**Supplemental Material**

**Synthesis of 4MU-GalNAc-4-S and 4MU-GalNAc-6-S.**

**(2R,3R,4R,5R,6S)-5-acetamido-2-(acetoxymethyl)-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-3,4-diyl diacetate (4)** Pyridine (15 mL) was added to nitrogen back flushed flask containing D-galactosamine hydrochloride (1 g, 4.63 mmol) and the resultant slurry was cooled on an ice bath. To the cooled mixture acetic anhydride (5 g, 48.9 mmol) was added dropwise and allowed to warm to room temperature followed by stirring at this temperature for 20 hours. The reaction mixture was quenched with the addition of methanol (5 mL) and let to stir for 20 minutes. The resultant mixture was concentrated under reduced pressure and the residue was dissolved in 20% methanol in chloroform with the aid of warming the mixture. This solution was washed with 1N HCl solution followed by brine solution. The resultant organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The residue was taken in nitrogen back flushed flask equipped with a dropping funnel. Anhydrous dichloromethane (20 mL) was added to this residue and the resultant slurry was cooled on an ice bath. In the dropping funnel titanium tetrachloride (1.4 g, 7.38 mmol) was dissolved in anhydrous dichloromethane (8 mL) and the resulting solution was added dropwise to the cooled slurry. The reaction mixture was warmed to 50°C in an oil bath and left to stir at this temperature for 48 hours. Then the mixture was cooled back on an ice bath and saturated sodium bicarbonate solution was added dropwise with vigorous shaking. The resultant mixture was extracted between chloroform and saturated sodium bicarbonate solution. The organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The resultant residue was dissolved in dichloromethane (14 mL) and added slowly to a cooled mixture of 4-methylumbelliferone (1.63 g, 9.25 mmol) and tetrabutylammonium hydrogensulfate (2.04 g, 6.01 mmol) in aqueous 1 N NaOH solution (11 mL) at 0°C. The reaction was warmed to room temperature and left to stir for 16 hours. The reaction mixture was extracted between 1N NaOH and dichloromethane and the dichloromethane layer was further washed with brine solution. The organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The crude product thus obtained was purified by silica flash chromatography using a gradient of 40 to 100% ethyl acetate in hexanes as the elution mixture. The fractions with the desired compound, as determined by TLC, were combined and concentrated under reduced pressure to get **4** (0.743 g, 32%). 1H NMR (300 MHz, CDCl3) *δ* 7.50 (d, *J* = 9.2 Hz, 1H), 6.99 – 6.91 (m, 2H), 6.17 (s, 1H), 5.70 (d, *J* = 8.6 Hz, 1H), 5.48 – 5.35 (m, 3H), 4.30 (dd, *J* = 18.9, 8.6 Hz, 1H), 4.23 – 4.06 (m, 3H), 2.40 (s, 3H), 2.18 (s, 3H), 2.10 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H). MS (ESI+) for [M + H]+; calculated: 506.2, found: 506.1.

