Glycosylation Hot Paper

How to cite: *Angew. Chem. Int. Ed.* **2022,** *61,* e202115433
International Edition: doi.org/10.1002/anie.202115433
German Edition: doi.org/10.1002/ange.202115433

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

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Abstract: Glycosidic bond formation is a continual challenge for practitioners. Aiming to enhance the reproducibility and efficiency of oligosaccharide synthesis, we studied the relationship between glycosyl donor activation and reaction temperature. A novel semi-automated assay revealed diverse responses of members of a panel of thioglycosides to activation at various temperatures. The patterns of protecting groups and the thiol aglycon combine to cause remarkable differences in temperature sensitivity among glycosyl donor building blocks. We introduce the concept of donor activation temperature to capture experimental insights, reasoning that glycosylations performed below this reference temperature evade deleterious side reactions. Activation temperatures enable a simplified temperature treatment and facilitate optimization of glycosyl donor usage. Isothermal glycosylation below the activation temperature halved the equivalents of building block required in comparison to the standard "ramp" regime used in solution- and solid-phase oligosaccharide synthesis to-date.

Introduction

Glycans are structurally and functionally diverse molecules with immense significance in biology and untapped potential in biomedical and materials applications.[1] However, the refractory nature of carbohydrate synthesis presents a major obstacle to the systematic study and utilization of glycans. The glycosylation reaction, which creates higher-order carbohydrates by linking a glycosyl donor (glycosylating agent) with a glycosyl acceptor (nucleophile), is infamous for its poor reproducibility and often erratic outcomes.[2] Practitioners create glycosidic bonds using a myriad of glycosyl coupling pairs (glycosylating agent and nucleophile), promoter systems, solvents, and concentrations, all while relying upon barely differing temperature regimes.[3] Reported yields can be difficult to reproduce, and crucial reaction conditions often go unreported. Despite recent advances in the standardization of glycosidic bond formation, as well as an improved physical organic and mechanistic understanding of the factors governing the outcomes,[4–9] irreproducibility and poor transferability stymie progress. Here, we aim to decrypt the fundamental role of temperature, a key parameter influencing the yield and selectivity of glycosylations. Our approach leverages a semi-automated platform to achieve process parametrization, improve standardization, and establish reproducibility. The ultimate goal is an experimentally established, "plug-and-play" temperature approach that provides practitioners with optimal conditions for a desired chemical glycosylation.

In the laboratory, most glycosylations begin at cryogenic temperatures (−78 °C) and are gradually warmed to ambient temperature overnight.[2,10] This uncontrolled manner of warming is fundamentally flawed and variable in rate because it is highly dependent upon the scale of the reaction, the specific heat capacity of the solvent, the properties of the vessel, the size of the cooling bath, and other largely unaccounted for thermal considerations.[2] Most protocols report the temperature of the thermal bath rather than the reaction solution, for instance.[11] Unfortunately, glycosylation conditions to-date are often matters of personal discretion, resulting in confounding differences from practitioner to practitioner.

Considering glycosylation reactions may proceed in seconds[8] and the rate of unwanted side reactions increases as the reaction temperature increases, current temperature treatment may contribute to irreproducibility in glycosylation systems, leading to poor conversion and prediction of selectivity.[12] Andreana and Crich recently noted that activation energy, selectivity, and complex rate regimes at play in glycosidic bond formation all rely upon temperature, and that single-temperature experiments would improve the reproducibility and quality of glycosylations by fixing a key variable.[2] We are interested in translating these insights to

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the practical arena by optimizing temperature treatment for a diverse panel of commercially available glycosyl donors capable of providing a high percentage of the linkages found in the human glycome.[13] An improved understanding of temperature would reduce the consumption of glycosyl donor and improve overall efficiency, expanding access to interesting and useful structures. Optimization is of special importance in the context of automated glycan assembly (AGA), where process automation strives to conduct glycosylations within the shortest possible time and, ideally, at near-ambient temperatures.

Thioglycosides serve as model glycosyl donors for unraveling confounding factors in carbohydrate synthesis.[14,15] Valued for their synthetic tractability and long shelf life, thioglycosides are easily activated with the *N*-iodosuccinimide (NIS) and triflic acid (TfOH) promoter system.[16] Oxidation of the anomeric sulfur atom by NIS (Figure 1A), is thought to induce formation of the glycosyl cation that may be stabilized by a solvent-separated interaction with the triflate counterion or by direct contact, forming either a glycosyl triflate or glycosyl halide intermediate.[17] The strength of interaction between the predominant intermediate and the nucleophile, as well as the electronic or steric participation by neighboring or remote protecting groups and the relative energies of the intermediates, dictate kinetics and the stereochemical outcome of the resultant glycosidic bond.[2,17,18] Since the oxidized adduct is required for elaboration, we reasoned that any approach to temperature optimization should begin with the activation of glycosyl donor by NIS/TfOH. The activation step is identical during solution- and solid-phase synthesis, as it involves only the dissolved glycosyl donor. In general, excessively low temperatures result in sluggish reactions, while inappropriately high temperatures or rapid heating might cause side reactions, reducing efficiency. Both extremes are undesirable and necessitate excess reactants or more time to drive the reaction to completion.[12,19] Herein, we present a semi-automated platform to examine and modulate the relationship between reaction temperature and glycosyl donor activation (Figure 1B).

