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# Microfluidic Reactor Technology in Oligosaccharide Synthesis

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## Acronysms and Abbreviations

| | |
|---|---|
| **Ac** | Acetyl |
| **Bn** | Benzyl |
| **Cbz** | Carbobenzoyl |
| **CPS** | Capsular polysaccharide |
| **DBU** | Diazabicycloundecene |
| **DCM** | Dichloromethane |
| **DMAP** | 4-Dimethylaminopyridine |
| **DMF** | *N,N*-Dimethylformamide |
| **Et** | Ethyl |
| **LPS** | Lipopolysaccharide |
| **Me** | Methyl |
| **MenX** | *N. meningitidis* type X |
| **MRT** | Microfluidic Reactor Technology |
| **NAc** | *N*-Acetyl |
| **NIS** | *N*-iodo succinimide |
| **NMR** | Nuclear Magnetic Resonance |
| **Ph** | Phenyl |
| **PivCl** | Trimethylacetyl chloride, or Pivaloyl chloride |
| **Py** | Pyridine |
| **TEA** | Triethylamine |
| **TBAF** | Tetra-*n*-butylammonium fluoride |
| **TEAB** | Triethylammonium bicarbonate buffer |
| **TDS** | Thexyldimethylsilyl |
| **TFA** | Trifluoroacetic acid |
| **TfOH** | Trifluoromethansulfonic acid |
| **THF** | Tetrahydrofurane |
| **TMSOTf** | Trimethylsilyl trifluoromethansulfonate |

# Introduction

# 1 Flow chemistry and Microfluidic reactor technology

For chemists working into a common synthetic laboratory most equipment would look very familiar: they all work with standardized glassware, use somewhat sophisticated ways of stirring and different ways to heat (such as the use of microwaves). Indeed chemists have mainly been focused on the chemistry occurring inside the flasks or reaction vessels, making it in a very elaborated and successful manner. Despite their many advantages continuous flow processes have commonly used only in the industrial environment of chemical and biotechnological production.[1] With continuous flow processes constant parameters (temperature, time, amount of reagents and solvent, efficient mixing *etc.*) can easily be assured. Over the past 15 years, miniaturization has entered the field of flow chemistry and modern developments commonly deal with micro- and minifluidic flow devices.[2] With respect to heat and mass transfer, efficient mixing as well as precise parameter control, miniaturized flow devices have proven very beneficial for performing chemistry that is difficult to conduct under batch conditions.

Flow chemistry will not completely or generally change the way chemists are performing synthesis in the future, despite the fact that the field experiences a dramatic increase in interest both in academia and industry. Flow chemistry will become a common enabling technology to perform synthesis in the laboratory.[3]

Micro reaction Technology is a term widely used to describe the performance of reactions in a continuous manner, within well-defined reaction channels, where typical dimension are of the order <1000μm and volumes, at a research level, span the microliter to milliliter range. Often, microreactors are constructed as planar devices with ducts or channels machined into a planar surface.

## 1.1 Micro *versus* minifluididc reactors

Common miniaturized flow devices rely on reactors that have channels or tubes sized between 10 μm to a few mm in inner diameter. The differences, advantages and disadvantages between micro- and minifluidic reactors are reported in Table 1.

**Table 1. Micro- *versus* minifluidic reactors**

| Reactor | Advantages | Disadvantages |
|---|---|---|
| Microfluidic (10-500 μm i.d.) | ✓ high heat transfer surface to product volume ratio<br>✓ good heat transfer capabilities<br>✓ ideally suited for optimizing reaction conditions<br>✓ efficient mixing | ✓ micro channel suffer from restricted flow capacity<br>✓ high pressure drop<br>✓ tendency to block |
| Minifluidic (500 μm-several mm i.d.) | ✓ improved flow capacities<br>✓ lower pressure drop<br>✓ no blocking of channel<br>✓ preparation of multigram to multikilogram quantities<br>✓ possibility to work with packed bed reactors | ✓ lower heat transfer surface<br>✓ poorer heat transfer capabilities |

The range of scale of these microreactors (micro- and mini-) is sufficiently broad to allow synthesis of approximately 10 mg to an annual production rate of a desired compound.

Microfluidic devices are mainly found in academic laboratories and in analytical and diagnostic applications. Mesofluidic devices are also of academic interest, particularly when combined with functionalized fixed bed materials, but have also become popular in the industrial context.

High heat transfer capacities are achieved with small diameter channels favouring micro over mini flow reactors. But high pressure drops, a limited flow capacity and a tendency to block, are common problems with micro reactors. Micro reactors also suffer from the fact that it is very difficult to

utilize them for the production of substantial amounts of material. Technically it is difficult to overcome this problem even when massive parallelization is achieved.
However, the scale of micro flow reactors makes them ideal tools for process development experiments.

## 1.2 Three key factors in flow chemistry: residence time, flow rate and reactor volume

With respect to stoichiometry and reaction time there are fundamental differences between batch and flow systems. In batch processes stoichiometry is defined by the concentration of chemical reagents and their volumetric ratio while in flow processes this is defined by the concentration of reagents and the ratio of their flow rate. In batch production the reaction time is determined by how long a vessel is held at a given temperature to reach full conversion. When chemistry is operated under flow conditions the reaction time is determined by the volume of the reactor and the bulk flow rate. It is desirable to reach full conversion inside the reactor with the highest possible flow rate.
As the flow rate correlates with the residence time these factors determine how much products is formed in a given time. Under optimized conditions a flow reactor can be operated continuously as long as starting material are available and are pumped through the reactor.

## 1.3 The relationship between flow, heat and pressure

Initially, miniaturized flow devices have been used to perform highly exothermic reactions that are carried out at ambient temperature. In that context safety issues are matched due to the excellent mixing and heat transfer properties of these type of reactors.
Lately, developments in this field have focused on reactions that require more than 20 minutes reaction time under classical batch conditions and

thus need to be heated in order to be rapidly carried out under continuous flow conditions. As a cold stream of reactants enters the reactor and residence time in a flow protocol ideally need to be in the order of a few minutes to allow high flow rates heating through the walls of a preheated reactor has to be very rapid. If “flash” heating can be guaranteed and pressure resistant micro-structured flow reactors are chosen the reaction temperature can be set well above the boiling point of the solvent even up to supercritical conditions thus leading to very short reaction times.

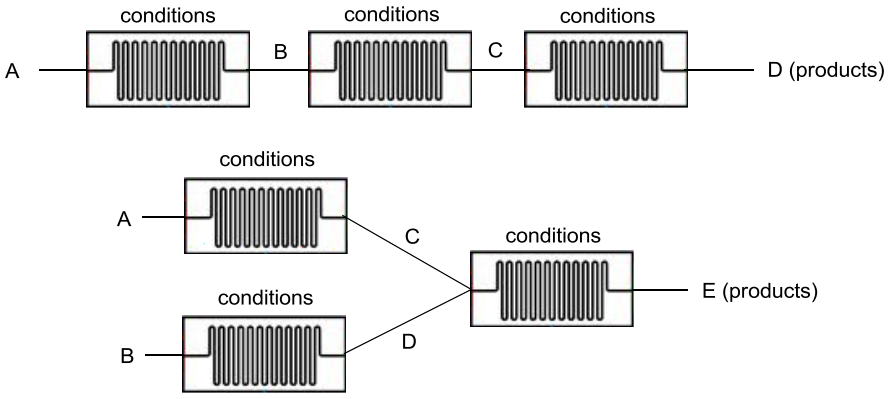
Several strategies have been pursued to achieve “flash” heating. Beside external ovens microwave irradiation (MW) has been combined with flow devices. This is a very practical approach because microwave chemistry is otherwise difficult to scale in a batch environment. Under flow conditions, however, only the reactor has to be heated and not the reservoirs containing the starting materials and the products.

## 1.4 Flow chemistry and Multistep synthesis

Synthesis under flow conditions is ideally suited to perform multistep process using linearly assembled flow devices. While synthesis under batch conditions means that each intermediate product of a multistep synthesis has to be isolated in bulk, the situation is totally different under flow conditions.

A continuously operated multistep synthesis uses linearly linked flow reactors, thereby work-up and isolation protocols are minimized. In addition, it is possible to create flow setup in which two short linear synthetic flow sequences converge into one flow device which allow production of complex molecules from two fairly complex building blocks (Figure 1).

**Figure 1. Linear and convergent multistep flow synthesis**




Multistep flow synthesis may also be very advantageous when highly unstable intermediates are formed during the synthetic process.

## 1.5 Scale up and Industrial application

For a long time the chemical industry has relied on the continuous production of chemicals, in general for commodities and less much so for fine chemicals and pharmaceuticals. As no flow equipment on the laboratory scale was available until recently, the chemistry developed in the laboratory was based on batch processes. This means that often research was disconnected from process chemistry which led to many problems and extra optimizations or even total redesign of the initial synthetic strategy.

With bench top flow systems some of these frustrations between process and research chemists will be reduced because an established flow process is readily scalable. The volume of a flow reactor is very small compared to the scale of the whole process; however, reaction parameter like temperature, concentration, composition of reactants established for a small scale flow process can directly be transferred to larger flow reactors, without the need for substantial alteration of the conditions.

When mini (meso) flow equipment is utilized for scale-up concepts the extension of product collection time or increase of the length or size of the microchannels or tube are valid strategy (*scale out*).

On the contrary, the parallel operation of many identical channels (numbering up principle) can be envisaged, but from a process engineering point of view this strategy is regarded to be far from ideal because the individual flow systems must show identical flow properties and the system requires highly complex online monitoring.

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# MRT and Glycosylation reactions

# 1 Carbohydrates and Glycosylation reaction

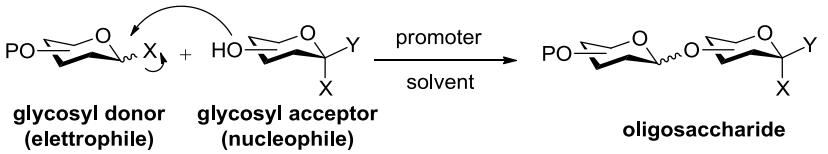
Carbohydrates play a fundamental role in signal transduction and vital molecular recognition phenomena. They are primarly involved in inflammatory processes, bacterial and viral invasions, tumor growth and metastasis, and many other crucial biological events.[1] The diversity of roles played by carbohydrates makes them attractive targets for the development of novel drugs to treat cancer, infectious diseases, inflammation and cardiovascular disorders.[2] However, there are still severe limitations to the development of saccharide-based drugs using carbohydrates synthesis: during the past decades only two pharmaceutical compound based on synthetic oligosaccharides have been approved for the market, the anticoagulant fondaparinux (Arixtra™) and the antiviral zanamivir (Relenza™).

Classical carbohydrate synthesis is a multistep process tipically afflicted by laborious protecting groups manipulations, often requiring tedious work-up and time-consuming purification steps.[3] The most important transformation in the chemical synthesis of carbohydrates is the glycosylation reaction, that remains a challenging task.

