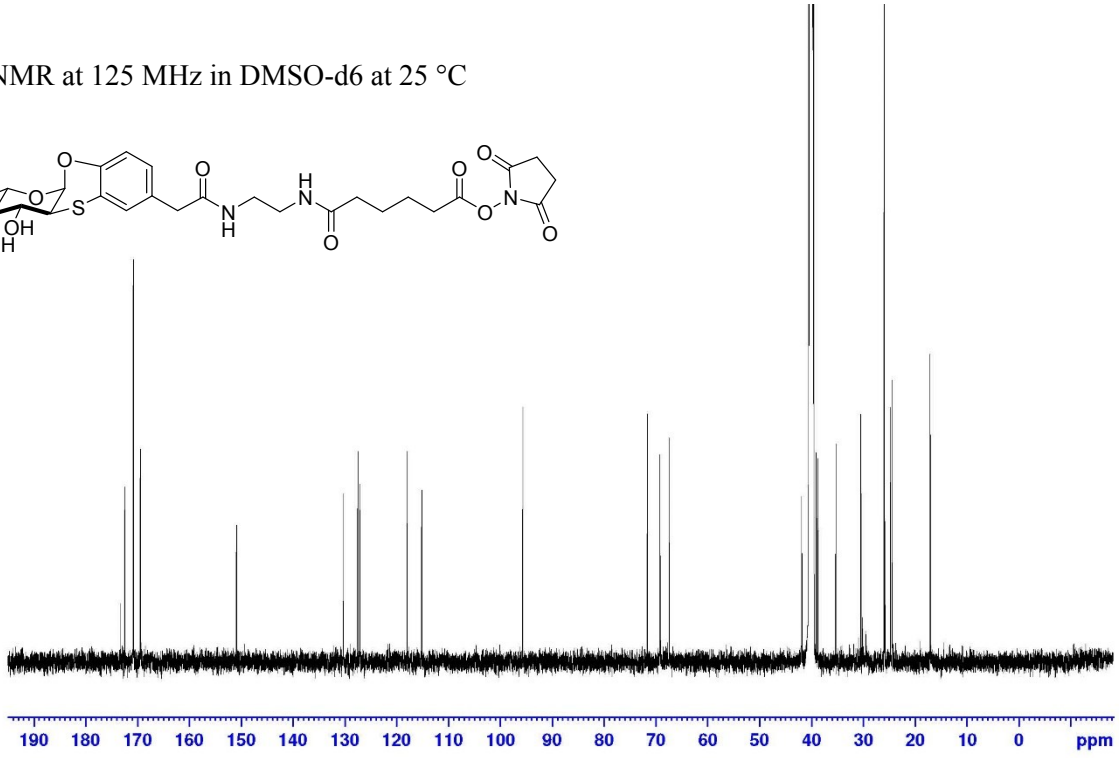


11

^{1}H NMR at 500 MHz in DMSO-d6 at 25 °C

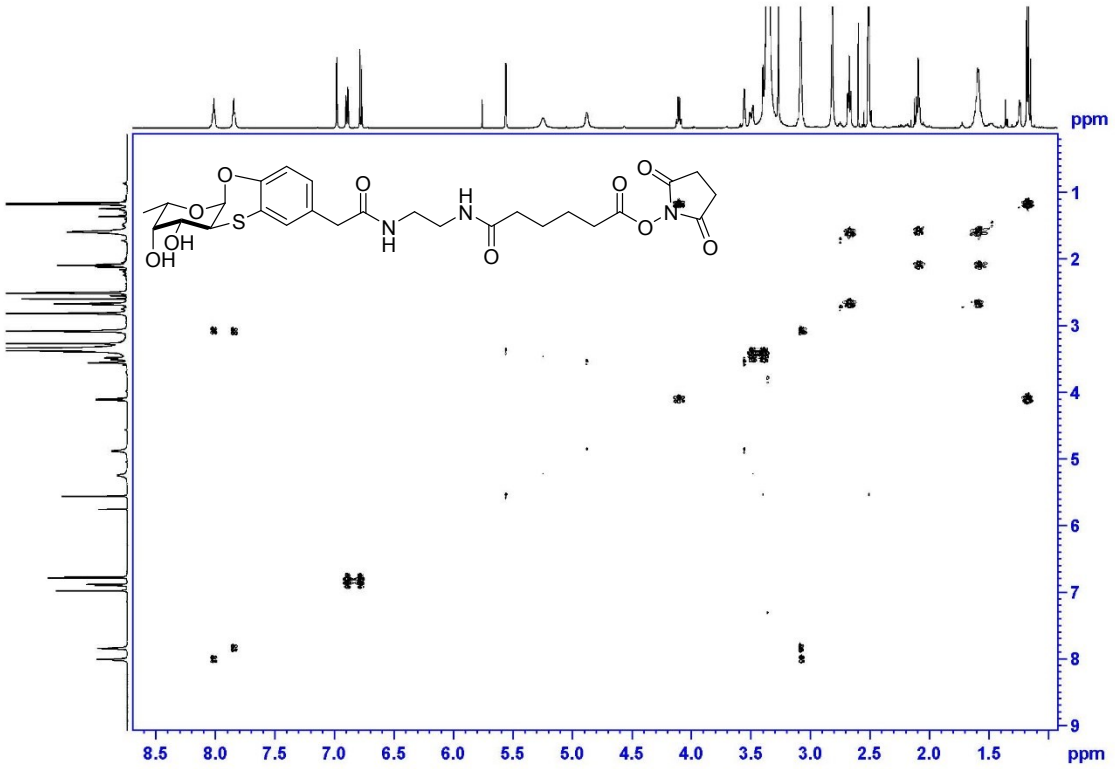


^{13}C NMR at 125 MHz in DMSO-d6 at 25 °C

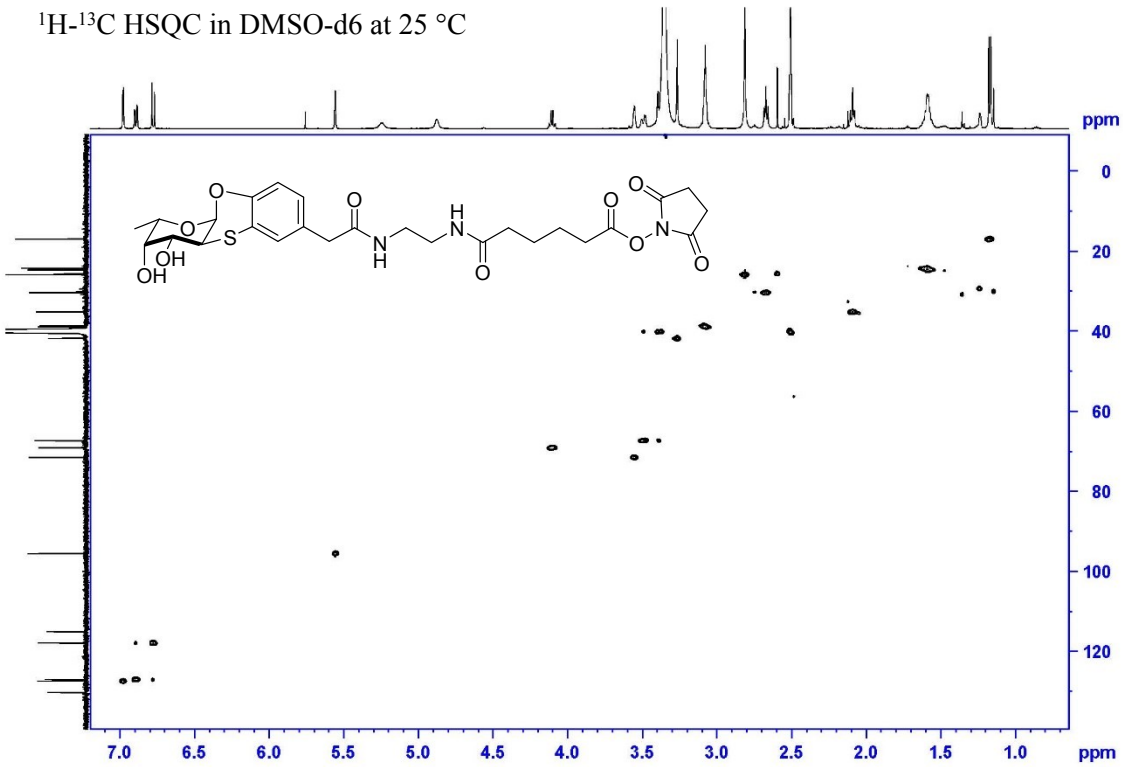


NMR of compound 7:

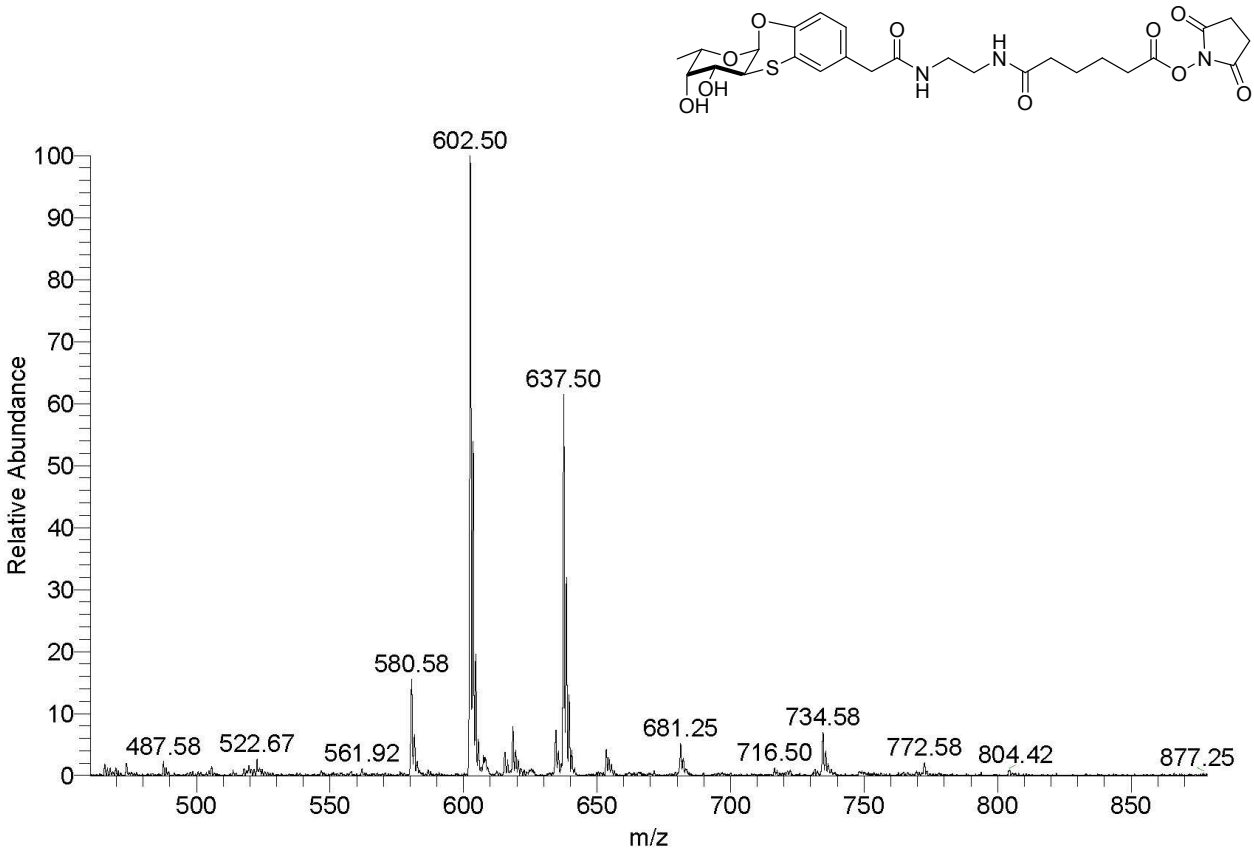
^{1}H-^{1}H COSY in DMSO-d6 at 25 °C



^{1}H-^{13}C HSQC in DMSO-d6 at 25 °C

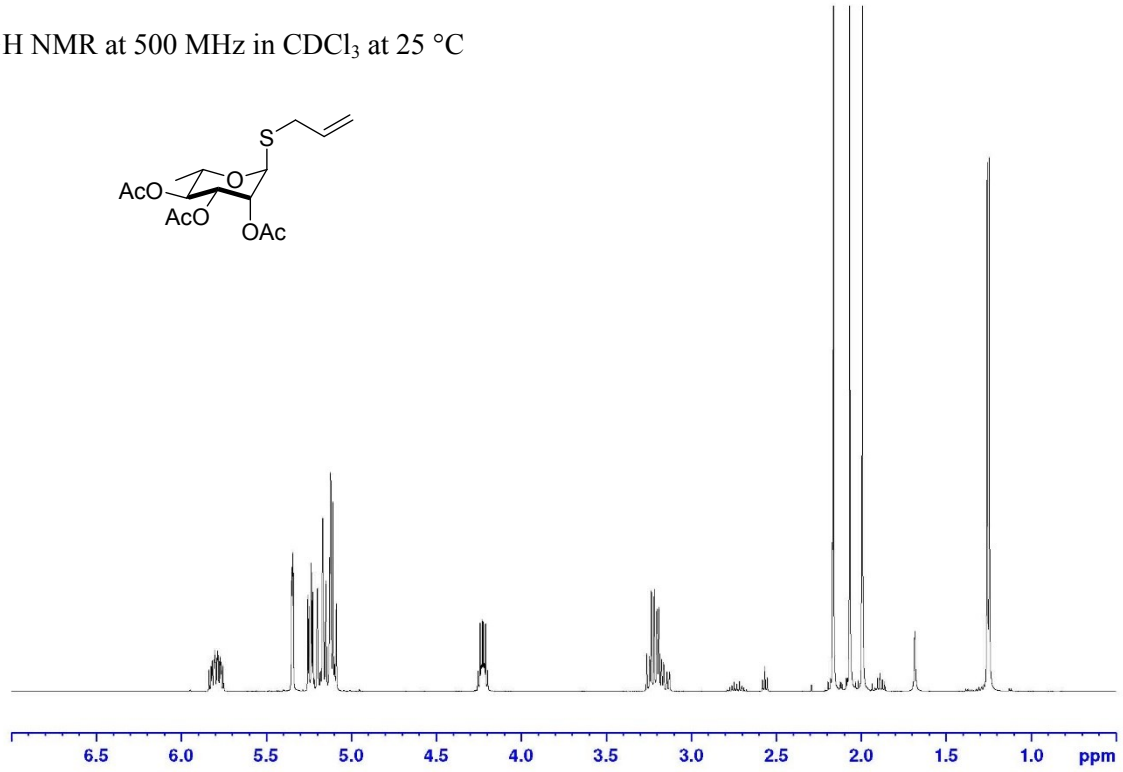


ESI-MS of compound 7:

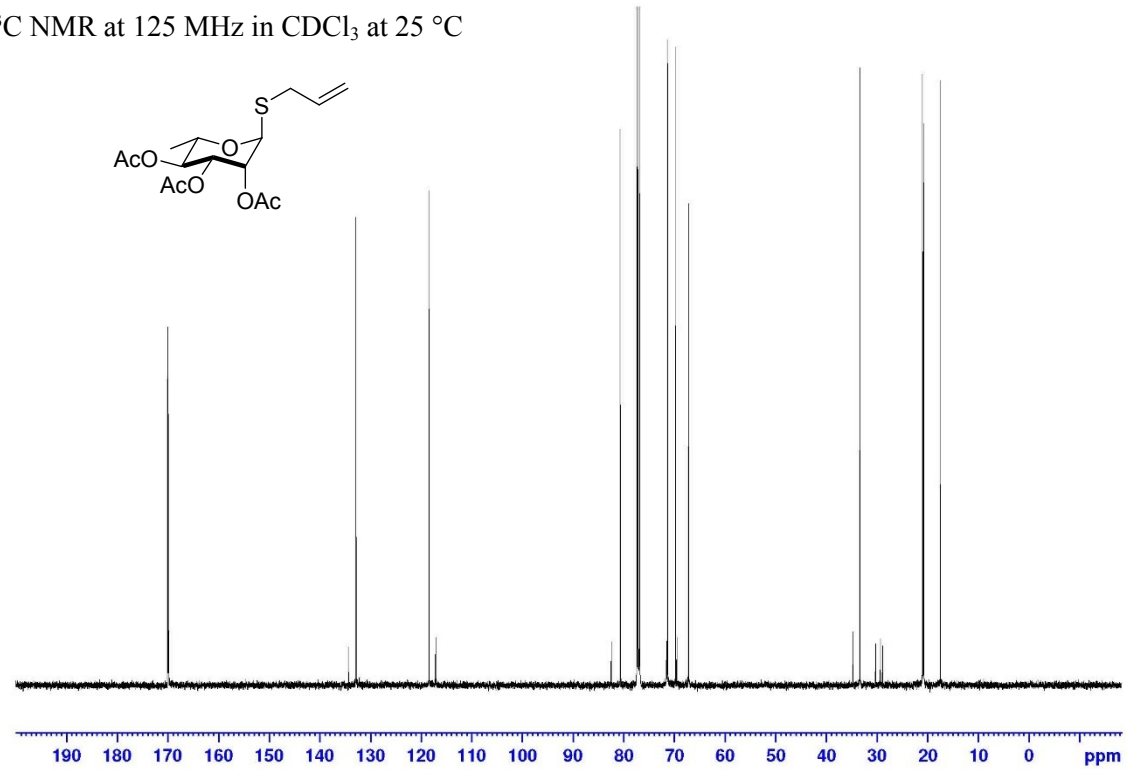


NMR of compound 14:

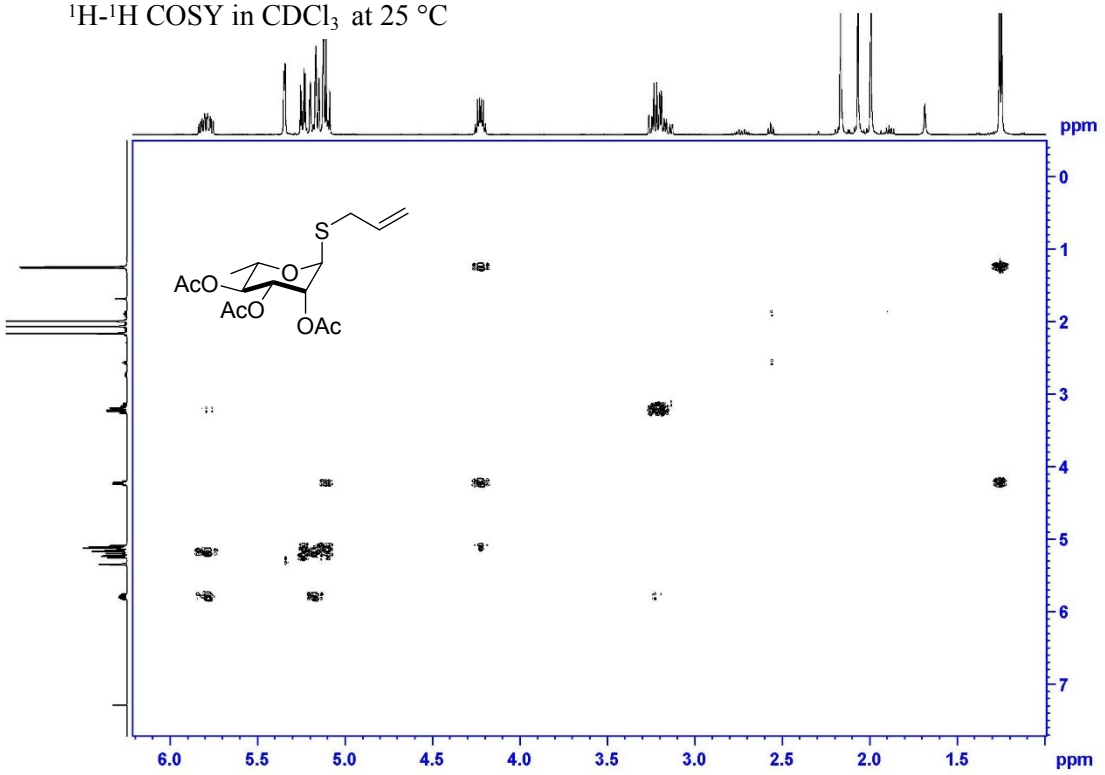
^{1}H NMR at 500 MHz in $CDCl_3$ at 25 °C