Results and Discussion

Semi-Automated Temperature Assay of Glycosyl Donor Activation

A semi-automated quench assay was devised to query the glycosyl donor-temperature relationship (Figure 2A) for a panel of commercially available thioglycosyl donors. We focused on the relationship between glycosyl donor and activation temperature in the absence of nucleophile. First, a glycosyl donor is dispensed into the reaction vessel and chilled to the set temperature. Once the desired temperature of the reaction solution is reached, as measured by an internal fiber optic probe, the donor solution is exposed to NIS/TfOH for five minutes under active cooling to maintain the narrowest possible internal temperature range (portion highlighted in red, Figure 2B). After five minutes, a solution of pyridine in DMF is added and the reaction solution is rapidly ejected into a vial containing sodium thiosulfate. The mixture is then worked-up while the system initiates the following cycle at the next temperature. In general, four different temperatures were probed for each glycosyl donor. Proton NMR was used to compare the crude reaction mixtures of glycosyl donors treated at varying temperatures, with each spectrum corresponding to a different temperature range (Figure 2B, see Supporting Information for additional glycosyl donor temperature data). To qualitatively evaluate the behavior of the glycosyl donor at these conditions, disappearance of the starting material anomeric (C-1) and C-2 protons were used as diagnostic chemical shifts (Figure 2B). The highest temperature at which the starting material was preserved over five minutes of reaction is defined as the temperature of activation ($\mathbf{T_A}$, bolded). Conversely, the lowest temperature where side reactions or activation completely consumed the starting material is

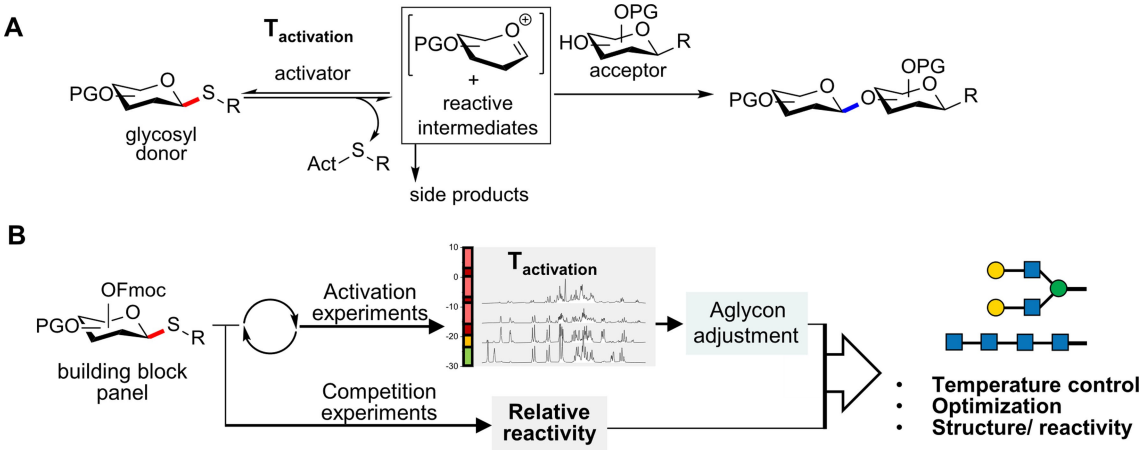


Figure 1. Simplified schematic of thioglycosylations. A) Temperature is influential at every step of the glycosylation reaction. B) This work uses an automated set-up to scan a range of temperatures conditions for an expanded panel of thioglycosides, incorporating relative reactivity measurements from a separate competition platform. PG = Protecting group, $T_{activation}$ = Temperature of Activation.