# 2 Glycosylation reaction

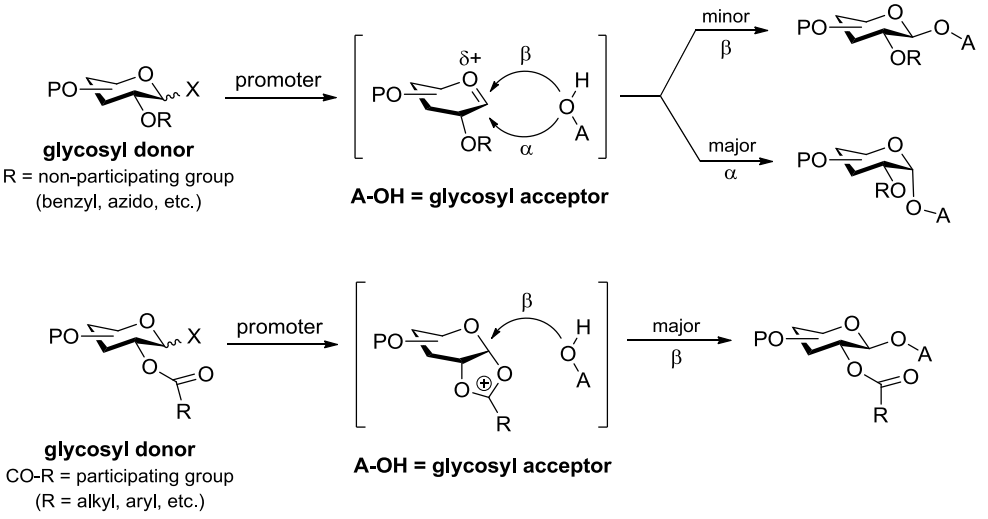
The glycosylation reaction is the transformation that connects two sugar residues. The glycosidic bond is formed by a nuclephilic displacement of a leaving group (X) attached to the anomeric bond of a sugar residue (called *glycosyl donor*) by an alcohol ROH, or by the OH group of a partially protected sugar moiety (called *glycosyl acceptor*). The reaction is generally performed in the presence of an acidic activator, called *promoter*. The role of the promoter, in general used in catalytic amount, is to assist the departure of the leaving group (Scheme 1).

**Scheme 1. General scheme of glycosylation reaction**




In the literature are present a lot of methods for glycosidic bond formation. The synthesis of disaccharides and oligosaccharides in general involves the linking of two polyfunctional compound. This kind of synthesis is much more complicated than the synthesis of other biopolymers because of the greater number of possibilities for the combination of monomeric units and because the glycosidic bond have to be formed in a stereoselective way.
In Scheme 2 are outlined the general pathways for glycosylation reaction: over 90% of all the glycosylation reported formally proceeds via this general mechanistic pathway.

**Scheme 2. General mechanistic pathway for glycodic bond formation**



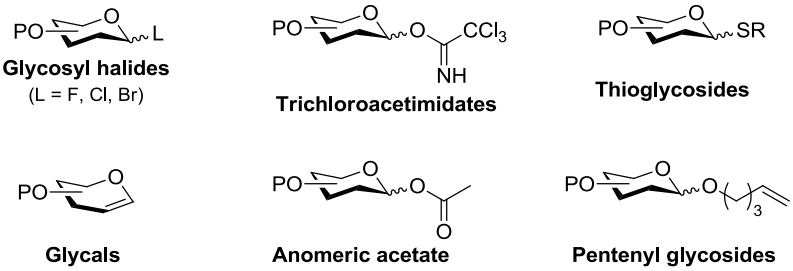

The good results of the glycosylation reactions depends on different issues:1) the structures and reactivity of glycosyl donors and acceptors; 2)

the nature of the promoter; 3) kind of substituents on both the saccharide units; 4) the preferred selectivity of the reaction toward the α- or the β-anomeric form.

## 2.1 Structure and reactivity of glycosyl donors and of glycosyl acceptor

There are numerous glycosylation methods involving different glycosyl donors: the name of glycosylation method generally reflects the functionality of the glycosyl donor.

**Figure 2. Some example of glycosyl donors used in oligosaccaride synthesis**




The reactivity at the anomeric center depends in a large extent on the choice of the protecting groups, especially those at C-2. Glycosyl donors are classified in two main groups: *armed donors* (with ether groups) and *disarmed donors* (with ester or amide groups). Ester groups exert an inductive electron-withdrawing effect at the anomeric carbon making the formation of the oxonium ion a slower process.

The reactivity of glycosyl donors may be controlled by different leaving groups: both the nature of the heteroatom and the substituent of the leaving group will affect the reactivity.

With regard to the reactivity of the glycosyl acceptor, this depends on the nucleophilicity of the hydroxyl groups in partially protected carbohydrates. In particular, it depends on their nature (primary more reactive than

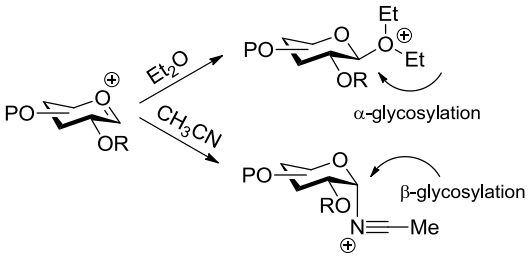
secondary), their spatial orientation (equatorial more reactive than axial), the conformation of the sugar and the presence of other protecting groups in the molecule. In general, electron-withdrawing effect and the steric hindrance of the protecting groups decrease the reactivity of the acceptor.

## 2.2 Promoters, Solvents and Experimental Conditions

The nature of the *promoter*, generally a Lewis acid, has an influence on the departure of the leaving group in the glycosyl donor. The nature of the promoter classifies the reactions as homogenous and heterogenous, with an implication for the stereoselectivity.

The *solvent* also has an influence on the overall rate of the process and in particular on the stereochemistry, especially in the case of non-participating groups in the glycosyl donor. Anhydrous solvents are required to avoid the competition from water. The most used solvents have low polarity, like dichloromethane or ether; sometimes polar aprotic solvents are used, such as acetonitrile or nitromethane. Some solvents may interacts with the oxonium ion forming an intermediate that drives the prevalent formation of a single anomer. In Scheme 3 is reported the formation of two different intermediates when acetonitrile or diethyl ether are used as solvents.

**Scheme 3. Influence of the solvent on the glycosylation stereochemistry**




The experimental conditions are very critical for the success of the glycosylation reaction. Generally, the use of extremely dry solvents, inert atmosphere and molecular sieves (that act as acid scavenger) are needed.

In some cases, the order in which the reagents are added is also important. In the normal procedure (appropriate for less reactive disarmed donors) the promoter is added over a mixture of acceptor and donor. In the inverse procedure (used for highly reactive armed donors) the donor is added to a mixture of acceptor and promoter.

It's important to notice that the stereoselective formation of a new glycosidic bond depends on different factors such as the type of the leaving group at the anomeric carbon, the protection and substitution on the rection partners, the promoter, the solvent and the temperature. So it should be realize that there are no general methods or strategies for oligosaccharide synthesis, which is one of its greatest difficulty.

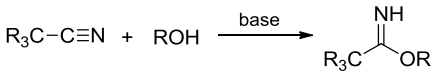
## 2.3 Different procedures of glycosylation reaction by different activation

From a chemical point of view, the synthesis of oligosaccharides still remains a challenge to synthetic chemists. In literature are reported a number of methods for glycoside bond formation. Although some methods are more popular than others, there is no universal protocol that can be applied to any combination of donor and acceptor. The two most popular and widespread are the glycosylations that involve trichloroacetimidates and thioglycosides.

### 2.3.1 The trichloroacetimidate method

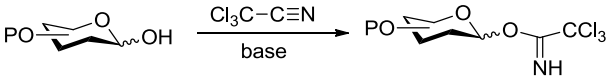
Electron deficient nitriles are known to undergo direct and reversible base-catalysed addition of alcohols to the triple bond system, providing *O*-alkyl imidates (Scheme 4). The free imidates can be directly isolated as stable adduct.

**Scheme 4. General synthesis of O-alkyl imidates**



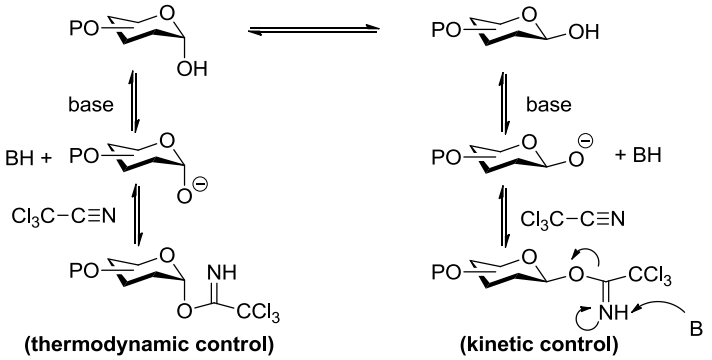

The reaction of a sugar hemiacetal in the presence of a base with trichloroacetonitrile gives the anomeric trichloroacetimidates (Scheme 5). In this way, the anomeric oxygen atom has been transformed into a good leaving group.[4]

**Scheme 5. General glycosyl trichloroacetimidates synthesis**




Taking into account the equilibrium between both anomers and the enhanced nuclophilicity of equatorial oxygen atoms (kinetic anomeric effect, Scheme 6), the equatorial (β)-trichloroacetimidate is generated with preference in a very rapid and reversible reaction. However, this product anomerizes in a slow base-catalyzed reaction through retro-anomerization of the oxide anion. A new trichloroacetonitrile addition by the more thermodynamically stable axial oxide anion provides the (α)-trichloroacetimidate (thermodynamic anomeric effect, Scheme 6).

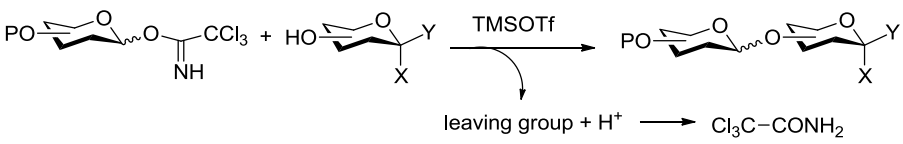
**Scheme 6. Thermodynamic and kinetic control in trichloroacetimidates synthesis**




The equilibrium between the two trichloroacetimidates can be speeded up by stronger base. Both *O*-activated anomers can be obtained in pure form and high yield using different bases. In particular, strong bases like NaH or DBU are appropriate for axial trichloroacetmidates while weaker bases such as $K_2CO_3$ are incapable of promoting the retro-anomerization mechanism.

Concerning the glycosylation step, reaction of donor and acceptor under very mild acidic conditions leads to the corresponding glycoside in an irreversible manner. In general, the reaction is promoted by a catalytic amount of a Lewis acid such as $BF_3{\cdot}OEt_2$ or TMSOTf (the most used). The proton liberated on the glycoside bond formation reacts with the forming leaving group leading to a stable, non-basic trichloroacetamide that provides the driving force of the glycosylation (Scheme 7).

**Scheme 7. Glycosylation with trichloroacetimidates**

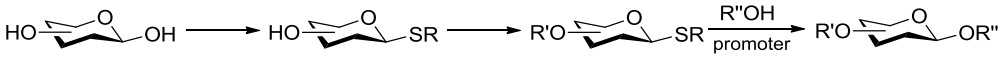


### 2.3.2 The thioglycoside method

There are several methods in which the anomeric carbon is activated by groups having sulphur in place of the exocyclic hemiacetal oxygen. The best known example of this type of protection/activation group is the alkyl(aryl)thio group (thioglycosides).

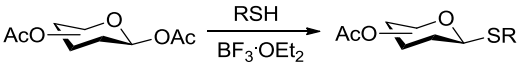
The sulphur atom in a thioglycoside is a soft nucleophile and is able to react selectively with soft electrophiles such as heavy metal cations, halogens and alkylating or acylating agents, making this kind of donors very versatile in carbohydrate chemistry. In addition, the hydroxyl and ring oxygen atom of carbohydrates are hard nucleophiles, which can be functionalized with strong reagents, without affecting alkyl(aryl)thio function (Scheme 8).