 **Sodium ((2R,3R,4R,5R,6S)-5-acetamido-3,4-dihydroxy-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-2-yl)methyl sulfate (4MU-GalNAc-6-S).** To a solution of **4** (0.172 g, 0.340 mmol) in anhydrous methanol (6 mL), cooled on an ice bath, 0.5 M sodium methoxide solution in methanol (90 µL, 45 µmol) was added dropwise and allowed to warm to room temperature. After 2 hours formic acid (50 µL) was added to the reaction mixture and concentrated to dryness under reduced pressure. The resultant residue was dissolved with anhydrous pyridine (8 mL) under nitrogen atmosphere and sulfur trioxide pyridine complex (106 mg, 0.666 mmol) was added to this solution and let to stir at 45°C for 5 hours. The reaction was quenched with the addition of methanol (0.5 mL) and subjected to semi-preparative reverse phase HPLC purification (gradient water/methanol system) to yield **4MU-GalNAc-6-S** as white amorphous solid (66 mg, 40%). The **4MU-GalNAc-6-S** was further purified by ion-exchange chromatography. A fritted thin column was loaded with 3 mL of Q-Sepharose fast flow (GE Healthcare, product code: 17-0510-01) and washed with methanol (50 mL). A solution of the **4MU-GalNAc-6-S** in methanol (2 mL) was loaded on to this column and the column was further washed with methanol (100 mL). The **4MU-GalNAc-6-S** was then eluted with 1M ammonium formate in methanol solution (100 mL) and concentrated under reduced pressure at temperature less than 25⁰C. The resultant residue was dissolved in DI-water and loaded on a C18 cartridge (Resprep cat # 26034, which was pre-activated with methanol and washed with DI-water) by applying negative pressure in the bottom. The cartridge was further washed with DI water (4x25 mL) and the substrate was eluted from the cartridge using methanol (4x25 mL). The methanol fraction was concentrated under reduced pressure at temperature less than 25⁰C to afford the pure **4MU-GalNAc-6-S**. 1H NMR (300 MHz, D2O) δ 7.36 (d, *J* = 8.9 Hz, 1H), 6.85 (dd, *J* = 8.9, 2.4 Hz, 1H), 6.62 (d, *J* = 2.4 Hz, 1H), 5.92 (s, 1H), 5.11 (d, *J* = 8.4 Hz, 1H), 4.34 – 4.20 (m, 4H), 4.12 (d, *J* = 3.3 Hz, 1H), 4.00 (dd, *J* = 10.9, 3.2 Hz, 1H), 2.19 (s, 3H), 2.12 (s, 3H). MS (ESI-) for [M - Na]-; calculated: 458.1, found: 458.2.

 **((2R,3R,4R,5R,6S)-5-acetamido-4-(benzoyloxy)-3-hydroxy-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-2-yl)methyl benzoate (5).** To a solution of **4** (0.24 g, 0.475 mmol) in anhydrous methanol (7 mL), cooled on an ice bath, 0.5 M sodium methoxide solution in methanol (100 µL, 50 µmol) was added dropwise and allowed to warm to room temperature. After 2 hours formic acid (50 µL) was added to the reaction mixture and concentrated to dryness under reduced pressure. The resultant residue was dissolved with anhydrous pyridine (6 mL) under nitrogen atmosphere and a portion of benzoyl chloride (66.0 µL, 0.568 mmol) was added dropwise at 0°C and left to stir at this temperature. Then another portion of benzoyl chloride (66.0 µL, 0.568 mmol) was added dropwise at 0°C and warmed to room temperature and left to stir for 2 hours. The reaction was extracted between 1 M HCl solution and chloroform. The chloroform layer was further washed with brine solution. The organic layer was concentrated and purified by flash silica column chromatography using a gradient of 2 to 8% methanol in DCM as the eluent. The desired fractions were concentrated under reduced pressure and further under high vacuum to yield **5** (0.187 g, 67%). 1H NMR (300 MHz, CDCl3) δ 8.04 (t, *J* = 8.5 Hz, 4H), 7.61 – 7.31 (m, 8H), 7.01 – 6.89 (m, 2H), 6.13 (s, 1H), 5.41 – 5.25 (m, 2H), 4.73 – 4.53 (m, 3H), 4.27 (d, *J* = 2.6 Hz, 1H), 4.24 – 4.15 (m, 1H), 2.33 (s, 3H), 1.86 (s, 3H). MS (ESI+) for [M + H]+; calculated: 588.2, found: 588.4.