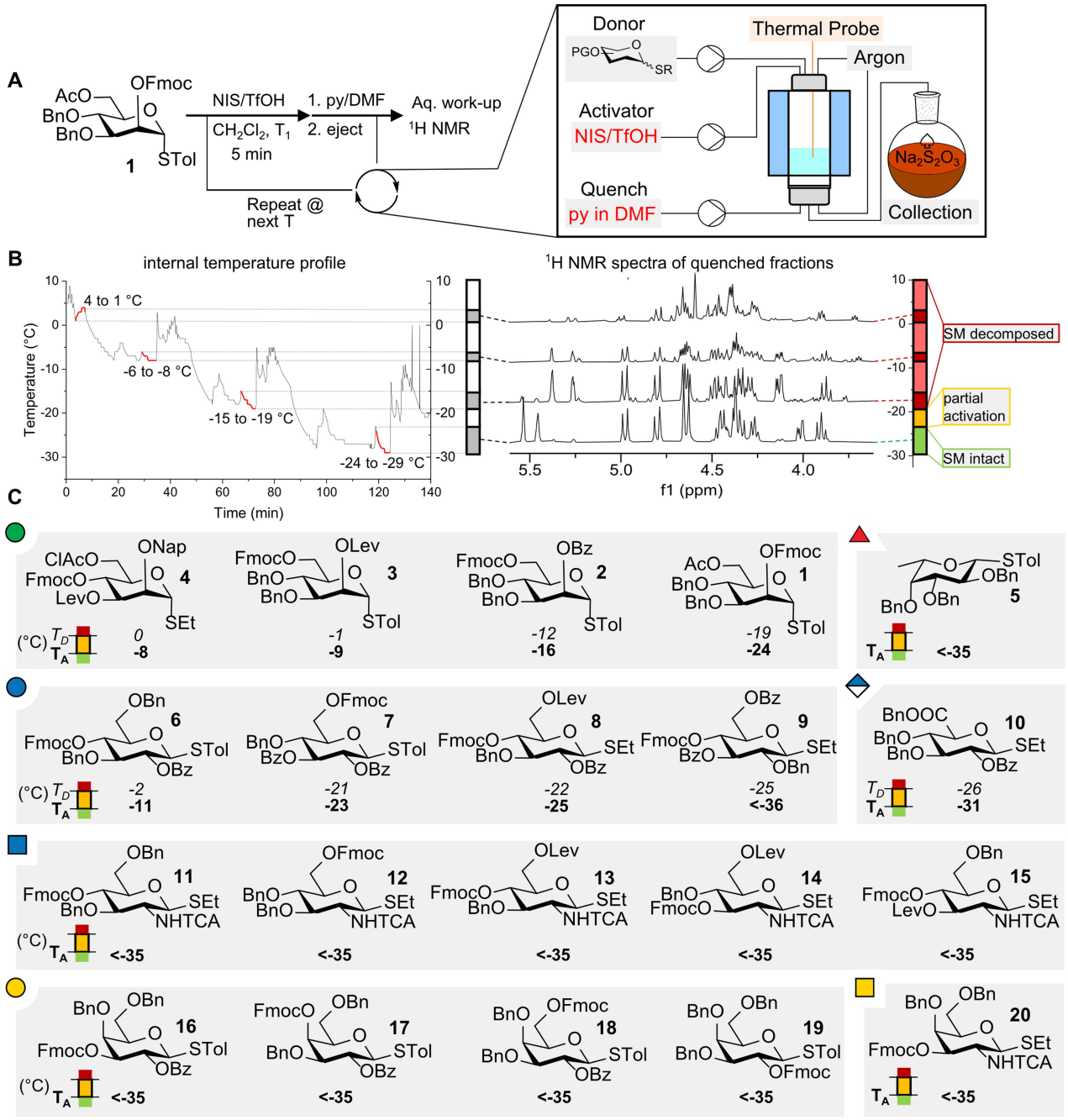


Figure 2. Systematic assessment of activation temperature of various thioglycoside monosaccharide glycosyl donors enabled by a semi-automated assay. A) General scheme of the activation assay. B) Representative temperature plot from an automated quench experiment and ^{1}H NMR (400 MHz) overlay of quenched fractions. Areas highlighted in red indicate the range of temperatures where the building block solution is exposed to NIS/TfOH. After activation, quenched and worked-up fractions are analyzed by ^{1}H NMR to evaluate decomposition at respective temperature ranges. 2-Fmoc-protected mannosyl thioglycoside **1** spectra shown. C) Assayed activation parameters for a panel of fully-protected glycosyl donors. Temperatures delineate the upper and lower limits of partial activation, illustrated in (B). Italicized temperatures indicate the upper limit, or where decomposition was observed in ^{1}H NMR ($T_{decomosition} = T_D$), while bolded values represent the highest temperature where reaction conditions afforded almost exclusively the starting material ($T_{activation} = \mathbf{T_A}$). STol = thiotoluene, SEt = thioethane, OBn = benzyl ether, OBz = benzoyl ester, OFmoc = 9-fluorenylmethyl carbonate, NHTCA = trichloroacetyl amide, OLev = levulinoyl ester, ClAcO = chloroacetyl ester, NapO = 2-naphthylmethyl ether.

defined as the decomposition temperature (T_D, italicized). The temperature range between $\mathbf{T_A}$ and T_D is the zone of partial activation (Figure 2B).

The semi-automated activation temperature assay elucidated temperature dynamics in the initial stages of glycosylation. Mannoside **1** (Figure 2A), for example, is completely intact after five minutes of reaction from −24 to

−29 °C, but at temperatures between −19 and −15 °C, a secondary product arises (δ = 5.25 ppm) concomitant with the disappearance of the anomeric proton resonance (δ = 5.55 ppm) of the starting material (Figure 2B). Mannoside **1** is rapidly activated at a temperature between −19 and −24 °C, presumably when sufficient thermal energy is provided to overcome the activation barrier, or more explicitly the activation entropy term $T\Delta S^{\ddagger}$, involved in transformation to intermediates.[2] Hence, if **1** were to be held at a lower temperature than its $\mathbf{T_A}$ in a glycosylation reaction, the reaction might proceed in a controlled manner. Conversely, treatment of **1** at elevated temperature could result in deleterious side reactions as the starting material is unrecognizable after five minutes.