**Scheme 8. General synthesis and glycosylation**



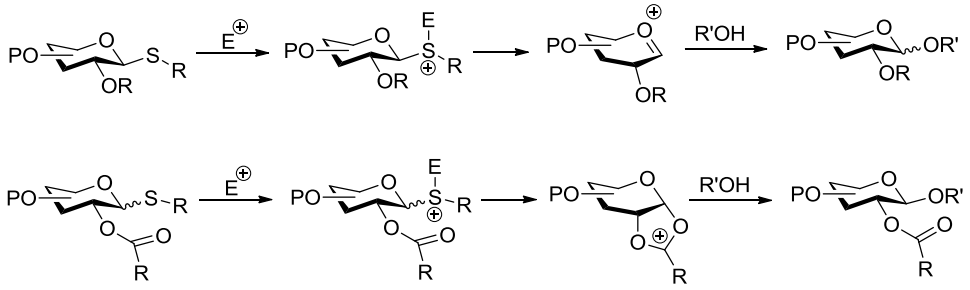

With regards to the synthesis of thioglycosides, they can be prepared by an acid-promoted displacement at the anomeric center. This implies the reaction between a peracetylated sugar and a thiol in the presence of a Lewis acid as promoter, in general $BF_3{\cdot}OEt_2$ (Scheme 9).

**Scheme 9. Thioglycosides preparation**




Concerning the glycosylation reaction, an electrophile activates the thioglycoside by producing intermediate sulfonium ion, which then gives rise to glycosylating carbocationic intermediates that can react with the alcohol or the glycosyl acceptor giving the glycoside (Scheme 10).

**Scheme 10. Activation and glycosylation of thioglycosides**




The first promoter used in a direct glycosylation involving thioglycosides was MeOTf: this promoter has disadvantages because it is toxic and, in the presence of slow reacting glycosyl donors, it can give rise to methyl ethers in addition to glycosides. For this reason, other thiophilic promoters have been developed. One of the most efficient promoter that produce fast reaction is the combination of *N*-iodosuccinimide and TfOH or TMSOTf.

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# 1 Flow Chemistry and Carbohydrates

Because of many advantages mentioned in the previous chapter (efficient mixing, good heat transfer, good control of reaction conditions, rapid screening of multiple reactions using a little amount of precious intermediates and simple scale-up), microreactors lend themselves particularly well for the accomplishment of organic transformations in which multiple factors determine the outcome of the reaction. In principle, the use microfluidic devices could speed up the saccharide drug discovery and development process, in order to adequately exploit the broad potential envisioned for this class of molecules in pharmaceutical and medicinal field. Nonetheless, only few applications of this technique to oligosaccharide synthesis have been reported in the literature, regarding both monosaccharide manipulations and glycosylation reactions.

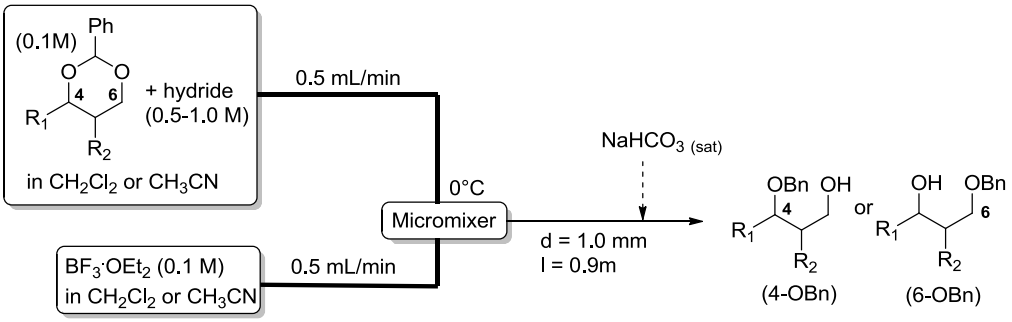
## 1.1 Protecting group manipulation

In 2009 Fukase and co-authors reported the use of a microfluidic device to perform the reductive opening of sugar 4,6-*O*-benzylidene acetals, a key functional group in carbohydrate chemistry.[1] Reductive opening of benzylidene acetals by the combination of acid/hydride reagents is one of the most useful transformation,[2] since the benzyl-protected derivatives at either the C4- or C6-hydroxyl can be selectively prepared by the choice of reagents and/or solvent systems.[3]

The typical procedure of the reductive opening of the acetals involves the very slow addition of the acid to a mixture of the substrate and excess hydride reagent at 0°C, followed by continuous stirring at room temperature for another few hours to ensure the completion of the reaction. To prevent the concomitant acid-catalyzed hydrolysis of the benzylidene ring it is important to control the addition speed of the acid (exothermic reaction). The yields of this reaction are not always reproducible, especially when it is performed on a large scale.

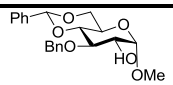
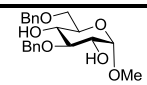
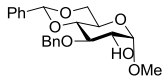
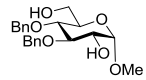
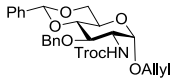
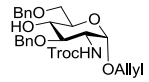
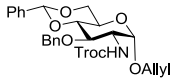
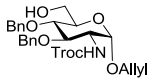
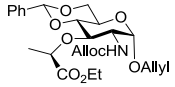
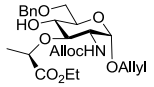
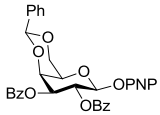
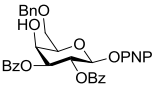
The authors decided to use a continuous flow microreactor to control the temperature, the mixing and the amount of hydride and acid (Figure 3).

**Figure 3. Schematic representation of reductive opening of benzylidene acetals under flow conditions**




Optimal conditions for reductive opening were examined using the glucose 4,6-*O*-benzylidene acetal (Table 2, Entry 1). Substrate (0.1 M) and hydride (1.0 M) dissolved in $CH_2Cl_2$ were mixed with $BF_3{\cdot}OEt_2$ in $CH_2Cl_2$ at 0°C at the flow rate of 0.5 mL/min, thereafter the reaction mixture was allowed to flow at room temperature for 90 seconds through a reactor tube and quenched by saturated $NaHCO_3$ solution at 0°C. Best reaction conditions were found using a solution of $BF_3{\cdot}OEt_2$ 1.0M, obtaining the desired product in almost quantitative yield. By applying these established conditions and using the appropriate hydride donor, glucose, glucosamine and galactose 4,6-*O*-benzylidene acetals were selectively transformed into the corresponding 6-O- or 4-O-benzyl derivatives (Table 2).

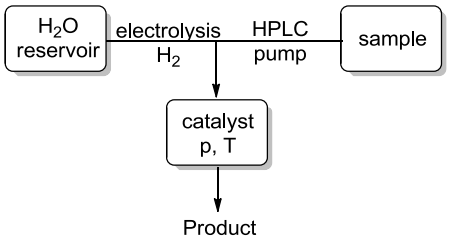
**Table 2. Results of reductive opening of benzylidene acetals under flow conditions**

| Entry | Substrate | Hydride | Solvent | Product | Yield (%, microfluidic) | Yield (%, batch) |
|---|---|---|---|---|---|---|
| **1** |  | $Et_3SiH$ (1.0 M) | $CH_2Cl_2$ |  | 93 | 58 |
| **2** |  | $BH_3{\cdot}Et_2NH$ (0.5 M) | $CH_2Cl_2$ |  | 100 | 90 |
| **3** |  | $Et_3SiH$ (1.0 M) | $CH_2Cl_2$ |  | 91 | 83 |
| **4** |  | $BH_3{\cdot}Et_2NH$ (0.5 M) | $CH_2Cl_2$ |  | 100 | 86 |
| **5** |  | $BH_3{\cdot}Et_2NH$ (0.5 M) | $CH_3CN$ |  | 100 | NA |
| **6** |  | $Et_3SiH$ (1.0 M) | $CH_2Cl_2$ |  | 91 | 62 |

Another application of flow chemistry to protecting group manipulation was explored by Leino group in 2011 in the deprotection of benzyl/benzylidene protected carbohydrates.[4] Benzyl ethers and benzylidene acetals are some of the most used protective group in carbohydrate chemistry, the main reasons being their stability toward a wide range of conditions and selective and mild removal conditions (mainly featuring hydrogenolyses). The commonly employed procedures to remove them utilizing batch reactors require long reaction times. The authors describes a rapid and efficient method of deprotection of different carbohydrates using a continuous flow hydrogenation reactor.

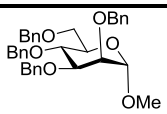
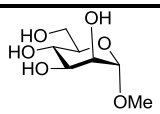
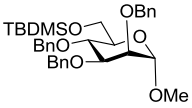
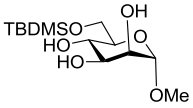
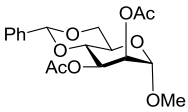
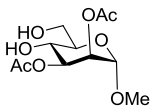
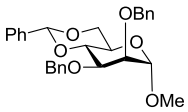
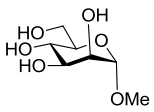
This consist of a $H_2O$-reservoir from which hydrogen gas is produced by electrolytic cleavage of water (this is a superior option for laboratory safety in comparison with classical batch reactor, in which hydrogen gas is taken from a bottle). The reaction was performed pumping the solution of the sample and the hydrogen gas through the catalyst (Pd/C) cartridge (Figure 4).

**Figure 4. Schematic representation of the continuous flow reactor**




The authors tested the flow conditions to different derivatives containing acid- and base labile protecting groups in order to ensure that the conditions would not influence other functional groups.
In all cases, they obtained the desired products in good yield (Table 3).

**Table 3. Results of the deprotection**

| Entry | Substrate | Product | Yield (%) |
|---|---|---|---|
| **1** |  |  | 95 |
| **2** |  |  | 95 |
| **3** |  |  | 95 |
| **4** |  |  | 90 |

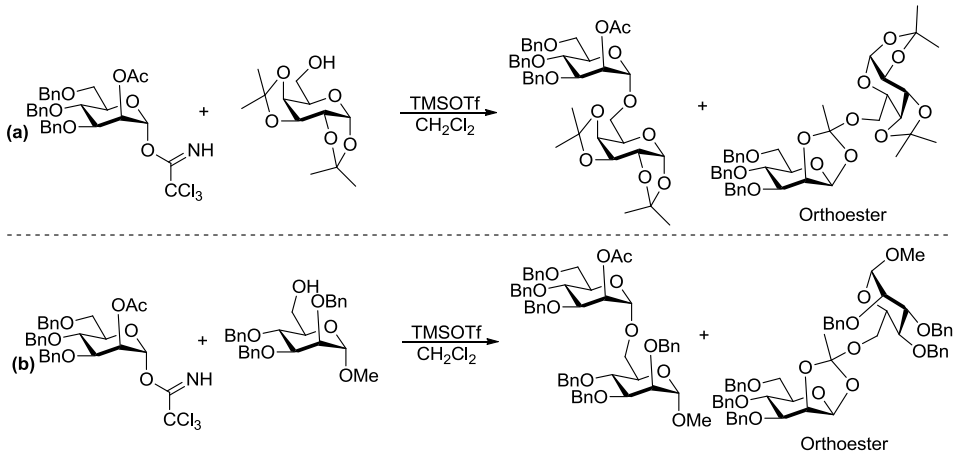
This method tolerates both acid- and base-sensitive functional groups (acetyl and silyl protective groups). The high efficiency, simple work-up and short reaction time should make this method appealing to a researcher working with carbohydrates.

## 1.2 Glycosylation reactions

The first example of glycosylation reaction performed in a microfluidic reactor was reported by Seeberger and co-authors in 2005.[5] The aim of the study was to find the best conditions in terms of temperature and reaction time to optimize the chemical yield and reduce the formation of side products. To perform the study they used a silicon microfluidic microreactor with an internal volume of 78.3 µL. The yield of the products were determined by HPLC using a UV-active compound (methyl 2,3,4,6-tetra-*O*-benzyl-α-D-mannopyranoside) as standard added at the end of the reaction in the quench solution.

The reactions studied involves the mannosyl trichloroacetimidate and two different acceptors (diisopropilidene galactose and methyl 2,3,4-tri-*O*-benzyl-α-D-mannopyranoside) shown in Scheme 11.

