Towards a Systematic Understanding of the Influence of Temperature on Glycosylation Reactions

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General information

All chemicals were reagent grade and used as supplied unless otherwise noted. All solvents for chemical reactions were commercially purchased in p.a. quality. If stated, they were dried in a Solvent Dispensing System (J.C. Meyer). For HPLC spectrometry, solvents with corresponding quality were used.

The automated syntheses were performed on a TempDUO home-built synthesizer developed at the Max Planck Institute of Colloids and Interfaces previously described. Thioglycosyl building blocks were purchased from GlycoUniverse GmbH & CO KGaA or synthesized if stated. Prior to automated synthesis, the building blocks were weighed and co-evaporated three times with anhydrous toluene and dried for at least 1 hour under high vacuum. All solutions were freshly prepared and kept under argon during the automation process. All reagent lines involved in automated syntheses were washed and primed prior to use.

Reaction completion, identity, and purity of all compounds were determined by analytical thin-layer chromatography (TLC) for solution-phase synthesis. TLC was performed on Merck silica gel 60 F254 plates (0.25 mm). Compounds were visualized by UV irradiation (254 nm) or stained (p-anisaldehyde stain: 3.7 mL p-anisaldehyde, 135 mL ethanol, 5 mL sulfuric acid, 1.5 mL glacial acetic acid or Hanessian's Stain: 235 mL of distilled water, 12 g of ammonium molybdate, 0.5 g of ceric ammonium molybdate, and 15 mL sulfuric acid). Flash column chromatography was performed on Kieselgel 60 with 230-400 mesh (Sigma-Aldrich, St. Louis, USA) or C18-reverse phased silica gel, fully endcapped (Sigma-Aldrich, St. Louis, USA). 1H, 13C, COSY and HSQC NMR spectra were recorded on a Varian 400-MR (400 MHz) spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to the respective residual solvent peaks (CHCl3: δ 7.26 in 1H and 77.16 in 13C; HDO δ 4.79 in 1H). Bidimensional and non-decoupled experiments were performed to assign identities of peaks showing relevant structural features. The following abbreviations are used to indicate peak multiplicities: s (singlet), d (doublet) dd (doublet of doublets), t (triplet), dt (doublet of triplets), td (triplet of doublets), q (quartet), p (pentet), m (multiplet). Coupling constants (J) are reported in Hertz (Hz). NMR spectra were processed using MestreNova 14.1 (MestreLab Research). Assignments were supported by COSY and HSQC experiments. High resolution mass spectra (HRMS) were obtained using 6210 ESI-TOF mass spectrometer (Agilent) and MALDI-TOF autoflexTM (Bruker) instruments. Analytical NP-HPLC was conducted on an Agilent 1200 Series system.
Materials and conditions for automated assay and syntheses

Preparation of Stock Solutions

**Building Block Solution**: Thioglycoside building block (0.09 mmol, 6.5 equiv. per cycle) was dissolved in 1 mL (per cycle) of anhydrous CH₂Cl₂.

**Dilute Building Block Solution**: Thioglucoside building block (0.04 mmol, 3.0 equiv. per cycle) was dissolved in 1 mL (per cycle) of anhydrous CH₂Cl₂.

**Acidic Wash Solution**: TMSOTf (0.45 mL, 0.31 mmol) was added to 40 mL of anhydrous CH₂Cl₂.

**Activator Solution**: Recrystallized NIS (1.58 g, 7.06 mmol) was dissolved in 45 mL of a 2:1 mixture of anhydrous CH₂Cl₂/dioxane, followed by addition of triflic acid (55 µL, 0.6 mmol). The solution was kept under ice-bath cooling for the duration of the automated run.

**Dilute Activator Solution**: For tetramer syntheses, a 0.5X version of the Activator Solution was prepared.

**Pre-capping Solution**: Pyridine (10 mL) was added to 90 mL of DMF.

**Capping Solution**: Methanesulfonic acid (2.4 mL, 37 mmol), acetic anhydride (12 mL, 127 mmol) were added to 50 mL anhydrous CH₂Cl₂.

**Fmoc Deprotection Solution**: Piperidine (20 mL) was added to 80 mL anhydrous DMF.

Modules

**Initiation**: For tetramer syntheses, resin loaded in the reaction vessel is washed with DMF, THF, and CH₂Cl₂ (3 x 3 mL for 15 s, respectively). The resin is then swollen in 2 mL CH₂Cl₂ for 20 minutes while the temperature of the reaction vessel is cooled to the lowest temperature required throughout the synthesis.