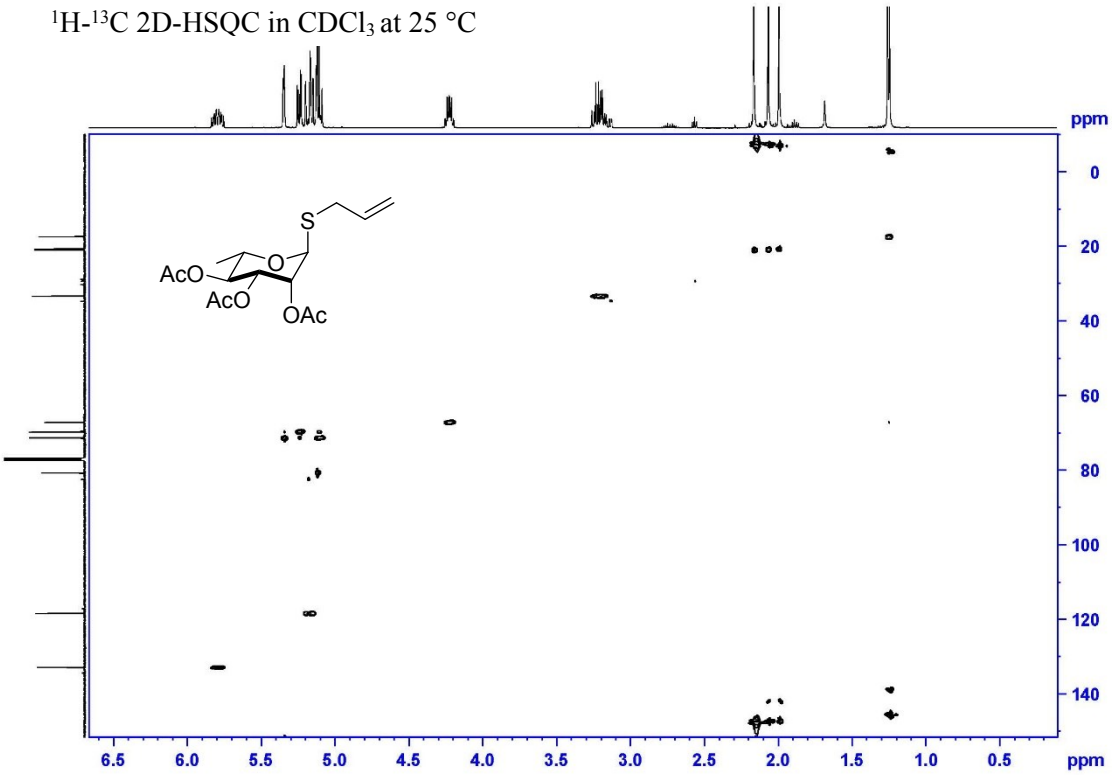
^{13}C NMR at 125 MHz in $CDCl_3$ at 25 °C