**Scheme 11. Glycosylation reactions studied by Seeberger**

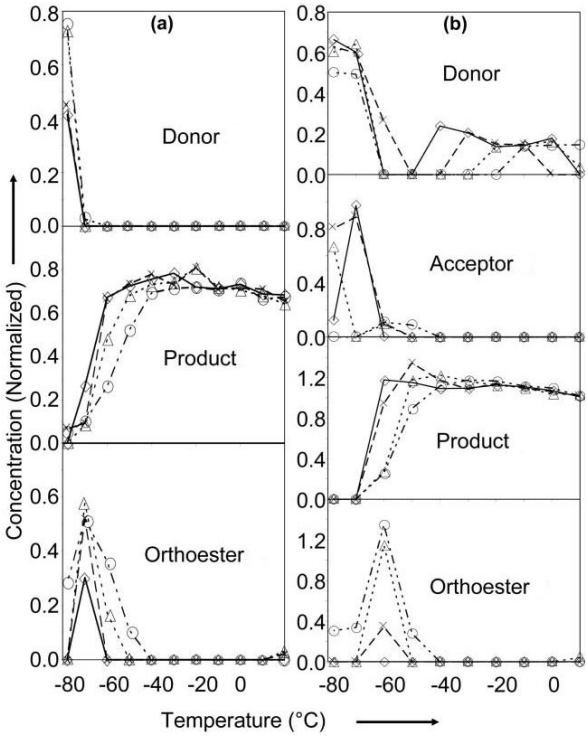



The reaction temperature was varied from -78 to 20°C using flow rates of 10, 20, 40 and 80 µL/min, that resulted in residence time of 26.7, 53.4, 106.8 and 213.5 seconds.

HPLC analysis of the crude samples showed a clear relationship between reaction temperature, reaction time and formation of products. The results

of the experiments are reported in Figure 5. In general, the yield of product increase with the temperature (for a given residence time) or with the residence time (at low temperature)

**Figure 5. Glycosylation results**

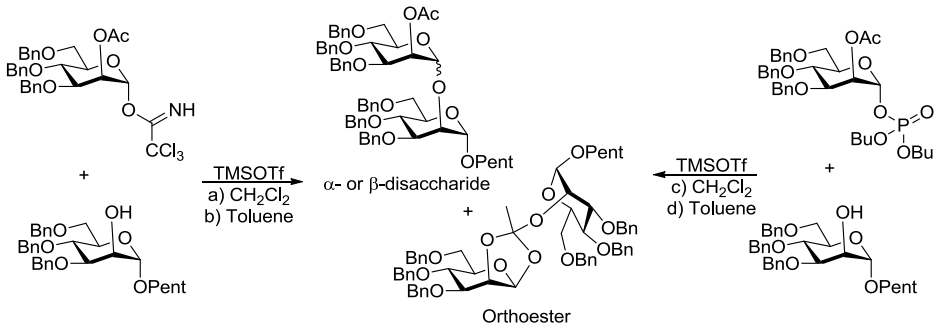



In this case the authors find that, unlike batch methods, the use of microreactor allow to obtain comprehensive information about a transformation (like the glycosylation reaction). They performed 44 reactions at varying temperature and reaction time using just over 2 mg of glycosylating agent for each reaction.

In 2007 the same group reported another study on glycosylation reactions performed in a microfluidic reactor.[6] In this case the authors used three mannose building blocks, pentenyl 3,4,6-tri-*O*-benzyl-α-D-mannopyranoside as acceptor and two different donors

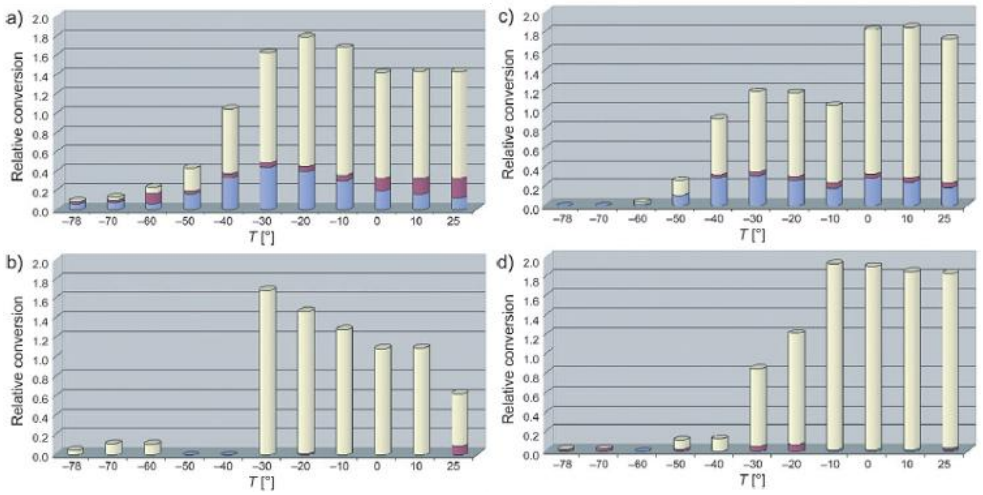
(trichloroacetimidate and phosphate, used for the first time in a microreactor, Scheme 12).

**Scheme 12. Glycosylations with trichloroacetimidate and phosphate**




The authors explored different reaction conditions, changing the flow rate, the solvent, the reagent quantities and the temperature. The conversion was determined by LC/MS analysis of the crude samples. The desired α-disaccharide was always the major product, although its distribution was highly dependent on reaction temperature and residence time; the formation of the orthoester was the main side reaction. The optimized reaction conditions for each glycosylating agent and solvent were determined and are reported in Figure 6.

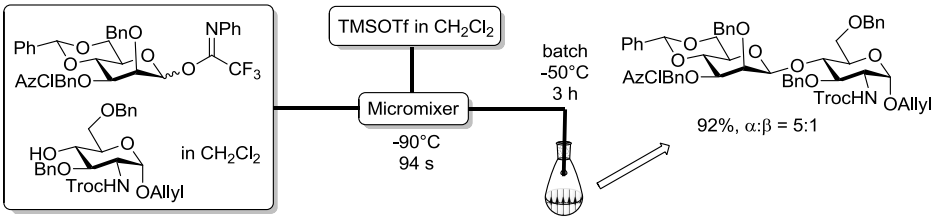
**Figure 6. Distribution for the optimized reaction conditions (yellow: α-disaccharide, red: β-disacharide, blue: orthoester)**




Also in this case the authors took advantages of the microreactor to compare different glycosylation parameters under various conditions, using only a little amount of starting materials.

The last example of the use of microfluidic reactors in oligosaccharide synthesis was reported in 2009 by Fukase group.[1] In this work the authors tried to perform β-mannosylation using a N-phenyl trifluoroacetimidate mannosyl donor. On milligram scale (20 mg) the reaction was carried out with good results in terms both of yield and stereoselectivity (84%, β:α 93:7); however, when the reaction was performed on 900 mg the yield decreases to 61% and the anomeric ratio decreases to β:α 4.9:1 (with an addition of TMSOTf in one portion). So the authors decided to examine the microfluidic conditions based on the observation that the success of the glycosylation depends on the fast addition and the efficient mixing with the Lewis acid to produce the active intermediate at low temperature. The experiment was performed with un apparatus composed by a micromixer and a second batch reactor (Figure 7).

**Figure 7. Glycosylation performer in microfluidic/batch system**




The optimal conditions in the integrated microfluidic/batch system were a micromixing at -90°C and a batch reaction at -50°C for 3 h: in these conditions the disaccharide was isolated in 92% yield and with an β:α ratio of 5:1. In addition, the authors performed a scale-up of the optimized conditions by simply preparing stock solutions of substrate and reagents, and then continuously pumping them into the integrated microfluidic/batch apparatus.

All these examples show that the microfluidic reactor technology is a really suitable technique to explore in detail the numerous experimental parameters determining the fate of a complex transformation such as the glycosylation reaction.

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[4] F. S. Ekholm, I. M. Mandity, F. Fulop, R. Leino *Tetrahedron Lett.* **2011**, 52, 1839
[5] D. M. Ratner, E. R. Murphy, M. Jhunjhunwala, D. A. Snyder, K. F. Jensen, P. H. Seeberger *Chem. Comm.* **2005**, 578
[6] K. Geyer, P. H. Seeberger *Helv. Chim. Acta* **2007**, 90, 395

# 3 Results and discussion

## 3.1 Paper

# Exploring Glycosylation Reactions under Continuous-Flow Conditions

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**Abstract.** Although carbohydrates offer new therapeutic opportunities in biomedical field, the industrial implementation of carbohydrate-based drugs is yet greatly thwarted by the difficulties and challenges inherent in oligosaccharide synthesis, especially for large scale preparation. Any tool or new technology enabling a cost-effective improvement of the lead generation process is therefore highly desirable in order to reduce the manufacturing costs of carbohydrate drugs. During last years, continuous-flow synthesis in microreactors has gained a great deal of attention featuring practical advantages such as high reproducibility, easy scalability and fast reaction optimization using small amounts of reagents or synthetic intermediates. This technique may therefore offer an effective support to make carbohydrates more attractive targets for drug discovery processes. In addition, also basic research in academia can benefit from microreactor technology as a tool to improve the organic synthesis of oligosaccharides. Here we report a systematic exploration of the glycosylation reaction, the most important and difficult transformation in oligosaccharide synthesis, carried out in microreactors under continuous-flow conditions. Various trichloroacetimidates and thioglycosides have been investigated as glycosyl donors in this study, using both primary and secondary glycosyl acceptors.

Each microfluidic glycosylation has been compared with the same reaction performed under traditional conditions, in order to highlight advantages and drawbacks of microreactors technology. As a significant example of multistep continuous-flow synthesis, we also describe the preparation of a trisaccharide by means of two consecutive glycosylations performed in two interconnected microreactors.

**Introduction**

Carbohydrates play a fundamental role in signal transduction and vital molecular recognition phenomena. They are primarily involved in inflammatory processes, bacterial and viral invasions, tumour growth and metastasis, and many other crucial biological events.[1] The diversity of roles played by carbohydrates makes them attractive targets for the development of novel drugs to treat cancer, infectious diseases, inflammation, and cardiovascular disorders.[2] However, there are still severe limitations to the development of saccharide-based drugs using traditional carbohydrate synthesis, even though some enormously successful pharmaceutical compounds based on synthetic oligosaccharides have been approved for the market during the past decade, such as the anticoagulant fondaparinux (Arixtra™) and the antiviral zanamivir (Relenza™).

Classical carbohydrate synthesis is a multistep process typically afflicted by laborious protecting groups manipulations, often requiring tedious work-up and time-consuming purification steps.[3] In addition the glycosylation, the most important transformation in the chemical synthesis of carbohydrates, is still a challenging task. Although multitudinous approaches for the stereoselective formation of glycosidic bonds have steadily emerged for the last two decades,[4] an efficient and general method to confidently prepare various glycoconjugates has yet been established, especially one that is applicable for large scale. As a consequence, the ideal reaction conditions (in terms of the protecting groups pattern of the condensing partners,

reagents concentration, stoichiometry, temperature and nature of the acidic promoter) have to be found, in theory, for each couple of glycosylation partners, leading to waste of time and high consumption of precious building blocks, which are valuable synthetic intermediates and they themselves require multistep synthesis. All of these drawbacks are inherent in carbohydrate synthesis and translate in huge hurdles for the industrial development of carbohydrate-based pharmaceutical compounds.

Continuous-flow organic synthesis performed in microreactors offers an attractive potential solution, as it should enable rapid early-stage reaction optimization and direct scale-up.[5] Due to the small dimensions and the increased surface to volume ratio of microreactors, mass and heat transport are significantly more efficient than in the classic round-bottomed flask. As a result, the reaction conditions in a continuous-flow microsystem are very homogenous, and variables such as temperature, pressure, concentration, and residence time (to be compared with the reaction time in a batch reaction) can be easily and precisely controlled, improving yield, purity and selectivity. In addition, the amount of starting material required to streamline a transformation of interest is dramatically reduced, so that a rapid screening of multiple reaction conditions is possible with a limited waste of precious intermediates. On the other hand, the scale-up of an optimized process can be achieved by continuous operation of the microreactor or running multiple reactors in parallel.