**Acidic Washing**: Once the temperature of the reaction vessel has adjusted to the desired temperature of the subsequent glycosylation by the cooling device, 1 mL of the Acidic Wash Solution is delivered to the reaction vessel through the precooling device (set at -20 °C). After three minutes, the solution is drained. Finally, the resin is washed with 3 mL CH₂Cl₂ (bubbling = 15 s) and drained.

**Glycosylation**: Upon draining the CH₂Cl₂ in the reaction vessel, 1 mL of Building Block Solution containing the appropriate building block is delivered from the building block storing component to the reaction vessel through the precooling device (set at -20 °C). After the temperature reaches the desired temperature (T₁), Activator Solution (1 mL) is delivered to the reaction vessel from the respective activator storing component to the reaction vessel through the precooling device (set at -20 °C). The glycosylation mixture is incubated for the selected duration (t₁) at the desired T₁. In control cases
microwave irradiation (max power = 120 W) is employed to ramp the reaction temperature to \( T_2 \) (rate = 4 °C/min). Once \( T_2 \) is reached, it is maintained by microwave irradiation and the reaction mixture is incubated for an additional time \( (t_2) \). Once the incubation time is finished, the reaction mixture is drained and the resin is washed with \( \text{CH}_2\text{Cl}_2 \) (1 x 2 mL for 15 s), then dioxane (1 x 2 mL for 15 s), and finally \( \text{CH}_2\text{Cl}_2 \) (2 x 2 mL for 15 s). During the module, the active cooling element is maintained at the lowest temperature required throughout the synthesis.

**Capping:** The resin is initially washed with DMF (2 x 3 mL for 15 s). Then *Pre-capping Solution* (2 mL) is delivered and under microwave irradiation the reaction temperature is adjusted to and maintained at 50 °C for one minute (max power = 5 W). The resin is then washed with \( \text{CH}_2\text{Cl}_2 \) (3 x 2 mL for 15). Upon washing, *Capping Solution* (4 mL) is delivered and the temperature is adjusted to and maintained at 25 °C by microwave irradiation (max power = 100 W). The resin and reagents are incubated for 8 min. The solution is then drained from the reactor vessel and the resin is washed with \( \text{CH}_2\text{Cl}_2 \) (2 x 3 mL for 15 s). Then, DMF (4 mL) is delivered and irradiated with microwaves (max power = 5 W) for 10 s and the solution is allowed to incubate for an additional 50 s before draining. During the module, the active cooling element is maintained at the lowest temperature required throughout the synthesis.

**Fmoc Deprotection:** The resin is first washed with DMF (3 x 3 mL for 15 s), and then *Fmoc Deprotection Solution* (2 mL) is delivered to the reaction vessel. The temperature of the reagents inside the reactor vessel is then adjusted to and maintained at 60 °C by microwave irradiation (max power = 60 W). After 1 min the reaction solution is drained and the resin is washed with DMF (3 x 3 mL for 15 s) and \( \text{CH}_2\text{Cl}_2 \) (5 x 3 mL for 15 s). During the module, the active cooling element is maintained at the lowest temperature required throughout the synthesis. After this module the resin is ready for the next glycosylation cycle.

**Cleavage from Solid Support:** After automated synthesis, the resin was removed from the reaction vessel, suspended in \( \text{CH}_2\text{Cl}_2 \) (20 mL), and photocleaved in a continuous-flow photoreactor. A Vapourtec E-Series easy-MedCHem, equipped with a UV-150 photochemical reactor having a UV-150 medium-pressure mercury lamp (arc length 27.9 cm, 450 W) surrounded by a long-pass UV filter (Pyrex, 50% transmittance at 305 nm). A Pump 11 Elite Series (Harvard Apparatus syringe pump at a flow rate of 0.7 mL/min was used to pump the mixture through a FEP tubing (i.d. 3.0 inch, volume: 12 mL) at 20 °C. The reactor was washed with 20 mL \( \text{CH}_2\text{Cl}_2 \) at a flow rate of 2.0 mL/min. The output solution was filtered to remove the resin and the solvent was evaporated *in vacuo*. The crude product was then analyzed by MALDI and analytical HPLC.
Temperature of activation experiments

To assay the temperature of activation for various thioglycoside building blocks, the glycosylation module above was adapted, with $t_1 = 5$ min and $T_1$ corresponding to the desired temperature for activation assay. Upon completion of 5 minutes of the modified glycosylation module, pre-capping solution is added, and the reaction mixture is ejected to an external fraction containing 10% aqueous Na$_2$S$_2$O$_3$. The reaction vessel is washed with CH$_2$Cl$_2$ (1 x 2 mL for 15 s), then dioxane (1 x 2 mL for 15 s), and finally CH$_2$Cl$_2$ (2 x 2 mL for 15 s). After washing, the adapted glycosylation protocol is repeated for the next desired temperature. Collected fractions are extracted twice with 2 ml CH$_2$Cl$_2$, then the combined organic phase is dried over Na$_2$SO$_4$, filtered, and concentrated, coevaporating thrice with anhydrous toluene to remove residual solvent. The residue is placed under high vacuum for at least eight hours before NMR analysis. References are included in select overlays to highlight donor differences. Regions marked red are time periods where modified glycosylation module occurred.