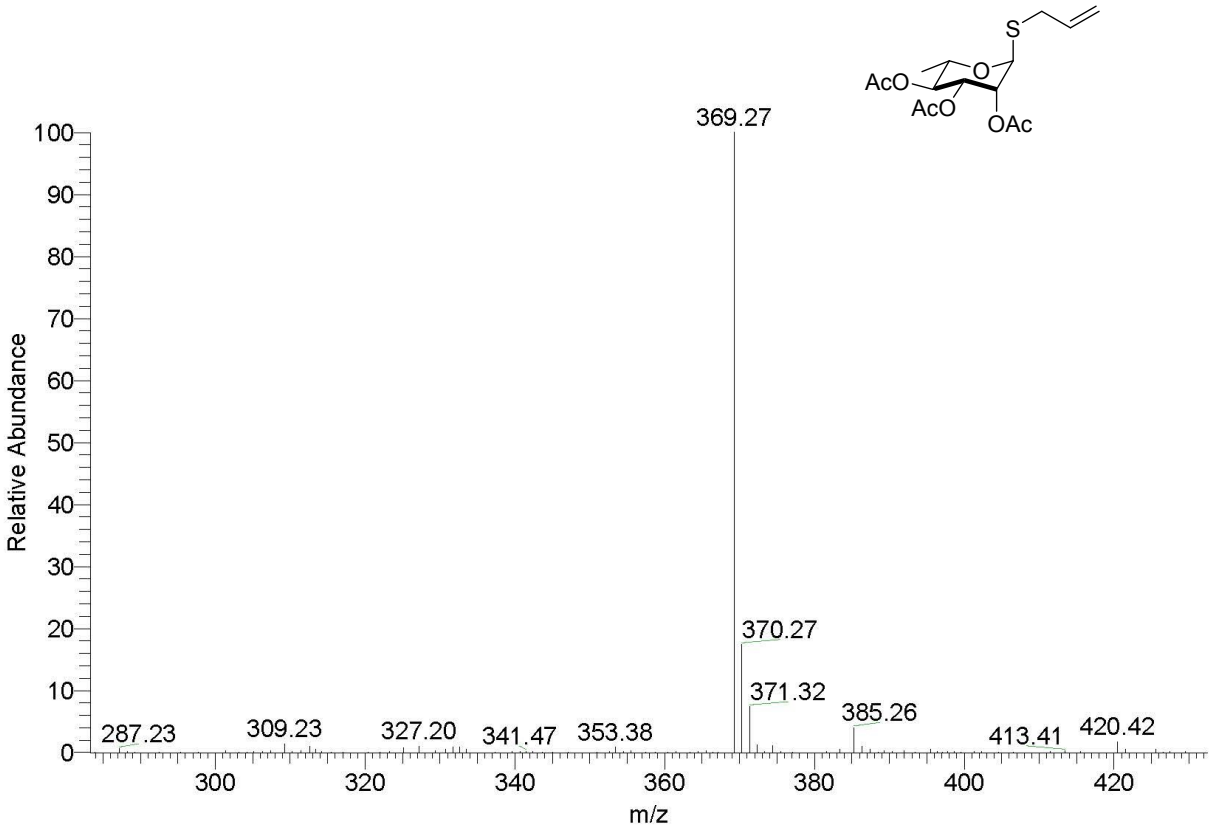
^{1}H-^{1}H COSY in $CDCl_3$ at 25 °C



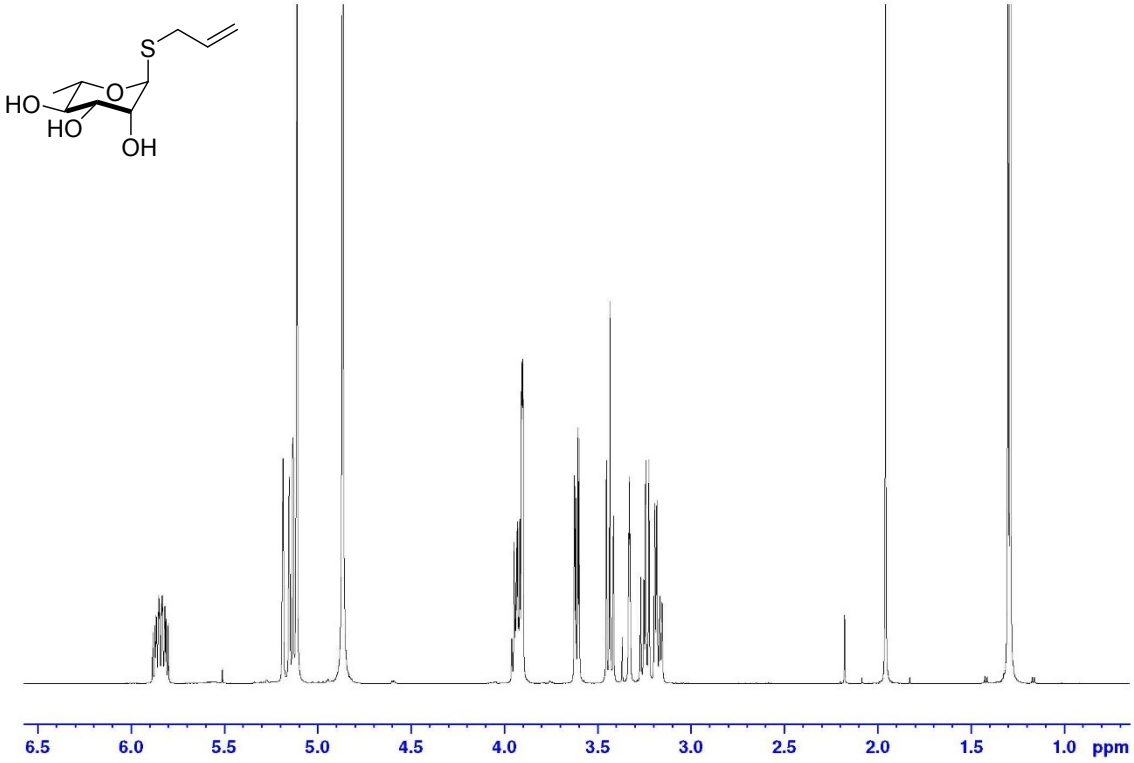