Because of many practical advantages mentioned above, the use of microfluidic reactors could speed up the saccharide drug discovery and development process, in order to adequately exploit the broad potential envisioned for this class of molecules in pharmaceutical and medicinal field. Nonetheless, only few applications of this technique to oligosaccharide synthesis have been reported in the literature.[6,5g]

With the purpose to gain more insights on the potential of microreactors in oligosaccharide synthesis, here we report a systematic study on the

glycosylation reaction performed under continuous-flow conditions using different glycosyl donors and acceptors. In particular, both primary and secondary acceptors (monosaccharides **1**-**3**, Figure 8) have been used in this study, while thioglycosides –employed for the first time in microreactors– and glycosyl trichloroacetimidates have been selected as glycosyl donors. Each glycosylation has been also carried out under traditional batch conditions, in order to compare the results both in terms of chemical yield and stereoselectivity.

Finally, we describe the straightforward preparation of a trisaccharide by means of two sequential microfluidic glycosylations, as a significant example of multistep synthesis that makes use of interconnected microreactors.

**Results and Discussion**

First, one primary (glucoside **1**, Figure 8) and two secondary acceptors (compound **2** and the more sterically hindered glucoside **3**, Figure 1) were synthesized from methyl α-D-glucopyranoside following known procedures.[7] Then, a panel of glycosyl donors differing for conformational rigidity and activating group at the anomeric carbon was synthesized according to literature procedures. In particular, the most popular and widespread classes of glycosyl donors, trichloroacetimidates and thioglycosides, were selected and employed in this study. Moreover, all the glycosyl donors do not have participating groups at C-2, so that a possible influence of the microfluidic conditions on the glycosylation stereoselectivity could emerge.

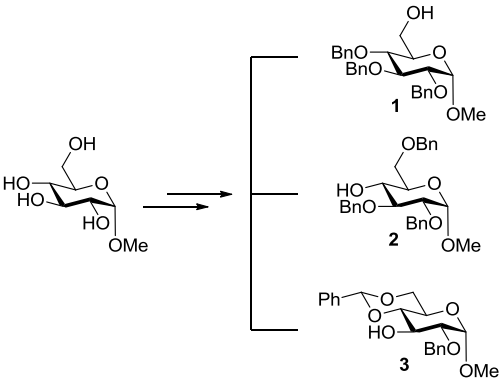



**Figure 8. Glucosyl acceptors employed in this study.**

Glucosyl trichloroacetimidates **4** and **5** (Figure 9) were prepared as previously described.[8] Ethyl- and *p*-tolyl- thioglycosides **6** and **7**, and their corresponding more conformationally rigid counterparts **8** and **9** (Figure 9) were synthesized from pentaacetylglucose.[9]

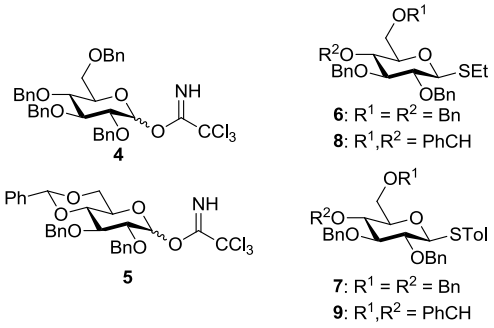



**Figure 9. Glucosyl donors employed in this study.**

All the batch glycosylations were carried out at room temperature in dry $CH_2Cl_2$ under inert atmosphere. Flow glycosylations were instead performed at room temperature pumping into a microreactor with an internal volume of 13 µl two distinct solutions in reagent grade $CH_2Cl_2$, containing the glycosyl donor and acceptor (1.2:1 ratio, Solution A) and the acidic promoter (0.01 M TMSOTf, Solution B). In the glycosylations performed with thioglycosyl donors, Solution A contained also the *N*-

iodosuccinimide (NIS) required for anomeric activation. A fine tuning of the hydrodynamic pumping (using a conventional double syringe pump) allowed to fix the flow rate, *i.e.* the residence time in the microreactor, corresponding to the reaction time. The device is completed by a reservoir connected to the outlet of the microreactor and containing a solution of triethylamine in $CH_2Cl_2$ to neutralize the promoter and quench the reaction (Figure 10).

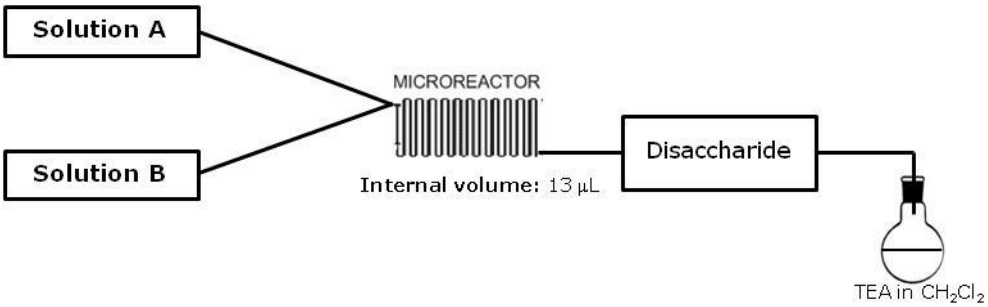
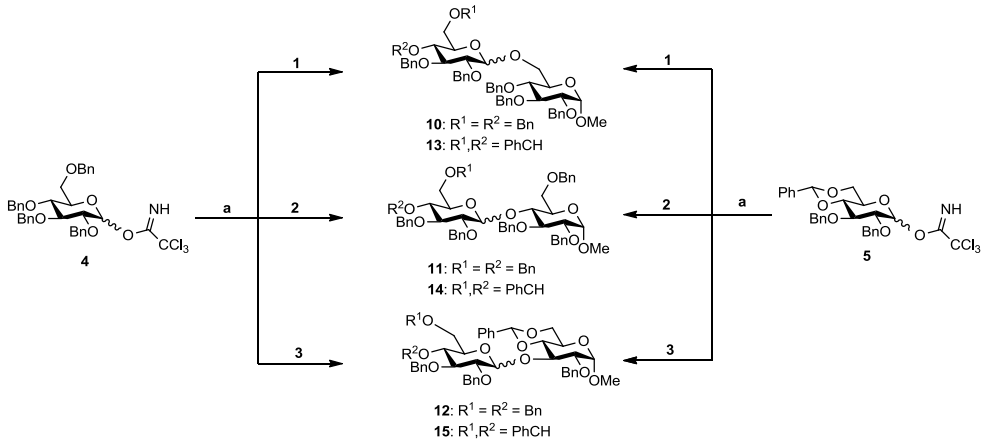



**Figure 10. Diagramatic representation of continous-flow glycosylations**

In the first set of experiments, the trichloroacetimidate donor **4** was reacted with acceptors **1**-**3** (Scheme 13). Initially, the reaction time of both batch and microfluidic reactions was set at 5 min.




**Scheme 13.** ***Reagents and reaction conditions*****: (a) 0.01 M TMSOTf, $CH_2Cl_2$**

Although the unusual reaction environment (use of reagent grade instead of dry solvent, room temperature) all the flow glycosylations afforded the corresponding disaccharides **10**-**12** in moderate to good yields, comparable or even higher than the batch reactions (Table 4, entries 1-2, 6-7, and 9-10). Interestingly, when the reaction time was further reduced to 1 min, a significant increase of the chemical yields was observed (Table 4, entries 3, 8, 11), while the stereoselectivity -in terms of α:β ratio- remained almost unchanged. The most sterically hindered acceptor **3** provided exclusively the α disaccharide **12** under all the conditions tested (Table 4, entries 9-11). In a separate experiment, we investigated the influence of the temperature on the course of the microfluidic glycosylation of acceptor **1** with donor **4** (Table 4, entries 4 and 5). However, going from room temperature to 0°C we observed only a slight increase of the chemical yield (and a decrease of the stereoselectivity), while at -20°C the precipitation of the trichloroacetamide caused the microchannels occlusion. All the following glycosylations were therefore performed at room temperature.

**Table 4. Glycosylations performed with trichloroacetimidate donors 4 and 5**

| entry | conditions | donor | acceptor | product | time (min) | yield (%)[a] | α:β[b] |
|---|---|---|---|---|---|---|---|
| **1** | batch | **4** | **1** | **10** | 5 | 66 | 2.4:1 |
| **2** | flow | **4** | **1** | **10** | 5 | 81 | 1.8:1 |
| **3** | flow | **4** | **1** | **10** | 1 | 95 | 1.6:1 |
| **4** | flow | **4** | **1** | **10** | 1[c] | 99 | 1:1 |
| **5** | flow | **4** | **1** | **10** | 1[d] | 26[e] | 1:1.8 |
| **6** | batch | **4** | **2** | **11** | 5 | 28 | 1:1.3 |
| **7** | flow | **4** | **2** | **11** | 5 | 50 | 1.3:1 |
| **8** | flow | **4** | **2** | **11** | 1 | 77 | 1.6:1 |
| **9** | batch | **4** | **3** | **12** | 5 | 58 | 1:0 |
| **10** | flow | **4** | **3** | **12** | 5 | 50 | 1:0 |
| **11** | flow | **4** | **3** | **12** | 1 | 85 | 1:0 |
| **12** | batch | **5** | **1** | **13** | 5 | 62 | 5:1 |
| **13** | flow | **5** | **1** | **13** | 1 | 92 | 3:1 |
| **14** | flow | **5** | **1** | **13** | 0.5 | 93 | 4:1 |
| **15** | batch | **5** | **2** | **14** | 5 | 71 | 6:1 |
| **16** | flow | **5** | **2** | **14** | 1 | 62 | 6.5:1 |
| **17** | flow | **5** | **2** | **14** | 0.5 | 65 | 6.5:1 |
| **18** | batch | **5** | **3** | **15** | 5 | 56 | 15:1 |
| **19** | flow | **5** | **3** | **15** | 1 | 60 | 9:1 |
| **20** | flow | **5** | **3** | **15** | 0.5 | 57 | 19:1 |

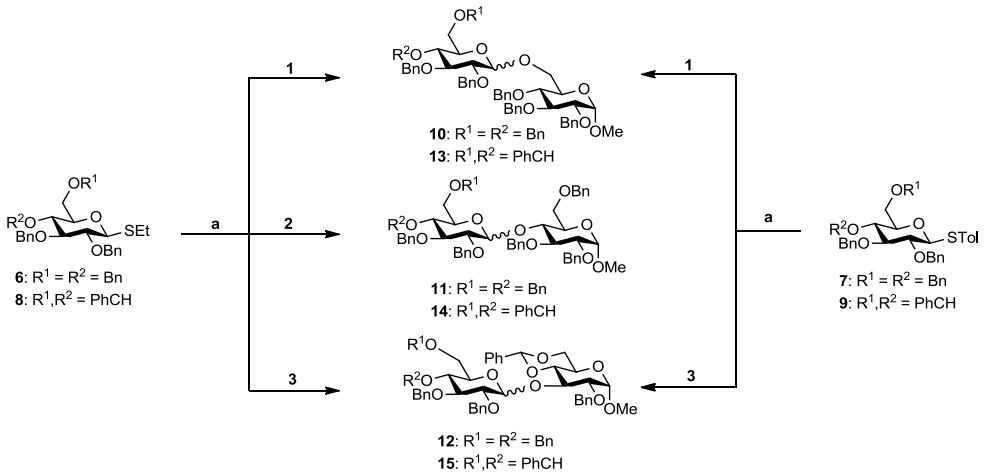
[a]Isolated yield; [b] Determined by $^{1}$H-NMR; [c]reaction performed at 0°C; [d]reaction performed at -20°C; [e]microchannels occlusion

Trichloroacetimidate **5** afforded very similar results to donor **4**, in terms of chemical yields (Scheme 13 and Table 4) and stereoselectivity. The only exception was again the glycosylation of acceptor **3**. This reaction showed high α stereoselectivity under batch conditions (15:1 α:β ratio), and reached an excellent 19:1 with 0.5 min reaction time in the microreactor (Table 4, entries 18 and 20, respectively). We deemed that the good α stereoselectivity achieved only with acceptor **3** should be ascribed to matching steric effects, rather than to a specific influence of the microfluidic

conditions. On the other hand, the reduction of the reaction time to 0.5 min did not bring any apparent advantage with acceptors **1** and **2** (Table 4, compare entries 13-14, and entries 16-17). We therefore considered 1 min as the optimal reaction time for our system, and this value was fixed in all the subsequent experiments.