Screened thioglycosides varied considerably in temperature sensitivity (Figure 2C). While mannosides are generally activated at higher temperatures, glucosides decomposed more readily. Glucosamine and galactoside glycosyl donors decomposed at every condition assayed, showing no trace of starting material after five minutes even at −35 °C (lowest temperature achievable in setup). In general, these data agree with trends observed to-date between configuration and reactivity of the glycosyl donor;[20] however, they represent the first systematic attempt to understand thioglycoside response to temperature. Moreover, a general trend in protecting group pattern and decomposition temperature is apparent. Placement of electron-withdrawing groups (EWGs) such as esters increases the activation temperature, whereas inclusion of electron-donating (EDGs) or electron-rich groups like ethers and carbonates leads to decomposition even at low temperatures.[21] The electronic effect is most apparent when comparing glycosyl donors **2** and **3**. The levulinoyl (Lev) substituent on **3** is presumably more electron-withdrawing than the benzyl ester (Bn) on **1**, meaning **1** is more reactive and can more readily overcome activation barriers to reactive intermediates. The reactivity is modulated even further by use of a third ester protecting group (**4**, $\mathbf{T_A}$ = 0 °C). The position of the bulky Fmoc group also appears to impact the temperature of activation.

Aglycon Modification Enables Tuning of Activation Temperature

A surprising result of the findings presented in Figure 2C was the low $\mathbf{T_A}$ of glucosamine glycosyl donors **11**–**15** that we rationalized by considering the aglycon leaving group. Glucosaminyl donors, in addition to glycosyl donors **4**, **8**, **9**, and **10**, contain thioethyl (SEt) moieties at their anomeric position, as opposed to thiotoluyl (STol) leaving groups in the remaining glycosyl donors. Oscarson[22] and Gildersleeve[23] showed that the nucleophilicity of the sulfur atom can be reduced via induction or by blocking incoming electrophiles through steric hinderance, thus stabilizing glycosyl donors and increasing their activation temperatures.[24,25] By the same token, STol may reduce the reactivity of glycosyl donors with respect to SEt. To test this hypothesis, we replaced the aglycon on glucosamine **11** and galactoside **16** with increasingly deactivating thiols in two steps, proceeding through a glycosyl phosphate intermediate (Figure 3A). Activation temperature assays of STol glucosamine **21** demonstrated that starting material was retained at the lowest temperature tested. Further tuning with the di-*ortho*-methyl thiophenol (**22**) decreased the temperature

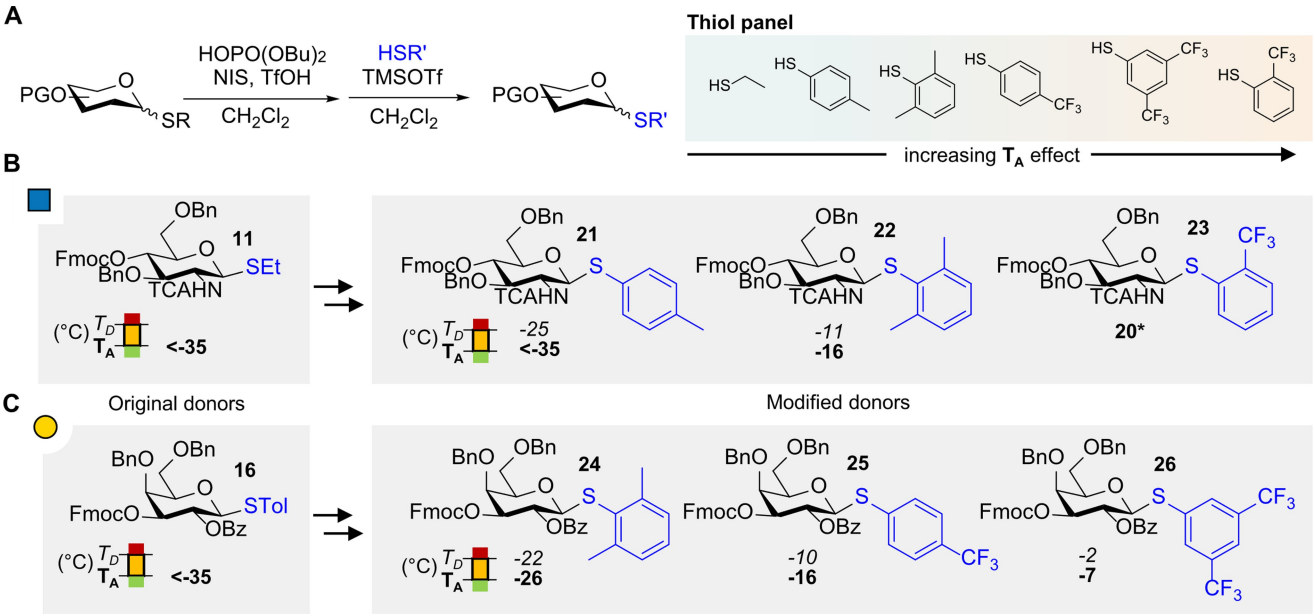


Figure 3. Aglycon replacement alters the temperature sensitivity of glycosyl donors. A) General scheme for replacement of the aglycon of glycosyl donors that are activated below the minimum temperature used for AGA and panel of replacement thiols. B, C) Activation parameters for glycosyl donors **11** and **16**, respectively, and new parameters after aglycon replacement. *) Glucosamine **11** with *ortho*-CF_3-thiophenol at the anomeric position had both starting material and activated adducts present at ambient temperature.