**Figure S1.** Systematic assessment of activation temperature of various Fmoc-protected monosaccharide building blocks enabled by automated glycan assembly (AGA) apparatus. Monosaccharides are chilled to set temperature $T$ then exposed to NIS/TfOH for five minutes. Reactive intermediates are captured by quenching with pyridine, ejection to a fraction, washing of the reaction vessel, and the initiation of a new activation cycle. Temperature is monitored by an in-vessel thermal probe. Reactions are carried out under inert Argon atmosphere. Collected fractions are worked up and analyzed with $^1$H NMR.
Activation experiment spectra and temperature profiles

4-Methylphenyl 6-O-acetyl-3,4-di-O-benzyl-2-O-(9-fluorenylmethoxycarbonyl)-1-thio-α-D-mannopyranoside (1)

4-Methylphenyl 2-O-benzoyl-3,4-di-O-benzyl-6-O-(9-fluorenylmethoxycarbonyl)-1-thio-α-D-mannopyranoside (2)
4-Methylphenyl 3,4-di-O-benzyl-6-O-(9-fluorenylmethoxycarbonyl)-2-O-levulinoyl-1-thio-α-D-mannopyranoside (3)

Ethyl 6-O-(2-chloroacetyl)-4-O-(9-fluorenylmethoxycarbonyl)-3-O-levulinoyl-2-O-(2-naphthylmethyl)-1-thio-α-D-mannopyranoside (4)
4-Methylphenyl 2,3,4-tri-O-benzyl-1-thio-β-L-fucopyranoside (5)

4-Methylphenyl 2-O-benzoyl-3,6-di-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-glucopyranoside (6)
4-Methylphenyl 2,3-di-O-benzylo-4-O-benzyl-6-O-(9-fluorenlymethoxycarbonyl)-1-thio-ß-D-glucopyranoside (7)

Ethyl 2-O-benzylo-3-O-benzylo-4-O-(9-fluorenlymethoxycarbonyl)-6-O-levulinoylo-1-thio-ß-D-glucopyranoside (8)
Methyl 2-O-benzyl-3,6-di-O-benzyl-4-O-(9-fluorenlymethoxycarbonyl)-1-thio-β-D-glucopyranoside (9)

Ethyl 2-O-benzoyl-3,4-di-O-benzyl-1-thio-β-D-glucopyranuronic acid (10)
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Ethyl 3,6-di-O-benzyl-2-deoxy-2-[(2,2,2-trichloroacetyl)amino]-4-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-glucopyranoside (11)

Ethyl 3,4-di-O-benzyl-2-deoxy-2-[(2,2,2-trichloroacetyl)amino]-6-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-glucopyranoside (12)
Ethyl 3-O-benzyl-2-deoxy-2-[(2,2,2-trichloroacetyl)amino]-6-O-levulinoyl-4-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-glucopyranoside (13)

Methyl 4-O-benzyl-2-deoxy-2-[(2,2,2-trichloroacetyl)amino]-6-O-levulinoyl-3-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-glucopyranoside (14)
Ethyl 6-O-benzyl-2-deoxy-2-[[2,2,2-trichloroacetyl]amino]-4-O-(9-fluorenylmethoxycarbonyl)-3-O-
OA[47x758]levulinoyl-1-thio-β-D-glucopyranoside (15)
Methylphenyl 2-O-benzoyl-4,6-di-O-benzyl-3-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-
galactopyranoside (16)
4-Methylphenyl 2-O-benzoyl-3,6-di-O-benzyl-4-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (17)

4-Methylphenyl 2-O-benzoyl-3,4-di-O-benzyl-6-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (18)
4-Methylphenyl 3,4,6-tri-O-benzyl-2-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (19)

Ethyl 4,6-di-O-benzyl-2-deoxy-2-[(2,2,2-trichloroacetyl)amino]-3-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (20)
Aglycon replacement experiments and compound characterization