In the next stage of our investigation we explored the behavior of thioglycosyl donors in microreactor. It should be emphasized that, to the best of our knowledge, this is the first example of use of thioglycosyl donors in glycosylations under continuous-flow conditions. The flow reactions were carried out by pumping in the microreactor a solution containing the donor, the acceptor and NIS, while a 0.01 M solution of TMSOTf was injected in the second inlet with a flow rate corresponding to 1 min reaction time. In this way, the activation of the glycosyl donor by $I^+$ takes place in the microreactor soon after mixing the two solutions.

Gratifyingly, glycosylations with thioglycosides **6**–**9** occurred smoothly at room temperature under microfluidic conditions, furnishing chemical yields and stereoselectivities absolutely comparable to batch reactions even if performed using reagent grade $CH_2Cl_2$ (Scheme 14 and Table 5).




**Scheme 14.** ***Reagents and reaction conditions*****: (a) NIS, 0.01 M TMSOTf, $CH_2Cl_2$.**

In particular, while flow glycosylations of acceptors **1** and **2** provided very similar or even higher yields than corresponding batch reactions (Table 5, entries 1–2, 3–4, 7–8, 9–10, 13–14, and 17–18), acceptor **3** afforded significantly lower yields (10-15% less) of disaccharides **12** and **15** under flow conditions with all thioglycosyl donors (Table 5, entries 5–6, 11–12, 15–16, and 19–20).

**Table 5. Glycosylations performed with thioglycosyl donors 6-9**

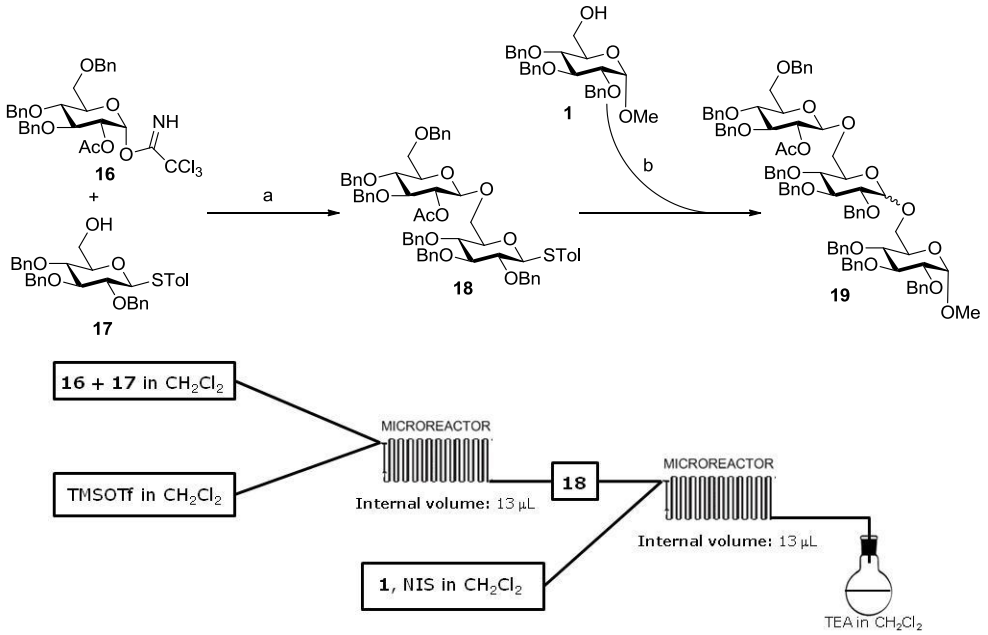
| entry | conditions | donor | acceptor | product | time (min) | yield (%)[a] | α:β[b] |
|---|---|---|---|---|---|---|---|
| **1** | batch | **6** | **1** | **10** | 5 | 71 | 1:1 |
| **2** | flow | **6** | **1** | **10** | 1 | 81 | 1.4:1 |
| **3** | batch | **6** | **2** | **11** | 5 | 69 | 2:1 |
| **4** | flow | **6** | **2** | **11** | 1 | 67 | 1.8:1 |
| **5** | batch | **6** | **3** | **12** | 5 | 90 | 3.5:1 |
| **6** | flow | **6** | **3** | **12** | 1 | 76 | 5:1 |
| **7** | batch | **8** | **1** | **13** | 5 | 80 | 4.5:1 |
| **8** | flow | **8** | **1** | **13** | 1 | 70 | 3.4:1 |
| **9** | batch | **8** | **2** | **14** | 5 | 55 | 4.5:1 |
| **10** | flow | **8** | **2** | **14** | 1 | 56 | 4.4:1 |
| **11** | batch | **8** | **3** | **15** | 5 | 88 | 2.6:1 |
| **12** | flow | **8** | **3** | **15** | 1 | 60 | 3:1 |
| **13** | batch | **7** | **1** | **10** | 5 | 68 | 1.6:1 |
| **14** | flow | **7** | **1** | **10** | 1 | 81 | 1.5:1 |
| **15** | batch | **7** | **3** | **12** | 5 | 86 | 3.9:1 |
| **16** | flow | **7** | **3** | **12** | 1 | 69 | 5:1 |
| **17** | batch | **9** | **1** | **13** | 5 | 65 | 4:1 |
| **18** | flow | **9** | **1** | **13** | 1 | 80 | 4:1 |
| **19** | batch | **9** | **3** | **15** | 5 | 88 | 2.9:1 |
| **20** | flow | **9** | **3** | **15** | 1 | 75 | 4.8:1 |

[a]Isolated yield; [b]Determined by $^1$H-NMR

Finally, we explored the possibility to accomplish two consecutive glycosylation reactions in two interconnected microreactors. This is a crucial issue to convincingly establish the microfluidic devices as valuable tools to speed up the synthesis of even complex oligosaccharides in order to expand the industrial development of saccharide-based drugs.[10]

Taking advantage of the well distinct mode of activation of trichloroacetimidates and thioglycosides, the experiment was designed and

carried out as follows (Scheme 15). A $CH_2Cl_2$ solution of 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate **16**[11] and acceptor **17** – obtained from thioglycoside **9** by reductive opening of the benzylidene acetal– and a 0.02 M $CH_2Cl_2$ solution of TMSOTf were pumped in the first microreactor, setting the reaction time at 2 min. The glycosylation occurred, then the solution containing the disaccharide thioglycoside **18** and TMSOTf flowed from the outlet of the microreactor and was directly conveyed to the inlet of the second microreactor, together with a $CH_2Cl_2$ solution of acceptor **1** and NIS. Eventually, the newly formed trisaccharide **19** was recovered at the outlet of the second microreactor after neutralization of TMSOTf with triethylamine. Chromatographic purification provided trisaccharide **19** in 51% overall yield. NMR analysis showed the exclusive formation of a β glycosidic bond in the first glycosylation (disaccharide **18**), demonstrating that anchimeric assistance of the neighboring acetyl ester in donor **16** is preserved under microfluidic conditions. On the other hand, disaccharide donor **18** afforded a mixture of anomers (estimated as 6.3:1 α:β ratio by NMR) at the second glycosidic linkage of trisaccharide **19**.




**Scheme 15.** ***Reagents and Reaction Conditions*****: (a) 0.02 M TMSOTf, $CH_2Cl_2$; (b) 0.05 M 1, NIS, $CH_2Cl_2$**

## Conclusions

We performed a systematic study to explore the application of microreactors technology in oligosaccharide synthesis. Two of the most widespread glycosyl donors, trichloroacetimidates and thioglycosides, were coupled with different glycosyl acceptors under batch and microfluidic conditions. We found that, in comparison with traditional batch reactions, the glycosylations carried out under continuous-flow conditions occur with very similar stereoselectivity and comparable or even higher chemical yield. However, microfluidic glycosylations have some practical advantages, such as the possibility to be performed in standard grade solvent at room temperature. Also thioglycosides, used for the first time in microreactors and which are notoriously highly sensitive to traces of moisture in the reaction mixture, afforded the corresponding disaccharides with high efficiency.

Further benefits of microreactors derive from their use for the multistep synthesis. Multistep flow reactions can indeed accelerate the development of efficient chemical processes for the discovery and production of saccharide-based lead compounds, enabling a drastic reduction of manufacturing costs. As a proof of concept, we synthesized a trisaccharide performing two consecutive glycosylations into interconnected microreactors. The large variety of glycosyl donors that can be activated in highly specific manner enables to design the multistep synthesis of even complex oligosaccharides profiting by the technical ease and flexibility of microreactors.

All these characteristics feature the microreactors as a promising technology for the industrial development of carbohydrate-based pharmaceutical compounds.

With the intent to explore drawbacks and potential of microfluidic devices in oligosaccharide synthesis, further efforts in this direction are currently underway in our laboratory.

## Experimental

**General procedures.** The reactions performed under microfluidic conditions were carried out in a glass microreactor with an internal volume of 13 μL purchased by Micronit Microfluidics®. All commercially available reagents including dry solvents were used as received. Nonvolatile materials were dried under high vacuum. Reactions were monitored by thin-layer chromatography on pre-coated Merck silica gel 60 F254 plates and visualized by staining with a solution of cerium sulfate (1g) and ammonium heptamolybdate tetrahydrate (27 g) in water (469 mL) and concentrated sulfuric acid (31 mL). Flash chromatography was performed on Fluka silica gel 60. NMR spectra were recorded at 300 K on spectrometer operating at 400 MHz. Proton chemical shifts are reported in

ppm (δ) with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard ($CDCl_3$ δ = 7.26 ppm). *J* values are given in Hz. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, δ = 77.0 ppm). High resolution mass spectra (HRMS) were performed with micro hybrid quadrupole time of flight (Q-Tof) Mass Spectrometer (Waters) with Electronspray Ionization (ESI) source and *MassLinx NT* software.

**Glycosylation with trichloroacetimidate donors under batch conditions (General procedure A)**

To a solution of the glycosyl acceptor (1 eq) and trichloroacetimidate (1.2 eq) in dry $CH_2Cl_2$ at room temperature and under Argon atmosphere, TMSOTf (0.1 eq) was added. After reaction completion (5 min), triethylamine was added to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Glycosylation with thioglycoside donors under batch conditions (General procedure B)**

To a solution of the glycosyl acceptor (1 eq), thioglycoside (1.2 eq) and *N*-iodosuccinimide (1.5 eq) in dry $CH_2Cl_2$ at room temperature and under Argon atmosphere, TMSOTf (0.1 eq) was added. After reaction completion (5 min), triethylamine was added to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Microfluidic glycosylation with trichloroacetimidate donors (General procedure C)**

A solution of the glycosyl acceptor (1 eq, 0.1 M) and trichloroacetimidate (1.2 eq, 0.12 M) in reagent grade $CH_2Cl_2$ (2 mL) was prepared (solution A).