 **sodium (2R,3R,4R,5R,6S)-5-acetamido-4-hydroxy-2-(hydroxymethyl)-6-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)tetrahydro-2*H*-pyran-3-yl sulfate (4MU-GalNAc-4-S).** To a solution of **5** (93.0 mg, 0.158 mmol) in pyridine (5 mL) under nitrogen atmosphere, sulfur trioxide pyridine complex (47 mg, 0.295 mmol) was added and warmed to 45°C and let to stir for 16 hours. The reaction was quenched with the addition of methanol (0.5 mL) and concentrated to dryness under reduced pressure. The resultant residue was dissolved in anhydrous methanol (16 mL), under nitrogen atmosphere, and cooled on an ice bath. To it 0.5 M sodium methoxide solution in methanol (1.6 mL, 0.800 mmol) was added dropwise and allowed to warm to room temperature and let to stir for 16 hours. The reaction was quenched with the addition of formic acid (0.1 mL) and subjected to semi-preparative reverse phase HPLC purification (gradient water/methanol system) to yield **4MU-GalNAc-4-S** as amorphous white solid (19.9 mg, 26%). The **4MU-GalNAc-4-S** was further purified by ion-exchange chromatography. A fritted thin column was loaded with 2 mL of Q-Sepharose fast flow (GE Healthcare, product code: 17-0510-01) and washed with methanol (50 mL). A solution of the **4MU-GalNAc-4-S** in methanol (2 mL) was loaded on to this column and the column was further washed with methanol (100 mL). The **4MU-GalNAc-4-S** was then eluted with 1M ammonium formate in methanol solution (100 mL) and concentrated under reduced pressure at temperature less than 25⁰C. The resultant residue was dissolved in DI-water and loaded on a C18 cartridge (Resprep cat # 26034, which was pre-activated with methanol and washed with DI-water) by applying negative pressure in the bottom. The cartridge was further washed with DI water (4x25 mL) and the substrate was eluted from the cartridge using methanol (4x25 mL). The methanol fraction was concentrated under reduced pressure at temperature less than 25⁰C to afford the pure **4MU-GalNAc-4-S.** 1H NMR (300 MHz, D2O) δ 7.42 (d, *J* = 8.9 Hz, 1H), 6.84 (dd, *J* = 8.9, 2.2 Hz, 1H), 6.66 (d, *J* = 2.2 Hz, 1H), 6.02 (s, 1H), 5.20 (d, *J* = 8.3 Hz, 1H), 4.86 (d, *J* = 2.9 Hz, 1H), 4.27 (dd, *J* = 10.9, 8.4 Hz, 1H), 4.15 – 4.04 (m, 2H), 3.98 – 3.83 (m, 2H), 2.25 (s, 3H), 2.12 (s, 3H). MS (ESI-) for [M - Na]-; calculated: 458.1, found: 458.3.

**Supplemental Table 1.** 4MU-Fluorimetric assay of GALNS in random newborn, MPS-IVA-affected patients, and quality control DBS.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sample**  | **Umbelliferone (1.0s) (Counts)** | **Ratio** | **pico moles** | **Activity** |
|  | **no NGA** | **with NGA** |  | **of 4MU** | **umol/hr/L** |
| Newborn-1 | 129995 | 361692 | 2.78 | 205.55 | 4.14 |
| Newborn-2 | 98277 | 263355 | 2.68 | 144.93 | 2.92 |
| Newborn-3 | 112303 | 319021 | 2.84 | 182.82 | 3.69 |
| Newborn-4 | 100641 | 304648 | 3.03 | 180.36 | 3.64 |