sensitivity further: the highly sterically hindered and electron-withdrawing *ortho*-trifluoromethyl thiophenol glycoside **23** showed starting material in approximately equivalent proportion to a quenched intermediate at ambient temperature (See Supporting Information), highlighting the kinetic control afforded by thiol replacement and its impact on activation temperature (Figure 3B). Deactivating thiols additionally brought the reactive galactoside **16** within range of our assay (Figure 3C, **24** and **25**). Correlations of aglycon modifications and temperature are useful insights for future temperature controlled sequential glycosylation reactions,[14] and might help rationalize stereoselectivity effects in the S_N1-S_N2 glycosylation continuum.[2]

Aglycon replacement experiments shed light on the structure–reactivity relationship for the panel of thioglycosides. Replacement of thioethyl on glucosaminyl donor **11** with a thiotoluyl aglycon (**21**) invites direct comparison to glucoside **6**, since the two differ only with respect to their C-2 protecting groups.[26,27] The trichloroacetyl amide (TCA) on the C-2 position of **21** appears to activate the glucosamine to a greater extent than the benzoyl group on **6**. Therefore, the general trend of glucosaminyl donors displaying a lower $\mathbf{T_A}$ than their glucoside counterparts may be attributed both to the SEt aglycon and the amide motif. The additional thioethyl-containing donors further contextualize their observed $\mathbf{T_A}$. Glucoside **8**, for example, contains a C-6 levulinoyl ester, but appears on first glance to be more active than its analog **6** because it decomposes at lower temperatures. However, the thioethyl aglycon on **8** clarifies this apparent exception. Modification of glucose at the C-2 position with an electron-rich non-participating benzyl ether (**9**) decreased the activation temperature even with two esters, while conversion to the glucuronic acid (**10**) had similar temperature stability as when the electron-withdrawing Lev protecting group was used (**8**). Additionally, aglycon $\mathbf{T_A}$ differences invite comparison between diester thiotoluyl mannoside **3** and triester thioethyl mannoside **4**. Contrasting galactosides to other donors elucidates a hierarchy of $\mathbf{T_A}$ among configurations: Fucose, N-acetyl galactosamine < galactose < glucosamine < glucose < mannose.[28]

This trend is especially notable because galactose, N-acetyl glucosamine, and fucose glycosyl donors have often proven cumbersome during AGA, requiring double coupling cycles or additional equivalents to achieve acceptable coupling efficiencies.[29] Aglycon replacement to modulate the reactivity of these problematic glycosyl donors and to bring their activation barriers within range of AGA presents an opportunity to enhance synthetic efficiency and to improve access to previously challenging linkages.

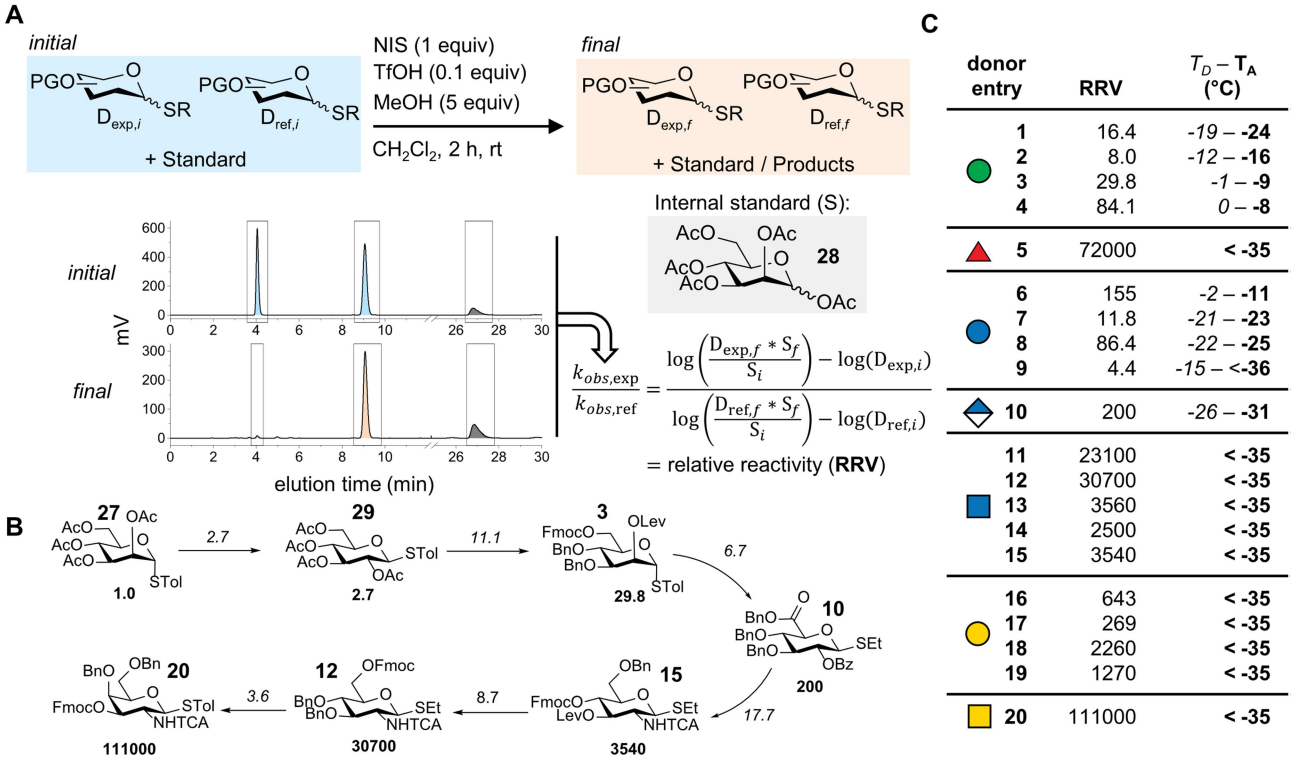
Relative Reactivity Values (RRV) of Thioglycosides