General procedure for thioglycoside aglycon replacement

Dibutyl hydrogen phosphate (2.0 equiv.) is added to a round bottom flask containing thioglycoside. The mixture is coevaporated thrice in anhydrous toluene. The residue is dissolved in anhydrous CH₂Cl₂ (0.1 M). The mixture is cooled to 0 °C and NIS (1.3 equiv.) is added. Following vigorous stirring for 15 min, triflic acid (0.3 equiv.) is added. The reaction is stirred for 30 min or until complete disappearance of starting material as judged by TLC. Upon completion, the reaction is diluted with CH₂Cl₂ and quenched with aq. sat. NaHCO₃. The organic layer is washed twice with Na₂S₂O₃ and once with water, followed by drying with Na₂SO₄, filtration, and evaporation to dryness. Purification by silica gel chromatography (hexanes/EtOAc 70:30) affords the glycosyl phosphate in yields from 72 to 86% as a colorless oil and α/β mixture. Phosphate compounds matched previously described spectra.¹

The glycosyl phosphate is combined with the desired thiol (1.5 equiv.), then the mixture is coevaporated thrice to dryness with anhydrous toluene. The residue is dissolved in anhydrous CH₂Cl₂ (0.1 M). The solution is cooled to 0 °C, then TMSOTf (1.1 equiv.) is added dropwise during vigorous stirring. The reaction is removed from ice and allowed to warm to room temperature or is gently heated until completion as judged by disappearance of the starting material spot in TLC, 30 min to 1 h. The reaction mixture is diluted with CH₂Cl₂ and quenched with aq. sat. NaHCO₃. The organic layer is washed twice with Na₂S₂O₃ and once with water, followed by drying with Na₂SO₄, filtration, and evaporation to dryness. Purification by silica gel chromatography (hexanes/EtOAc 70:30) affords the glycosyl phosphate in yields from 60 to 82% as an opaque solid.

Figure S2. Method for replacement of thioglycoside aglycon. The temperature of activation of reactive glycosyl donors was tuned by replacement of the original thiol leaving group with a more deactivating thiol. Replacement was achieved in two steps via the glycosyl phosphate.
Yield in two steps from 11: 70.5%

**$^1$H NMR (400 MHz, CDCl$_3$)** δ 7.74 (d, $J = 7.6$ Hz, 2H), 7.56 (d, $J = 7.5$ Hz, 1H), 7.50 (d, $J = 7.6$ Hz, 1H), 7.44 – 7.27 (m, 10H), 7.25 – 7.13 (m, 6H), 7.05 (d, $J = 8.1$ Hz, 2H), 6.89 (d, $J = 7.5$ Hz, 1H), 5.23 (d, $J = 10.2$ Hz, 1H), 4.86 (t, $J = 9.0$ Hz, 1H), 4.60 (d, $J = 1.7$ Hz, 2H), 4.54 (d, $J = 2.7$ Hz, 2H), 4.36 – 4.24 (m, 3H), 4.11 (t, $J = 7.2$ Hz, 1H), 3.81 – 3.73 (m, 1H), 3.69 – 3.66 (m, 2H), 3.40 (td, $J = 10.1, 7.5$ Hz, 1H), 2.32 (s, 3H).

**$^{13}$C NMR (101 MHz, CDCl$_3$)** δ 161.7, 154.4, 143.3, 143.1, 141.4, 141.4, 139.0, 138.1, 137.3, 134.0, 130.1, 128.6, 128.5, 128.1, 128.1, 128.0, 127.8, 127.4, 127.3, 127.3, 125.2, 125.1, 120.2, 120.2, 92.4, 84.1, 78.6, 77.4, 76.1, 75.2, 73.7, 70.3, 69.5, 57.1, 46.7, 21.3.

**HRMS (QToF):** Calcd for C$_{44}$H$_{40}$Cl$_3$NNaO$_7$S [M+Na]$^+$ 854.1489, found 854.1380

$^1$H NMR (400 MHz, CDCl$_3$) of 21:
$^{13}$C NMR (101 MHz, CDCl$_3$) of 21:

$^1$H, $^1$H COSY of 21:
$^1$H, $^{13}$C HSQC of 21:

Activation experiment spectra and temperature profile for 21:
2,4-Dimethylphenyl 3,6-di-O-benzyl-2-deoxy-2-[(2,2,2-trichloroacetyl)amino]-4-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-glucopyranoside (22)

Yield in two steps from 11: 63.7%

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.74 (d, $J = 7.6$ Hz, 2H), 7.54 (d, $J = 7.5$ Hz, 1H), 7.49 (d, $J = 7.5$ Hz, 1H), 7.40 – 7.34 (m, 2H), 7.32 – 7.13 (m, 12H), 7.12 – 7.06 (m, 3H), 5.04 (d, $J = 10.4$ Hz, 1H), 4.99 – 4.92 (m, 1H), 4.65 (s, 2H), 4.42 (s, 2H), 4.34 – 4.22 (m, 3H), 4.09 (t, $J = 7.2$ Hz, 1H), 3.76 – 3.67 (m, 1H), 3.62 – 3.49 (m, 3H), 2.54 (s, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 161.9, 154.4, 144.5, 143.3, 143.2, 141.4, 141.4, 138.0, 137.4, 130.1, 129.5, 128.6, 128.4, 128.4, 128.0, 127.9, 127.7, 127.6, 127.3, 127.3, 125.2, 125.1, 120.2, 120.2, 92.4, 86.1, 78.4, 77.0, 76.0, 74.8, 73.7, 70.2, 69.8, 58.4, 46.7, 22.8.