| Newborn-5 | 108448 | 953189 | 8.79 | 763.43 | 15.39 |
| Newborn-6 | 110367 | 323572 | 2.93 | 188.73 | 3.80 |
| Newborn-7 | 109755 | 347667 | 3.17 | 211.21 | 4.26 |
| Newborn-8 | 142487 | 547957 | 3.85 | 363.69 | 7.33 |
| Newborn-9 | 126418 | 293366 | 2.32 | 146.63 | 2.96 |
| Newborn-10 | 94677 | 225125 | 2.38 | 113.42 | 2.29 |
| Newborn-11 | 107630 | 312494 | 2.90 | 181.14 | 3.65 |
| Newborn-12 | 113305 | 286397 | 2.53 | 152.22 | 3.07 |
| Newborn-13 | 105083 | 364444 | 3.47 | 230.73 | 4.65 |
| Newborn-14 | 107284 | 244100 | 2.28 | 119.21 | 2.40 |
| Newborn-15 | 113512 | 341679 | 3.01 | 202.34 | 4.08 |
| Newborn-16 | 103154 | 354547 | 3.44 | 223.48 | 4.51 |
| Newborn-17 | 117641 | 257116 | 2.19 | 121.63 | 2.45 |
| Newborn-18 | 97173 | 291018 | 2.99 | 171.11 | 3.45 |
| Newborn-19 | 113790 | 324926 | 2.86 | 186.84 | 3.77 |
| Newborn-20 | 107822 | 340066 | 3.15 | 206.05 | 4.15 |
| Newborn-21 | 107181 | 262289 | 2.45 | 135.86 | 2.74 |
| Newborn-22 | 88925 | 241143 | 2.71 | 133.23 | 2.69 |
| Newborn-23 | 210013 | 334375 | 1.59 | 107.88 | 2.17 |
| Newborn-24 | 96896 | 347451 | 3.59 | 222.72 | 4.49 |
| Newborn-25 | 91491 | 275861 | 3.02 | 162.49 | 3.28 |
| Newborn-26 | 93429 | 268196 | 2.87 | 153.75 | 3.10 |
| Newborn-27 | 96308 | 281300 | 2.92 | 163.05 | 3.29 |
| Newborn-28 | 92446 | 260046 | 2.81 | 147.23 | 2.97 |
| Newborn-29 | 97101 | 239195 | 2.46 | 124.02 | 2.50 |
| Newborn-30 | 90804 | 225647 | 2.48 | 117.42 | 2.37 |
| Newborn-31 | 84044 | 190267 | 2.26 | 91.37 | 1.84 |
| Newborn-32 | 108089 | 305604 | 2.83 | 174.45 | 3.52 |
| Newbonr-33 | 90327 | 314893 | 3.49 | 199.07 | 4.01 |
| Newborn-34 | 91067 | 229977 | 2.53 | 121.12 | 2.44 |
| Newborn-35 | 93232 | 275984 | 2.96 | 161.01 | 3.25 |
| Newborn-36 | 87775 | 259169 | 2.95 | 150.68 | 3.04 |
| Newborn-37 | 93033 | 298210 | 3.21 | 181.42 | 3.66 |
| Newborn-38 | 104766 | 295470 | 2.82 | 168.25 | 3.39 |
| Newborn-39 | 105586 | 187062 | 1.77 | 68.85 | 1.39 |
| Newborn-40 | 82664 | 135284 | 1.64 | 42.59 | 0.86 |
| Newborn-41 | 94328 | 222370 | 2.36 | 111.23 | 2.24 |
| Newborn-42 | 108786 | 234675 | 2.16 | 109.27 | 2.20 |
| Newborn-43 | 98065 | 374927 | 3.82 | 246.66 | 4.97 |
| Newborn-44 | 132445 | 222221 | 1.68 | 76.41 | 1.54 |
| Newborn-45 | 102708 | 240915 | 2.35 | 120.48 | 2.43 |
| Newborn-46 | 128632 | 257681 | 2.00 | 112.15 | 2.26 |
| MPS-IVA patient - 1 (age 12 years) | 79841 | 120775 | 1.51 | 31.96 | 0.64 |
| MPS-IVA patient - 2 (age 1 year) | 84787 | 106322 | 1.25 | 14.31 | 0.29 |

**Supplemental Table 2.**  4MU-Fluorimetric assay of ARSB in random newborn, MPS-VI-affected patients, and quality control DBS.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Well** | **Umbelliferone (1.0s) (Counts)** | **Ratio** | **pico moles** | **Activity** |
|  | **no NGA** | **with NGA** | **of 4MU** | **umol/hr/L** |
| Newborn-1 | 92071 | 499892 | 5.43 | 365.83 | 7.38 |
| Newborn-2 | 90844 | 398619 | 4.39 | 274.79 | 5.54 |