To improve our understanding of donor characteristics, temperature, and reactivity, we sought to relate our findings to the concept of relative reactivity.[20,22,30,31] The relative reactivity value (RRV),[20] introduced by Wong, is derived from a competition reaction between equimolar amounts of two glycosyl donors, D_{exp} and D_{ref} (Figure 4A). Thiotoluyl peracetylated mannoside **27** is set to an arbitrary RRV = 1, and tracing reactivity back to this glycosyl donor provides the "absolute" RRV for each experimental glycosyl donor (Figure 4B). However, the task of elucidating RRVs is not trivial.[31] Employing an inert internal standard, peracetylated mannose **28**, and comparing only glycosyl donors with no more than twenty-fold differences in experimental reactivity gave the most reproducible results (Figure 4A).[31] These data represent the first report of RRV for Fmoc-containing thioglycosides.

Relative reactivities parallel activation temperature results in some respects but differ in others (Figure 4C). Mannosides and glucosides generally had the highest $\mathbf{T_A}$, suggesting that their reactivity is low. RRV experimentation supported this trend, but within monosaccharide groups, results were unexpected and variable. Disparate, competing mechanisms may be at play depending on protecting group pattern. Nonlinear trends between $\mathbf{T_A}$ and RRV could suggest values that describe competing aspects of the mechanistic continuum (see Supporting Information).[18,32,33] Interestingly, galactosides, though more temperature sensitive than N-acetyl glucosamine glycosyl donors (Figure 4C), had lower RRVs. The reactivity of galactose glycosyl donors with respect to Fmoc position was C-6 > C-2 > C-3 > C-4. N-acetyl glucosamine glycosyl donors **13**–**15** were the next most reactive; since they contain electron-withdrawing substituents, it follows that **11** and **12** are more reactive, though the order of magnitude difference between **11** and **13** is curious. Finally, direct competition between perbenzylated fucose **5** and the N-acetyl galactosamine thioethyl glycoside **20** indicated the galactosamine is approximately 1.5 times more reactive than fucose. In general, we note that regression of the natural logarithm of RRVs against the chemical shifts of the anomeric proton of the glycosyl donors we screened do not suggest linearity as others have reported (see Supporting Information). RRVs assume glycosyl donors react in their standard states (i.e. ambient temperature), but most glycosylations are carried out in different conditions. Moreover, the relative energy difference between donor pairs changes as a function of temperature. Future experimentation should quantify relative reactivities at lower, nonambient temperatures. In general, glycosyl donors with RRV greater than 250 exhibit $\mathbf{T_A}$ lower than −35 °C, meaning low $\mathbf{T_A}$ may be roughly inferred from RRV. An interesting outlier in this data set is, **9**. Though its $\mathbf{T_A}$ is relatively low, the measured RRV was also very small, opposite to the general trend.

Temperature Control Improves Model Tetramer AGA Syntheses

AGA enables facile access of complicated oligosaccharides through iterative couplings of glycosyl donors to nucleophiles attached to a solid-phase. Orthogonal protecting group modification exposes nascent acceptors to ensure chemoselectivity. The coupling step (glycosylation) is the most crucial, but least well-understood component of AGA. It determines both the yield and the stereochemical outcome



donor entry	RRV	$T_D - T_A$ (°C)
1	16.4	*-19* – **-24**
2	8.0	*-12* – **-16**
3	29.8	*-1* – **-9**
4	84.1	*0* – **-8**
5	72000	**< -35**
6	155	*-2* – **-11**
7	11.8	*-21* – **-23**
8	86.4	*-22* – **-25**
9	4.4	*-15* – **<-36**
10	200	*-26* – **-31**
11	23100	**< -35**
12	30700	**< -35**
13	3560	**< -35**
14	2500	**< -35**
15	3540	**< -35**
16	643	**< -35**
17	269	**< -35**
18	2260	**< -35**
19	1270	**< -35**
20	111000	**< -35**

Figure 4. Comparison of glycosyl donor activation temperatures to relative reactivity. A) Schematic representation of a competitive HPLC experiment. Representative trace from reaction of 2-O-Lev, 6-O-Fmoc thiotoluylmannoside against glucose benzylidene reference shown. The inert internal standard **28** is used to correct for volumetric losses after work-up. B) Method for determination of absolute reactivity, where the peracetylated thiotoluylmannoside **27** is arbitrarily set to 1.0. Each arrow represents an RRV experiment. C) Table of experimental RRV values for panel of thioglycosides set alongside activation experiment data.