A solution of TMSOTf (0.1 eq, 0.01 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in a separate flask (solution B). Equal volumes (0.5 mL) of the two solutions were taken and injected into the microreactor (internal volume = 13 µL) via a double syringe pump, setting the desired flow rate (corresponding to the reaction time, see Table 1). The mixture flowed from the microreactor was dropped in a $CH_2Cl_2$ solution of triethylamine to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Microfluidic glycosylation with thioglycoside donors (General procedure D)**

A solution of the glycosyl acceptor (1 eq, 0.1 M), thioglycoside (1.2 eq, 0.12 M) and *N*-iodosuccinimide (1.5 eq, 0.15 M) in reagent grade $CH_2Cl_2$ (2 mL) was prepared (solution A). A solution of TMSOTf (0.1 eq, 0.01 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in separate flask (solution B). Equal volumes (0.5 mL) of the two solutions were taken and injected into the microreactor (internal volume = 13 µL) via a double syringe pump, setting the desired flow rate (corresponding to the reaction time, see Table 2). The mixture flowed from the microreactor was dropped in a $CH_2Cl_2$ solution of triethylamine to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Two-steps synthesis of trisaccharide 19**

A solution of donor **16** (76 mg, 0.12 M) and thioglycoside acceptor **17** (56 mg, 0.1 M) in reagent grade $CH_2Cl_2$ (1 mL) was prepared (solution A, Scheme 3). A solution of TMSOTf (0.018 mL, 0.02 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in a separate flask (solution B, Scheme 3). Equal volumes (0.5 mL) of the two solutions were taken and injected into the first microreactor (internal volume = 13 µL) via a double syringe pump,

setting the flow rate at 3.25 μL/min (total flow rate = 6.5 μL/min, corresponding to a residence time = 2 min). The reaction mixture (containing the disaccharide donor **18**) flowed from the microreactor was pumped into the second microreactor together with a solution of acceptor **1** (47 mg, 0.05 M) and NIS (34 mg, 0.075 M) in reagent grade $CH_2Cl_2$ (2 mL). The flow rate of the second solution was set at 6.5 μL/min (total flow rate in the second microreactor = 13 μL/min), in order to have an overall residence time = 1 min. The reaction mixture flowed from the microreactor was eventually quenched in a $CH_2Cl_2$ solution of triethylamine The mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient), furnishing trisaccharide **19** (51%, mixture of anomers at the newly formed glycosidic bond) as a colourless oil. $\delta_H$ (400 MHz; $CDCl_3$) 7.61-7.05 (45H, m, $H_{Ar}$), 5.07 (t, 1H, t, $J_{2,3}$ 8.8, H2"), 5.03-4.48 (20H, m, $CH_2Ph$, H1', H1), 4.38 (1H, d, $J_{1,2}$ 8.0 Hz, H1"), 4.10-3.42 (17H, m, 2H6, 2H6', 2H6", H5, H5', H5", H4, H4', H4", H3, H3', H3", H2, H2'), 3.36 (3H, s, $OCH_3$-α), 3.31 (s, 3H, OCH3-β), 1.87 (s, 3H, $CH_3$-Ac); $\delta_C$ (100 MHz; $CDCl_3$) 128.4-127.4, 101.0, 98.0, 97.1, 78.1, 75.7, 75.4, 75.3, 75.0, 74.7, 73.4, 73.0, 72.3, 70.6, 69.8, 68.9, 67.8, 65.7, 55.1, 20.9; HRMS (Q-Tof) $[M+Na]^+$ *m/z* calc 1393.6076 for $C_{84}H_{90}O_{17}Na$, found 1393.6066.

Electronic Supplementary Information (ESI) available: Experimental procedures for the synthesis of compounds **1**-**9** and **16**-**18**, copies of $^1$H-NMR spectra of compounds **10**-**15** and $^1$H-NMR and $^{13}$C-NMR spectra of compounds **18** and **19**.

**Acknowledgements**

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# Exploring Glycosylation Reactions under Continous-Flow Conditions

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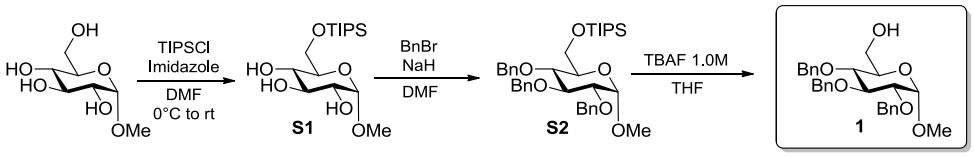
## Supplementary Informations

# Synthesis of glycosyl acceptors

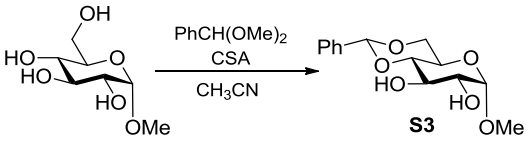
### Methyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside (1)




To a cooled solution (0°C) of methyl α-D-glucopyranoside (2.50 g, 12.88 mmol) and imidazole (2.63 g, 38.64 mmol) in DMF (20 mL), TIPSCl (3.03 mL, 14.17 mmol) was added dropwise over a period of 15 minutes. After 17 h at rt, the reaction mixture was concentrated *in vacuo*, then the crude was diluted with water (100 mL) and extracted with $CH_2Cl_2$ (3 x 60 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and dried under high vacuum. To a solution of crude **S1** and BnBr (7.60 mL, 64.40 mmol) in DMF (100 mL) NaH (1.46 g, 64.40 mmol) was slowly added. After 16 h at rt the reaction mixture was carefully quenched with MeOH (100 mL). The reaction mixture was concentrated *in vacuo*, then the crude was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 100 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, and concentrated *in vacuo*. To a solution of crude **S2** in dry THF (25 mL), TBAF 1.0M in THF (25.8 mL, 25.76 mmol) was added. The reaction mixture was stirred at rt for 16 h, diluted with $H_2O$, and extracted with $CH_2Cl_2$ (3x100 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 6:4) to obtain compound **1** as a white solid (4.85 g, 81%, over 3 steps).

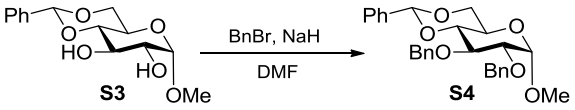
The spectroscopic data of acceptor **1** were in agreement with those previously reported.[1]

**Methyl 4,6-*O*-benzylidene-α-D-glucopyranoside (S3)**




To a solution of methyl α-D-glucopyranoside (5.00 g, 25.7 mmol) and $PhCH(OMe)_2$ (11.6 mL, 77.1 mmol) in $CH_3CN$ (85 mL), a catalytic amount of camphorsulfonic acid (pH = 2) was added. After 48 h the reaction was neutralized with TEA and concentrated *in vacuo*. The crude was purified by flash chromatography (Hexane:AcOEt 2:8) to obtain the **S3** (6.25 g, 86%).
The spectroscopic data of **S3** were in agreement with those previously reported.[2]

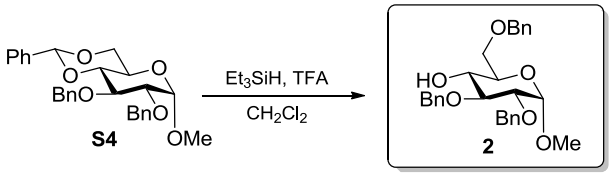
**Methyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (S4)**




To a solution of **S3** (2.00 g, 7.08 mmol) and BnBr (2.1 mL, 17.7 mmol) in DMF (45 mL), NaH (0.37 g, 15.6 mmol) was carefully added. After 1 h the reaction mixture was carefully quenched with MeOH (20 mL). The reaction mixture was concentrated *in vacuo*, then the crude was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 20 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 8:2) to obtain **S4** as a white solid (2.86 g, 87%).

The spectroscopic data of **S4** were in agreement with those previously reported.[2]

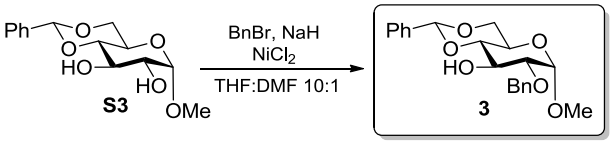
**Methyl 2,3,6-tri-*O*-benzyl-α-D-glucopyranoside (2)**




To a cooled solution (0°C) of **S4** (2.86 g, 6.18 mmol) and $Et_3SiH$ (4.93 mL, 30.9 mmol) in dry $CH_2Cl_2$ (40 mL), trifluoroacetic acid (2.35 mL, 30.9 mmol) was slowly added (15 min). After 3.5 h the mixture was diluted with $CH_2Cl_2$ and neutralized with a satd aqueous solution of $NaHCO_3$. The two phases were separated, and the organic layer was washed with a satd aqueous solution of $NaHCO_3$ and brine, dried over $Na_2SO_4$ and concentrated *in vacuo*. The crude was purified by flash chromatography (Hexane:EtOAc 8:2) to obtain compound **2** as a colourless oil (2.49 g, 87%).

The spectroscopic data of **2** were in agreement with those previously reported.[3]

**Methyl 2-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (3)**

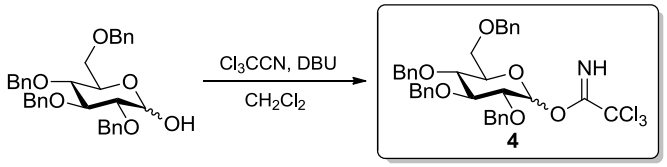



To a solution of **S3** (2.00 g, 7.08 mmol) in a mixture THF:DMF 10:1 (77 mL), NaH (0.34 g, 14.23 mmol) was added. After 1 h $NiCl_2$ (0.92 g, 7.08 mmol) was added. After 1 h BnBr (0.92 mL, 7.08 mmol) was added. The reaction mixture was allowed to react for 48 h, thereafter it was quenched by addition of $H_2O$ and 5 drops of AcOH. The solvents were removed *in vacuo*, and the crude residue was diluted with $H_2O$ and extracted with a $CH_2Cl_2$/$CHCl_3$ mixture (3 x 50 mL). The combined organic layers were washed with $H_2O$, a satd aqueous solution of $NaHCO_3$ and brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 8:2→7:3) to obtain compound **3** as a white solid (1.14 g, 43%).

The spectroscopic data of compound **3** were in agreement with those previously reported.[4]

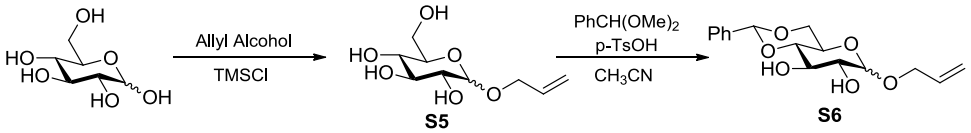
# Synthesis of glycosyl donors: Trichloroacetimidates

### 2,3,4,6-tetra-*O*-benzyl-D-glucopyranosyl trichloroacetimidate (4)




To a solution of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (1.00 g, 1.85 mmol) and $Cl_3CCN$ (1.85 mL, 18.5 mmol) in dry $CH_2Cl_2$ (20 mL), a catalytic amount of DBU was added. After 2 h the reaction mixture was concentrated *in vacuo* and purified by flash chromatography (Hexane:EtOAc 9:1→8:2 + 1% TEA) to obtain compound **4** as a white solid (1.22 g, 96%, α:β = 18:1). The spectroscopic data of donor **4** were in agreement with those previously reported.[5]

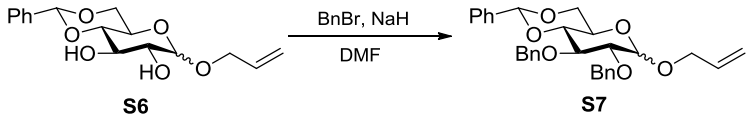
**Allyl 4,6-*O*-benzylidene-D-glucopyranoside (S6)**




To a solution of D-glucopyranose (1.00 g, 5.55 mmol) in allyl alcohol (35 mL), TMSCl (7.00 mL, 55.5 mmol) was added. After 12 h the allyl alcohol was removed *in vacuo*. The crude residue was diluted with toluene and concentrated *in vacuo* two times, and dried under high vacuum. To a solution of crude **S5** and $PhCH(OMe)_2$ (1.25 mL, 8.33 mmol) in $CH_3CN$ (35 mL), p-TsOH (0.11 g, 0.56 mmol) was added. After 5 h the reaction was neutralized with TEA and concentrated *in vacuo*. The crude residue was purified by flash chromatography (Hexane:EtOAc 1:1) to obtain **S6** as a colourless oil (1.44 g, 85%).