HRMS (QToF): Calcd for C$_{45}$H$_{42}$Cl$_3$NNaO$_7$S [M + Na]$^+$ 868.1645, found 868.1461

$^1$H NMR (400 MHz, CDCl$_3$) of 22:
$\text{^1}^3\text{C NMR (101 MHz, CDCl}_3\text{) of 22:}$

$\text{^1}H$, $\text{^1}H$ COSY of 22:
 Activation experiment spectra and temperature profile for 22:
Yield in two steps from 11: 43.2%

H NMR (400 MHz, CDCl3) δ 7.90 (d, J = 7.8 Hz, 1H), 7.75 (d, J = 7.5 Hz, 2H), 7.64 (dd, J = 7.9, 1.5 Hz, 1H), 7.56 (d, J = 6.6 Hz, 1H), 7.52 (d, J = 6.5 Hz, 1H), 7.43 – 7.14 (m, 15H), 7.02 (d, J = 7.6 Hz, 1H), 5.29 (d, J = 10.4 Hz, 1H), 4.93 (dd, J = 9.9, 8.9 Hz, 1H), 4.64 (s, 2H), 4.53 (s, 2H), 4.39 – 4.27 (m, 4H), 4.13 (t, J = 7.1 Hz, 1H), 3.80 (d, J = 4.8 Hz, 1H), 3.68 (d, J = 4.6 Hz, 3H).

13C NMR (101 MHz, CDCl3) δ 162.0, 154.4, 143.3, 143.1, 141.4, 141.4, 138.0, 137.2, 135.0, 132.5, 128.7, 128.6, 128.5, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 127.3, 127.3, 126.8, 126.7, 125.2, 125.1, 120.3, 120.2, 92.2, 84.6, 78.4, 77.4, 77.3, 75.8, 75.1, 73.7, 70.3, 69.5, 57.6, 46.8.

HRMS (QToF): Calcd for C_{44}H_{37}Cl_{3}F_{3}NNaO_{7}S [M + Na]^+ 908.1206, found 908.1144

H NMR (400 MHz, CDCl3) of 23:
$^{13}$C NMR (101 MHz, CDCl$_3$) of 23:

$^1$H, 1H COSY of 23:
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$^1$H, $^{13}$C HSQC of 23:

Activation experiment spectra and temperature profile for 23:
2,4-Dimethylphenyl 2-O-benzoyl-4,6-di-O-benzyl-3-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (24)

Yield in two steps from 16: 69.4%

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.02 (dd, \(J = 8.4, 1.3\) Hz, 2H), 7.60 (d, \(J = 7.4\) Hz, 2H), 7.51 – 7.45 (m, 1H), 7.39 – 7.21 (m, 15H), 7.19 – 7.15 (m, 3H), 7.07 – 6.94 (m, 5H), 5.76 (t, \(J = 10.1\) Hz, 1H), 4.94 (dd, \(J = 10.0, 3.1\) Hz, 1H), 4.70 (d, \(J = 11.4\) Hz, 1H), 4.45 (dd, \(J = 10.8, 6.7\) Hz, 2H), 4.34 (dd, \(J = 11.7, 9.0\) Hz, 2H), 4.21 (dd, \(J = 10.4, 7.1\) Hz, 1H), 4.11 (dd, \(J = 10.4, 7.8\) Hz, 1H), 4.02 – 3.95 (m, 2H), 3.58 – 3.50 (m, 3H), 2.34 (s, 6H).

\(^13\)C NMR (101 MHz, CDCl\(_3\)) \(\delta\) 165.4, 154.7, 144.2, 143.4, 142.9, 141.3, 141.2, 137.9, 137.9, 133.4, 131.8, 130.1, 129.5, 129.2, 128.7, 128.6, 128.5, 128.3, 128.0, 127.9, 127.9, 127.3, 127.2, 125.3, 125.1, 120.1, 89.4, 79.2, 75.3, 74.0, 73.6, 70.3, 69.2, 68.6, 46.6, 22.7.