of a given oligosaccharide synthesis. During glycosylation, the interacting moieties are different for practically every reaction. Structure and electronics of the glycosyl donor impart distinct reactivity (Figure 4),[20,21] and each hydroxyl nucleophile (acceptor) in the growing glycan differs from the previous acceptor depending on the desired linkage.[28]

To date, a general coupling protocol that is applicable to a range of glycosyl donors is used that evolved during the development of AGA.[34] In a standard cycle, 6.5 equiv. of glycosylating reagent react for 5 min at −20 °C, then the reaction vessel is allowed to warm to 0 °C over the course of five minutes and maintained at that temperature for 15 min, before draining of the reaction mixture and washing to prepare for successive cycles. Challenging linkages may require a double coupling, whereby the entire process is repeated. Employing this general procedure, AGA was used to procure polysaccharides as long as a 151-mer[35] and glycogen oligosaccharides that contain multiple challenging *cis*-glycosidic linkages.[36] However, to avoid the excessive consumption of valuable building blocks, reaction conditions must be frequently adjusted in response to new synthetic challenges. Recent advances in AGA hardware, such as the addition of an internal probe to precisely measure and control the reaction temperature,[29] combined with the need to develop a model for reproducible isothermal glycosylations, motivated us to interrogate the role of temperature in complete AGA syntheses.

Previously, mannoside **2** has been coupled using a standard temperature regime: five minutes at −20 °C followed by 20 minutes at 0 °C.[35] Temperature assays suggest these AGA conditions could therefore result in needless decomposition of **2** at elevated temperatures, where the initial five minutes at −20 °C suffices for complete coupling. This observation is consistent with previous AGA preparation of an α-(1,6)-octamannoside using comparatively short coupling times (8 min at −20 °C and ramped 5 min to 0 °C).[29] We hypothesize that maintaining a temperature below $\mathbf{T_A}$ will preserve the glycosyl donor, leading to more effective glycosylations. The activation temperate $\mathbf{T_A}$ therefore offers a reference parameter potentially representing the ceiling or highest operating temperature for efficient coupling.

Application of insights gained from $\mathbf{T_A}$ experiments improved the synthesis of a model β-(1–4) glucose tetramer **31** (Figure 5A). Glucoside **6** can be activated in a controlled fashion up to −11 °C (Figure 2C), but standard AGA conditions (−20 °C, 5 min→0 °C, 20 min) hold the glycosyl donor above this temperature during most of the coupling time. To test the utility of $\mathbf{T_A}$ assays, we performed three AGA syntheses of model β-(1,4)-glucose tetramer **31** using three equivalents of glycosyl donor rather than the standard 6.5 equivalents. Large excesses help achieve desired structures, but are costly and engender glycosyl donor decomposition. Three equivalents of glucoside **6** per cycle yielded