^{1}H-^{13}C 2D-HSQC in $CDCl_3$ at 25 °C



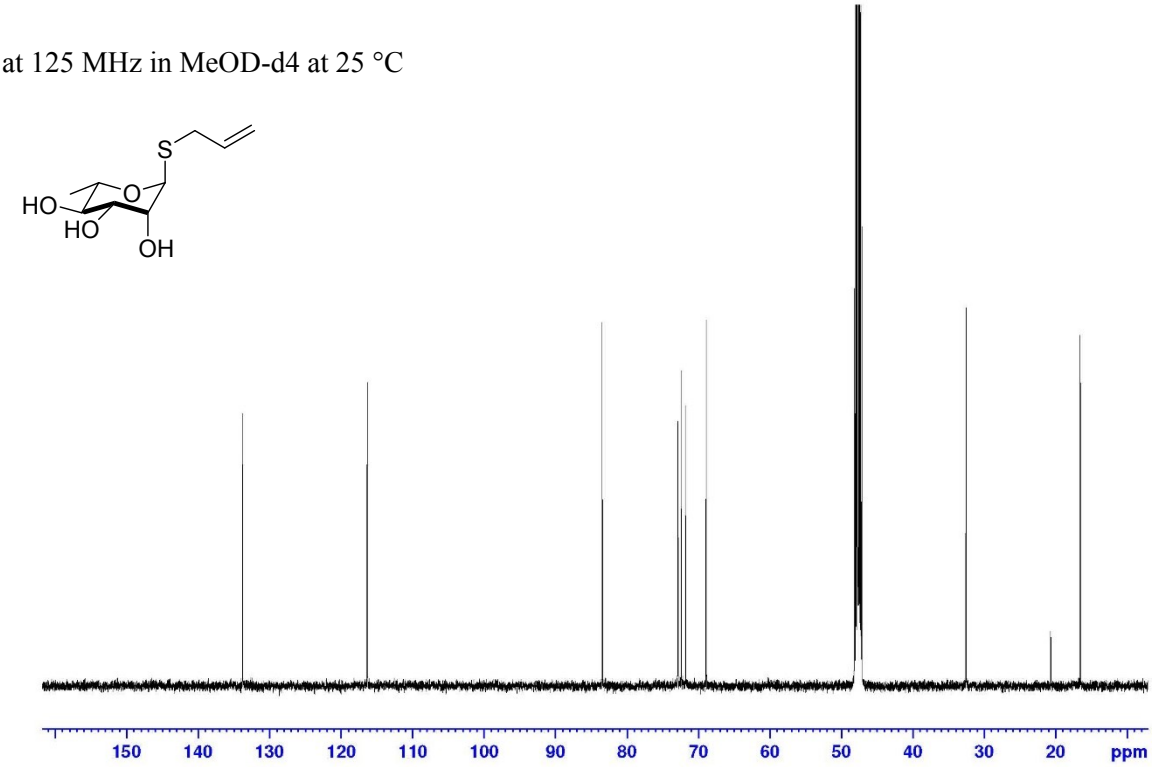
ESI-MS of compound 14:



^{1}H NMR at 500 MHz in MeOD-d4 at 25 °C

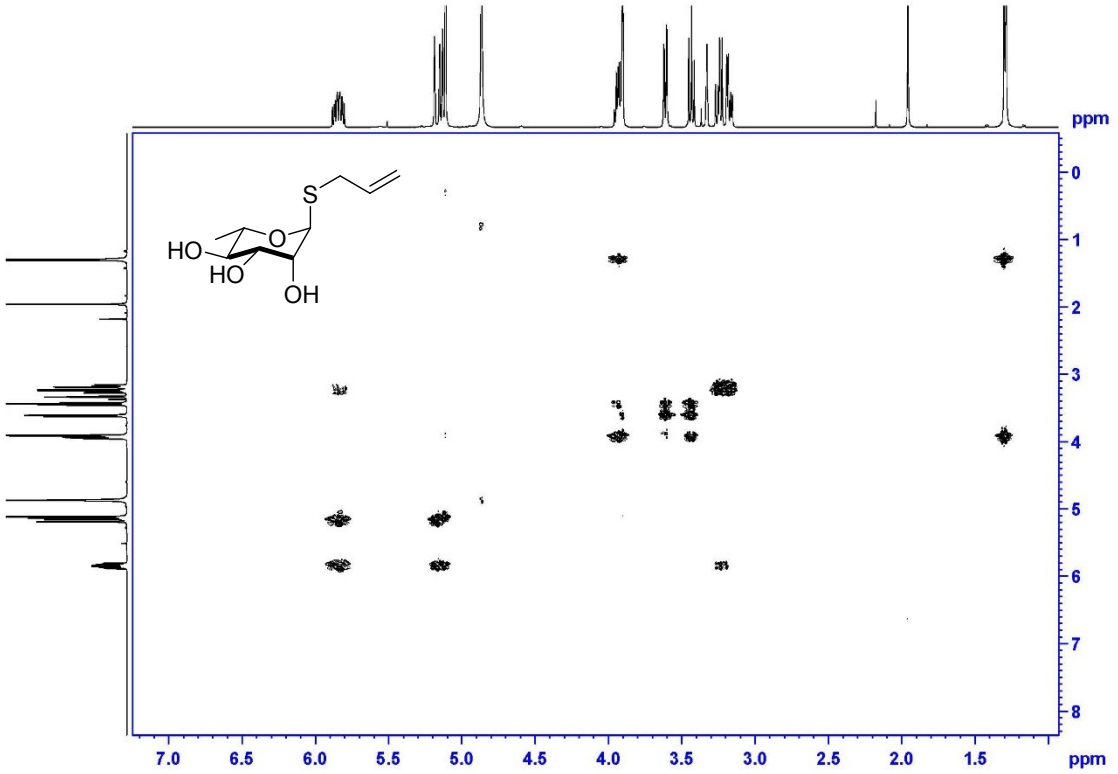


^{13}C NMR at 125 MHz in MeOD-d4 at 25 °C

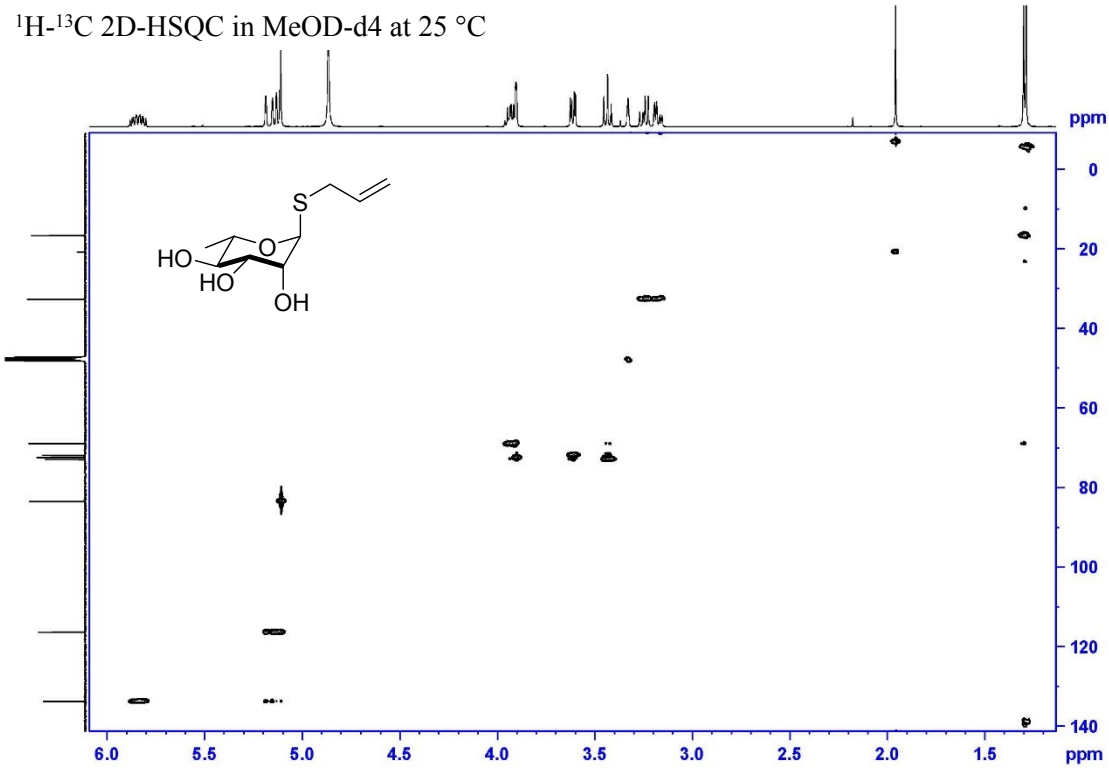


NMR compound 13:

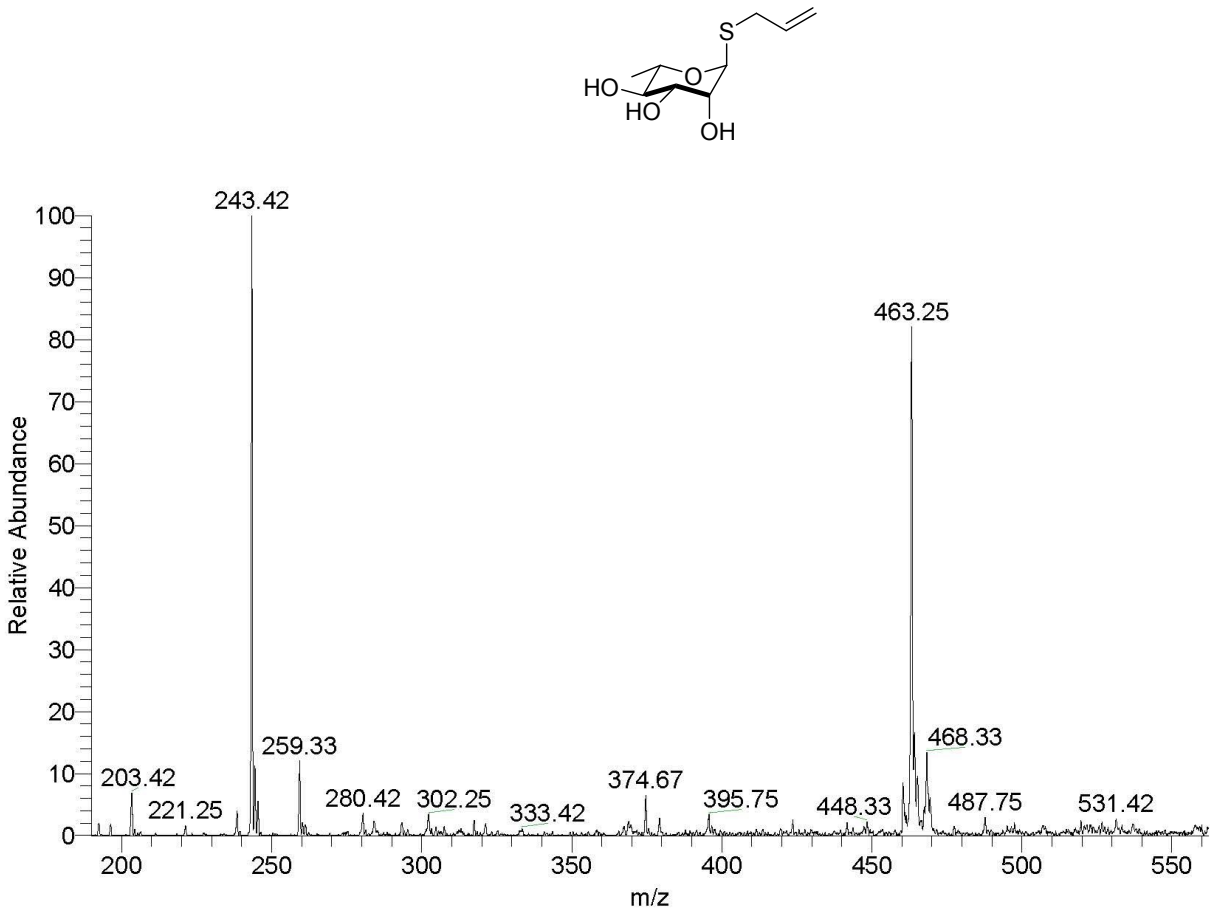
^{1}H-^{1}H COSY in MeOD-d4 at 25 °C



^{1}H-^{13}C 2D-HSQC in MeOD-d4 at 25 °C



ESI-MS of compound 13:



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