HRMS (QToF): Calcd for C\(_{50}\)H\(_{50}\)NO\(_8\)S [M + NH\(_4\)]\(^+\) 824.3257, found 824.3105

\(^1\)H NMR (400 MHz, CDCl\(_3\)) of 24:
$^{13}$C NMR (101 MHz, CDCl$_3$) of 24:

$^1$H, 1H COSY of 24:
 Activation experiment spectra and temperature profile for 24:
4-Trifluoromethylphenyl 2-O-benzyloxy-4,6-di-O-benzyloxy-3-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (25)

Yield in two steps from 16: 66.1%

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.02 (d, $J = 7.0$ Hz, 2H), 7.71 – 7.62 (m, 3H), 7.58 – 7.51 (m, 3H), 7.48 – 7.26 (m, 17H), 7.13 (td, $J = 7.5$, 1.1 Hz, 1H), 7.08 (td, $J = 7.5$, 1.2 Hz, 1H), 5.78 (t, $J = 9.9$ Hz, 1H), 5.09 (dd, $J = 10.0$, 2.9 Hz, 1H), 4.91 (d, $J = 9.9$ Hz, 1H), 4.77 (d, $J = 11.2$ Hz, 1H), 4.57 – 4.43 (m, 3H), 4.31 (dd, $J = 10.4$, 7.1 Hz, 1H), 4.23 (dd, $J = 10.4$, 7.7 Hz, 1H), 4.14 (d, $J = 2.3$ Hz, 1H), 4.06 (t, $J = 7.3$ Hz, 1H), 3.88 (t, $J = 6.5$ Hz, 1H), 3.73 (dd, $J = 9.4$, 6.0 Hz, 1H), 3.65 (dd, $J = 9.3$, 6.8 Hz, 1H).

$^{13}$C NMR (101 MHz, CDCl$_3$) 165.1, 154.5, 143.2, 142.8, 141.2, 141.1, 137.6, 133.4, 130.7, 130.0, 129.3, 128.5, 128.5, 128.4, 128.2, 128.0, 128.0, 128.0, 127.9, 127.1, 127.1, 125.6, 125.2, 124.9, 120.0, 85.4, 78.8, 75.2, 73.7, 73.7, 70.2, 68.3, 68.2, 46.4.

HRMS (QToF): Calcd for C$_{49}$H$_{45}$F$_3$NO$_8$S [M + NH$_4$]$^+$ 864.2818, found 864.2818

$^1$H NMR (400 MHz, CDCl$_3$) of 25:
$^{13}$C NMR (101 MHz, CDCl$_3$) of 25:

$^1$H, $^1$H COSY of 25:
Activation experiment spectra and temperature profile for 25:
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3,5-di-Trifluoromethylphenyl 2-O-benzoyl-4,6-di-O-benzyl-3-O-(9-fluorenylmethoxycarbonyl)-1-thio-β-D-galactopyranoside (26)

Yield in two steps from 16: 47.8%

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.04 (d, $J$ = 7.0 Hz, 2H), 7.97 (s, 2H), 7.73 (s, 1H), 7.67 (dd, $J$ = 7.7, 5.0 Hz, 2H), 7.58 – 7.54 (m, 1H), 7.46 – 7.41 (m, 3H), 7.39 – 7.21 (m, 13H), 7.12 (td, $J$ = 7.5, 1.1 Hz, 1H), 7.07 (td, $J$ = 7.5, 1.1 Hz, 1H), 5.66 (t, $J$ = 9.9 Hz, 1H), 5.06 (dd, $J$ = 10.0, 2.9 Hz, 1H), 4.87 (d, $J$ = 9.8 Hz, 1H), 4.71 (d, $J$ = 11.5 Hz, 1H), 4.53 – 4.41 (m, 4H), 4.28 (dd, $J$ = 10.4, 7.1 Hz, 1H), 4.19 (dd, $J$ = 10.4, 7.7 Hz, 1H), 4.10 (d, $J$ = 2.9 Hz, 1H), 4.04 (t, $J$ = 7.4 Hz, 1H), 3.86 (t, $J$ = 6.5 Hz, 1H), 3.65 (dd, $J$ = 9.3, 5.9 Hz, 1H), 3.57 (dd, $J$ = 9.3, 6.9 Hz, 1H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ 165.1, 154.5, 143.3, 142.9, 141.3, 141.2, 137.6, 137.6, 135.8, 133.7, 132.4, 132.0, 131.7, 130.1, 129.2, 128.7, 128.6, 128.6, 128.5, 128.5, 128.1, 128.0, 128.0, 127.2, 127.2, 125.3, 125.0, 124.5, 121.7, 120.1, 85.2, 78.7, 75.2, 73.7, 73.5, 70.3, 68.2, 68.0, 46.5.