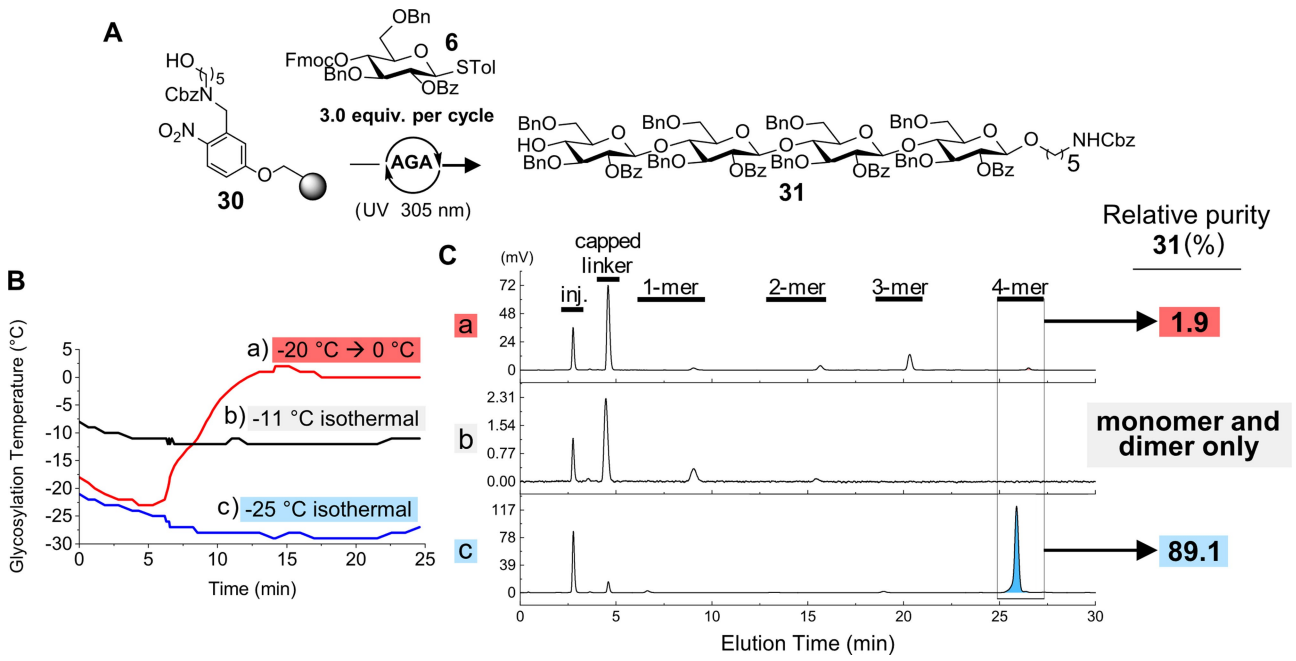


Figure 5. Optimization of cellulose tetramer synthesis using $T_{activation}$. A) Scheme of AGA of protected cellulose tetramer. B) Comparison of internal temperature of single glycosylation cycles. C) HPLC traces of crude photocleaved products of AGA syntheses in (B). Relative purity is defined as the ratio of the integral of the product peak (elution time = 26 min) to the sum of the integrals of the product peak and additional deletion sequences.

the tetramer product **31** in significantly higher relative purity (89.1 %) when isothermal conditions were employed (25 min at −25 °C, Figure 5B, C). Since the coupling temperature was below the $\mathbf{T_A}$ for **6** (−11 °C), the total reaction time was kept at 25 min to accommodate slower kinetics. Conversely, the control synthesis using standard AGA coupling temperatures (−20 °C, 5 min→0 °C, 20 min) yielded only 1.9 % of the desired tetramer relative to deletion sequences (Figure 4B, C), illustrating the adverse effects of decomposition at elevated temperatures on overall synthetic success. Interestingly, the synthesis of **31** using isothermal glycosylations at −11 °C, the $\mathbf{T_A}$ of **6**, resulted only in monomer, dimer, and capped linker (**30**). At this condition, **6** is at a temperature that causes decomposition and poor conversion. By comparison, even a brief interval in the control synthesis at −20 °C, below the ceiling temperature, sufficed to produce some target tetramer. Thus, glycosylation below the respective activation temperature is necessary to achieve efficient couplings in the synthesis of **31**.

Modification of the standard "ramp" regime significantly improved access to model tetramer **31**, validating optimization efforts. Taken together, these experiments demonstrate the applicability of $\mathbf{T_A}$ measurements and its potential to optimize coupling conditions and drastically reduce building block usage. Commonly-used solution-phase treatments—cryogenic initiation followed by warming to ambient temperature—may overshoot an important activation threshold, thereby limiting synthetic success and unnecessarily lengthening reaction times. Despite the significant gains in efficiency afforded by $\mathbf{T_A}$-guided temperature control, we acknowledge that activation alone paints an incomplete picture of oligosaccharide synthesis. The coupling step also involves the nucleophile glycosyl acceptor, and interactions between the activated glycosyl donor and acceptor likely require a distinct temperature for optimal conversion to the product.[28] Further studies will help to elucidate the delicate balance between nucleophilicity of acceptor, donor reactivity, and temperature.

Conclusion

A semi-automated assay was developed to probe the relationship between glycosyl donor, activation, and reaction temperature. Activation temperature ranges were assigned for a panel of thioglycosides. The glycosyl donors we screened varied greatly in $\mathbf{T_A}$ and can be manipulated by the placement of EDG or EWG protecting groups, or by adjusting the thiol aglycon. The latter strategy is advantageous because it enables tuning of highly reactive, previously intractable glycosyl donors to more manageable temperature conditions, both in the solid phase and in solution. This approach yielded significant synthetic advantages, including the optimization of glycosyl donor usage and simplification of temperature treatment. The efficiency and purity of the automated assembly of a tetraglucoside was greatly improved, reducing glycosyl donor equivalents by more than half (6.5→3 equiv) when compared to the standard thermal "ramp" regime currently used for AGA, amounting to significant cost reduction. Future studies will have to explore the coupling step and methods to manipulate the coupling temperature to achieve optimal conversion. For example,

aglycon modification could elevate the $\mathbf{T_A}$ of the glycosyl donors, enabling coupling at higher temperatures, in turn improving turnover for linkages suffering from poor acceptor nucleophilicity.[37] Generally, we note that the activation temperatures are measured in solution and are broadly applicable to solution-phase glycosylation techniques. Combined with isothermal glycosylations and potential machine learning applications, we anticipate this approach will help tame the seemingly unruly character of glycosyl donor treatment by providing experimentally established setpoints for glycosylations. Systematic optimization efforts, especially ones aided by automated technology, show great promise in advancing oligosaccharide synthesis and the glycosciences.

Acknowledgements

We gratefully acknowledge the Max-Planck Society for generous financial support. O.T.T. acknowledges financial support from a Fulbright Study/Research Award administered by the Fulbright Commission and Fulbright Germany. E.T.S. acknowledges financial support from the Alexander von Humboldt Foundation. Open Access funding enabled and organized by Projekt DEAL.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

Keywords: Activation Temperature · Carbohydrates · Glycans · Glycosylation · Thermal Stability

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Manuscript received: November 12, 2021
Accepted manuscript online: January 15, 2022
Version of record online: February 15, 2022