| Newborn-3 | 99268 | 751801 | 7.57 | 588.52 | 11.87 |
| Newborn-4 | 84184 | 637081 | 7.57 | 497.85 | 10.04 |
| Newborn-5 | 89704 | 406697 | 4.53 | 283.17 | 5.71 |
| Newborn-6 | 83239 | 450933 | 5.42 | 329.31 | 6.64 |
| Newborn-7 | 96487 | 380632 | 3.94 | 253.28 | 5.11 |
| Newborn-8 | 97458 | 636417 | 6.53 | 485.16 | 9.78 |
| Newborn-9 | 78682 | 526841 | 6.70 | 402.54 | 8.12 |
| Newborn-10 | 72252 | 254051 | 3.52 | 160.15 | 3.23 |
| Newborn-11 | 76037 | 1080561 | 14.21 | 908.83 | 18.32 |
| Newborn-12 | 79282 | 866210 | 10.93 | 710.82 | 14.33 |
| Newborn-13 | 79868 | 332819 | 4.17 | 224.90 | 4.53 |
| Newborn-14 | 82474 | 374099 | 4.54 | 260.09 | 5.24 |
| Newborn-15 | 80008 | 738675 | 9.23 | 594.10 | 11.98 |
| Newborn-16 | 70776 | 412130 | 5.82 | 305.34 | 6.16 |
| Newborn-17 | 85593 | 561997 | 6.57 | 428.24 | 8.63 |
| Newborn-18 | 87315 | 798228 | 9.14 | 641.64 | 12.94 |
| Newborn-19 | 78336 | 446687 | 5.70 | 329.91 | 6.65 |
| Newborn-20 | 81220 | 342811 | 4.22 | 232.76 | 4.69 |
| Newborn-21 | 72799 | 656546 | 9.02 | 525.92 | 10.60 |
| Newborn-22 | 84833 | 455832 | 5.37 | 332.32 | 6.70 |
| Newborn-23 | 82832 | 331925 | 4.01 | 221.39 | 4.46 |
| Newborn-24 | 82678 | 497576 | 6.02 | 372.27 | 7.51 |
| Newborn-25 | 82364 | 455310 | 5.53 | 334.09 | 6.74 |
| Newborn-26 | 79962 | 395659 | 4.95 | 281.99 | 5.69 |
| Newborn-27 | 84685 | 775918 | 9.16 | 623.73 | 12.58 |
| Newborn-28 | 92725 | 616653 | 6.65 | 471.49 | 9.51 |
| Newborn-29 | 80132 | 336956 | 4.21 | 228.42 | 4.61 |
| Newborn-30 | 67087 | 497855 | 7.42 | 386.71 | 7.80 |
| Newborn-31 | 93299 | 678959 | 7.28 | 527.66 | 10.64 |
| Newborn-32 | 89008 | 627042 | 7.04 | 484.32 | 9.76 |
| Newborn-33 | 85184 | 450599 | 5.29 | 327.24 | 6.60 |
| Newborn-34 | 100578 | 948950 | 9.43 | 766.73 | 15.46 |
| Newborn-35 | 79993 | 370318 | 4.63 | 258.91 | 5.22 |
| Newborn-36 | 85542 | 659568 | 7.71 | 517.07 | 10.42 |
| Newborn-37 | 80054 | 478265 | 5.97 | 357.08 | 7.20 |
| Newborn-38 | 81025 | 645093 | 7.96 | 508.01 | 10.24 |
| Newborn-39 | 79550 | 409803 | 5.15 | 295.24 | 5.95 |
| Newborn-40 | 78081 | 414820 | 5.31 | 301.14 | 6.07 |
| Newborn-41 | 78301 | 467677 | 5.97 | 349.04 | 7.04 |
| Newborn-42 | 86093 | 797326 | 9.26 | 641.93 | 12.94 |
| Newborn-43 | 79781 | 478570 | 6.00 | 357.61 | 7.21 |
| Newborn-44 | 98185 | 926058 | 9.43 | 748.08 | 15.08 |
| Newborn-45 | 69407 | 317333 | 4.57 | 220.32 | 4.44 |
| Newborn-46 | 79261 | 294503 | 3.72 | 190.58 | 3.84 |
| Newborn-47 | 82376 | 381145 | 4.63 | 266.59 | 5.37 |
| Newborn-48 | 63625 | 474967 | 7.47 | 369.03 | 7.44 |
| Newborn-49 | 100853 | 782209 | 7.76 | 614.74 | 12.39 |
| Newborn-50 | 84963 | 446551 | 5.26 | 323.76 | 6.53 |
| Newborn-51 | 64882 | 469262 | 7.23 | 362.70 | 7.31 |
| MPS-VI\_Patient-1 (age not mentioned) | 64460 | 92642 | 1.44 | 20.36 | 0.41 |
| MPS-VI\_Patient-2 (age 20 years) | 59027 | 111459 | 1.89 | 42.42 | 0.86 |
| MPS-VI\_Patient-3 (age 2 years) | 59697 | 87446 | 1.46 | 19.96 | 0.40 |