HRMS (QToF): Calcd for C$_{50}$H$_{44}$F$_6$NO$_8$S [M + NH$_4$]$^+$ 932.2692, found 932.2538

$^1$H NMR (400 MHz, CDCl$_3$) of 26:

![NMR spectrum of 26]
$^{13}$C NMR (101 MHz, CDCl$_3$) of 26:

$^1$H, $^1$H COSY of 26:
Supporting Information

$^1$H, $^{13}$C HSQC of 26:

Activation experiment spectra and temperature profile for 26:
Model tetramer syntheses

5-Amino-pentyl β-(1→4)-D-tetraglucopyranoside

Tetraglucoside 28 was obtained using the following sequence of AGA modules: **Initiation** with functionalized aminopentanol resin 27 → **Acidic Washing** → **Glycosylation** at a select temperature/temperature range with **Dilute Building Block Solution** containing 6 and **Dilute Activator Solution** → **Capping** → **Fmoc Deprotection**. Modules **Acidic Washing** to **Fmoc Deprotection** were repeated three times, then **Cleavage from Solid Support** was performed. Three separate AGA syntheses of 28 were carried out, each with different temperature conditions during the **Glycosylation** module. Crude photo-deprotected glycans from each condition were analyzed with NP-HPLC. Analytical NP-HPLC was conducted on an Agilent 1200 Series system. A YMC-Diol-300-NP column (150 mm x 4.60 mm I.D.) was used with a flow rate of 1.00 mL/min and hexane/EtOAc as eluent (20% EtOAc in hexane for 5 min, 20 → 100% EtOAc in hexane over 35 min, 100% EtOAc for 10 min).
NP-HPLC of crude 28 (ELSD trace) with Glycosylation at -20 °C for 5 min, then 0 °C for 20 min:

MALDI of crude 28 with Glycosylation at -20 °C for 5 min, then 0 °C for 20 min:
NP-HPLC of crude 28 (ELSD trace) with Glycosylation at -11 °C for 25 min:

MALDI of crude 28 with Glycosylation at -11 °C for 25 min:
NP-HPLC of crude 28 (ELSD trace) with Glycosylation at -25 °C for 25 min:

MALDI of crude 28 with Glycosylation at -25 °C for 25 min:

A single run at the -25 °C isothermal condition, followed by Cleavage from Solid Support, afforded 28 (21 mg, 0.0104 mmol, 76%).

$^1$H NMR (400 MHz, CDCl$_3$) δ 7.85 – 7.74 (m, 8H), 7.58 – 7.46 (m, 3H), 7.45 – 7.15 (m, 32H), 7.12 – 6.79 (m, 30H), 5.19 – 5.06 (m, 2H), 5.05 – 4.95 (m, 4H), 4.89 – 4.74 (m, 3H), 4.64 (dd, $J$ = 13.8, 11.7 Hz, 2H), 4.57 – 4.31 (m, 10H), 4.25 (dd, $J$ = 16.4, 8.2 Hz, 2H), 4.17 (d, $J$ = 7.9 Hz, 1H), 4.12 – 3.82 (m, 6H), 3.73 (t, $J$ = 9.0 Hz, 1H), 3.65 (dt, $J$ = 10.9, 5.8 Hz, 1H), 3.56 – 3.37 (m, 7H), 3.36 – 3.21 (m, 6H), 3.16 (q, $J$ = 7.2 Hz, 1H), 3.04 (d, $J$ = 9.4 Hz, 1H), 2.81 – 2.72 (m, 3H), 1.40 – 0.90 (m, 6H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ 165.1, 165.0, 164.9, 156.2, 138.8, 138.7, 138.7, 138.1, 138.1, 138.1, 137.7, 137.6, 137.5, 136.7, 133.4, 133.3, 133.1, 132.9, 130.0, 129.7, 129.7, 129.6, 129.5, 128.6, 128.6, 128.5, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 128.0, 127.9, 127.9, 127.9, 127.8, 127.8, 127.7, 127.6, 127.6, 127.1,
127.0, 126.9, 101.1, 100.0, 99.8, 81.8, 80.0, 79.8, 76.4, 76.1, 75.9, 74.6, 74.5, 74.4, 74.3, 74.2, 74.1, 73.8, 73.5, 73.4, 73.0, 71.2, 69.4, 67.3, 67.2, 66.5, 40.8, 29.3, 28.8, 23.0.

$^1$H NMR (400 MHz, CDCl$_3$) of 28:

$^{13}$C NMR (101 MHz, CDCl$_3$) of 28:
$^1$H, $^3$H COSY of 28:

$^1$H, $^13$C HSQC of 28:
To determine the relative reactivity of thioglycosides, 0.03 mmol each of an experimental donor ($D_{\text{exp}}$) and reference donor ($D_{\text{ref}}$) and 50 mg of an inert internal standard (S), 4-methylphenyl 2,3,4,6-tetra-O-acetyl-1-thio-\(\alpha\)-D-mannopyranoside, are combined and co-evaporated thrice with anhydrous toluene before placement over high vacuum overnight. The donor mixture is then dissolved in anhydrous CH\(_2\)Cl\(_2\) (3 mL). Two 1 mL aliquots are transferred to pre-dried vials under Argon atmosphere. The initial aliquot for each reaction (20 \(\mu\)L) is transferred to a separate vial, to which 80 \(\mu\)L 1:1 ethyl acetate to n-hexane (v/v) is added. Anhydrous MeOH (2.04 \(\mu\)L, 5 eq) is added, followed by 0.5 M NIS solution in anhydrous acetonitrile (20 \(\mu\)L) and 0.1 M TfOH in anhydrous Et\(_2\)O (10 \(\mu\)L). Reactions are stirred at ambient temperature. After 2 hours, the reaction mixture is immediately diluted with CH\(_2\)Cl\(_2\) (2 mL), washed with saturated aqueous Na\(_2\)S\(_2\)O\(_3\) solution containing 10% NaHCO\(_3\), dried over Na\(_2\)SO\(_4\), and evaporated to dryness. The resultant residue is then dissolved in CH\(_2\)Cl\(_2\) (1 mL), then the final aliquot is prepared as above. Initial and final aliquots are run separately and compared for donor concentration via HPLC using a Luna 3 \(\mu\)m Silica-NP Column (50 x 4.6 mm I.D.) with a flow rate of 1.00 mL/min and hexane/EtOAc as eluent (30% EtOAc in hexane → 100% EtOAc in hexane over 45 min). Relative reactivities are calculated according to the following equation:

\[
\frac{k_{\text{obs,exp}}}{k_{\text{obs,ref}}} = \frac{\log \left( \frac{D_{\text{exp}} \times S_e}{S_i} \right) - \log(D_{\text{exp}})}{\log \left( \frac{D_{\text{ref}} \times S_e}{S_i} \right) - \log(D_{\text{ref}})}
\]

To establish absolute RRVs, each building block is compared to another with known reactivity. The relative reactivity of 4-Methylphenyl 2,3,4,6-O-acetyl-1-thio-\(\alpha\)-D-mannopyranoside.\(^3\) To avoid peak integration issues, reactivities are included only if the relative difference in RRV is less than 20-fold. Values reflect agreement of at least two independent experiments.
Figure S3. Comparison of relative reactivities to determine RRV. Arrows represent individual competitive assays between two donors. Numbers over arrows are ratio of reactivity between donors, while retraced absolute values are shown bolded under donor structures. RRV differences greater than 20-fold were found to increase error in measurements, so RRV experiments were restricted to donors with similar reactivity.
Figure S4. Comparison of thioglycoside $^1$H anomeric shift (ppm) with the natural logarithm of their experimentally determined RRV. Regressions are shown purely to qualitatively demonstrate the absence of a uniform linearity of ln(RRV) versus $^1$H anomeric shift between glycoside configuration.
**Figure S5.** Comparison of activation temperature with natural logarithms of RRVs. Red dashes represent $T_{\text{decomposition}}$, green dashes are $T_{\text{activation}}$, and yellow points are the geometric mean of these values. A light trend could be seen between RRV and activation temperature for mannosides and glucosides, where less temperature sensitive donors reacted were more reactive as measured by competitive experiments.
References

1. M. Guberman, M. Bräutigam, P. H. Seeberger, *Chem. Sci.* **2019**, *10*, 5634–5640.
2. K. Le Mai Hoang, A. Pardo-Vargas, Y. Zhu, Y. Yu, M. Loria, M. Delbianco, P. H. Seeberger, *J. Am. Chem. Soc.* **2019**, *141*, 9079–9086.
3. Z. Zhang, I. R. Ollmann, X.-S. Ye, R. Wischnat, T. Baasov, C.-H. Wong, *J. Am. Chem. Soc.* **1999**, *121*, 734–753.
Notes and Author Contributions

Notes
Conflict of interest statement: P.H.S. declares a significant financial interest in GlycoUniverse GmbH & Co. KGaA, the company that commercialized most of the building blocks described.

Author Contributions
P.H.S. conceived the overall project and supervised the effort. O.T.T., E.T.S., and J.D.F. designed the experiments and ran the experiments protocols. O.T.T. and E.T.S. characterized the products. O.T.T. determined RRVs. J.D.F constructed the temperature controlled semi-automated set-up. P.H.S. wrote the paper together with O.T.T., E.T.S., and J.D.F.