The spectroscopic data of **S6** were in agreement with those previously reported.[6]

**Allyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-D-glucopyranoside (S7)**

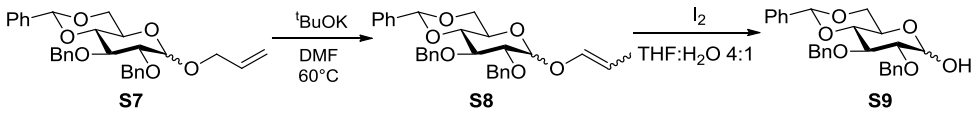



**S6** **S7**

To a solution of **S6** (1.44 g, 4.70 mmol) and BnBr (1.40 mL, 11.75 mmol) in DMF (40 mL), NaH (0.25 g, 10.34 mmol) was slowly added. After 3 h the reaction mixture was carefully quenched with MeOH (10 mL). The reaction mixture was concentrated *in vacuo*, then the crude residue was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 20 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 9:1) to obtain **S7** as a white solid (2.01 g, 87%).

The spectroscopic data of **S7** were in agreement with those previously reported.[7]

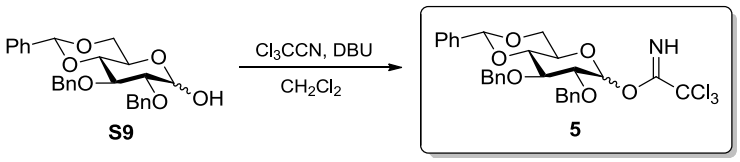
**2,3-di-*O*-Benzyl-4,6-*O*-benzylidene-D-glucopyranose (S9)**




To a solution of **S7** (1.00 g, 2.05 mmol) in DMF (20 mL), $^{t}$BuOK (0.46 g, 4.10 mmol) was added and the solution was warmed to 60°C. After 0.5 h the reaction mixture was cooled at room temperature and quenched by addition of 5% aqueous HCl, then the DMF was removed *in vacuo*. The crude residue was diluted with diethyl ether (30 mL) and washed with 5% aqueous HCl (20 mL). The aqueous layer was washed with diethyl ether (2 x 20 mL) and the combined organic layers were dried over $Na_2SO_4$, concentrated *in vacuo*, and dried under high vacuum. To a solution of crude **S8** in THF:$H_2O$ 4:1 (20 mL), $I_2$ (1.04 g, 4.10 mmol) was added. After 15 minutes, the reaction was quenched by addition of a satd aqueous solution of $Na_2S_2O_3$ (20 mL) and extracted with AcOEt (2 x 20 mL). The combined organic layers were dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 7:3) to obtain **S9** as a colourless oil (0.79 g, 86%).

The spectroscopic data of **S9** were in agreement with those previously reported.[8]

**2,3-di-*O*-Benzyl-4,6-*O*-benzylidene-D-glucopyranosyl trichloroacetimidate (5)**

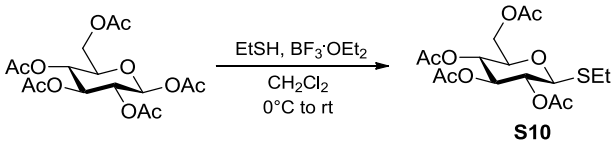



To a solution of **S9** (0.79 g, 1.77 mmol) and $Cl_3CCN$ (1.8 mL, 17.7 mmol) in dry $CH_2Cl_2$ (20 mL), a catalytic amount of DBU was added. After 0.5 h the reaction mixture was concentrated *in vacuo* and purified by flash chromatography (Hexane:EtOAc 9:1 + 1% TEA) to obtain compound **5** as a white solid (0.81 g, 77%, α:β = 5:1).

The spectroscopic data of donor **5** were in agreement with those previously reported.[9]

# Synthesis of glycosyl donors: Thioglycosides

**Ethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (S10)**

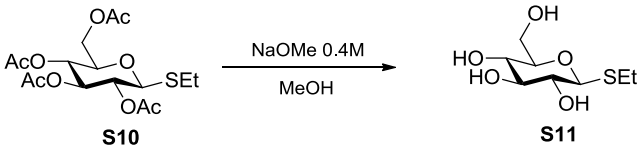



**S10**

To a cooled solution (0°C) of β-D-pentaacetylglucose (5.00 g, 12.85 mmol) and ethanethiol (4.73 mL, 64.05 mmol) in dry $CH_2Cl_2$ (80 mL), $BF_3{\cdot}OEt_2$ (2.44 mL, 19.22 mmol) was slowly added. After 20 h the reaction was neutralized with TEA and the solvent was removed *in vacuo*. The crude residue was purified by flash chromatography (Hexane:EtOAc 7:3) to obtain **S10** as a white solid (3.80 g, 76%).

The spectroscopic data of **S10** were in agreement with those previously reported.[10]

**Ethyl 1-thio-β-D-glucopyranoside (S11)**

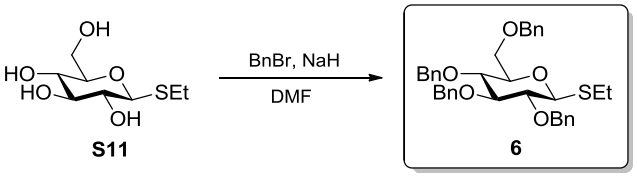



**S10** **S11**

To a solution of **S10** (3.80 g, 9.68 mmol) in dry MeOH (90 mL), NaOMe 0.4M in MeOH (3.63 mL, 1.45 mmol) was added. After 15 min the reaction was diluted with MeOH and neutralized with AMBERLITE® resin ($H^+$ form). The mixture was filtered and the solvent was removed *in vacuo*, obtaining **S11** as a white solid (2.17 g, qu).

The spectroscopic data of **S11** were in agreement with those previously reported.[11]

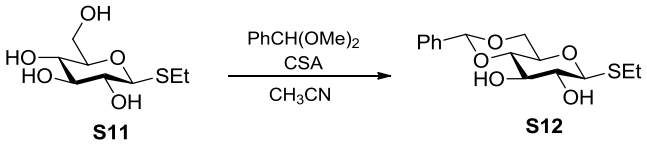
**Ethyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-glucopyranoside (6)**




To a solution of **S11** (0.59 g, 2.63 mmol) and BnBr (1.41 mL, 11.84 mmol) in DMF (17.5 mL), NaH (0.25 g, 11.05 mmol) was slowly added. After 6 h the reaction mixture was carefully quenched with MeOH (10 mL). The reaction mixture was concentrated *in vacuo*, then the crude residue was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 20 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 9:1) to obtain compound **6** as a white solid (0.90 g, 80%).

The spectroscopic data of **6** were in agreement with those previously reported.[12]

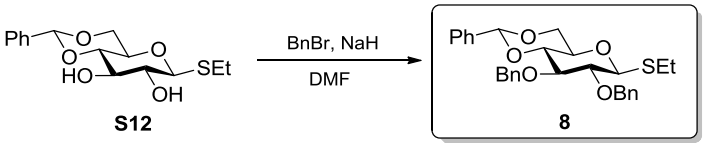
**Ethyl 4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (S12)**




To a solution of **S11** (1.14 g, 5.09 mmol) and $PhCH(OMe)_2$ (2.30 mL, 15.27 mmol) in $CH_3CN$ (35 mL), a catalytic amount of camphorsulfonic acid (pH = 2) was added. After 2 h the reaction was neutralized with TEA and concentrated *in vacuo*. The crude was purified by flash chromatography (Hexane:AcOEt 1:1) to obtain **S12** (1.20 g, 76%).

The spectroscopic data of **S12** were in agreement with those previously reported.[13]

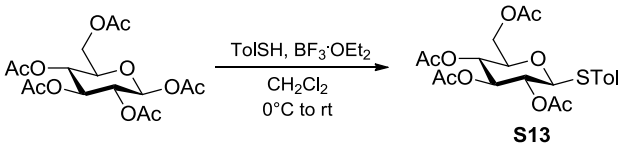
**Ethyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (8)**




To a solution of **S12** (0.60 g, 1.92 mmol) and BnBr (0.57 mL, 4.80 mmol) in DMF (20 mL), NaH (0.10 g, 4.22 mmol) was slowly added. After 1 h the reaction mixture was carefully quenched with MeOH (5 mL). The reaction mixture was concentrated *in vacuo*, then the crude residue was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 10 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 9:1) to obtain compound **8** as a white solid (0.76 g, 82%).

The spectroscopic data of **8** were in agreement with those previously reported.[14]

**4-Methylphenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside (S13)**

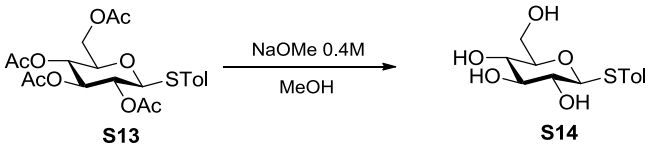


**S13**

To a cooled solution (0°C) of β-D-pentaacetylglucose (5.00 g, 12.85 mmol) and 4-methylbenzenethiol (7.90 g, 63.71 mmol) in dry $CH_2Cl_2$, $BF_3{\cdot}OEt_2$ (2.5 mL, 19.72 mmol) was slowly added. After 22 h the reaction was neutralized with TEA and the solvent was removed *in vacuo*. The crude residue was purified by flash chromatography (Hexane:EtOAc 7:3) to obtain **S13** as a white solid (5.29 g, 91%).

The spectroscopic data of **S13** were in agreement with those previously reported.[15]

**4-Methylphenyl 1-thio-β-D-glucopyranoside (S14)**

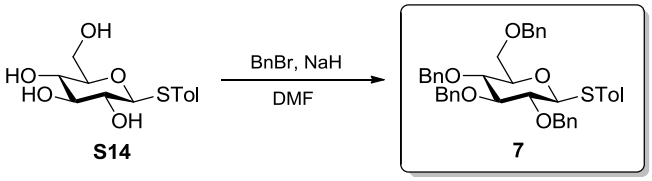



**S13** **S14**

To a solution of **S13** (4.00 g, 8.80 mmol) in dry MeOH (60 mL), NaOMe 0.4M in MeOH (3.30 mL, 1.32 mmol) was added. After 15 min the reaction mixture was diluted with MeOH and neutralized with AMBERLITE® resin ($H^+$ form). The mixture was filtered and the solvent was removed *in vacuo*, obtaining **S14** as a white solid (2.52 g, qu).

The spectroscopic data of **S14** were in agreement with those previously reported.[16]

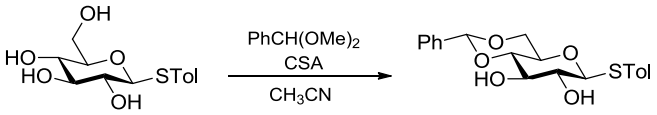
**4-Methylphenyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-glucopyranoside (7)**




To a solution of **S14** (1.35 g, 4.71 mmol) and BnBr (2.54 mL, 21.37 mmol) in DMF (47 mL), NaH (0.83 g, 19.94 mmol) was slowly added. After 15 h the reaction mixture was carefully quenched by addition of MeOH (10 mL). The reaction mixture was concentrated *in vacuo*, then the crude residue was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 20 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 9:1) to obtain compound **7** as a white solid (2.49 g, 82%).

The spectroscopic data of **7** were in agreement with those previously reported.[16]

**4-Methylphenyl 4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (S15)**

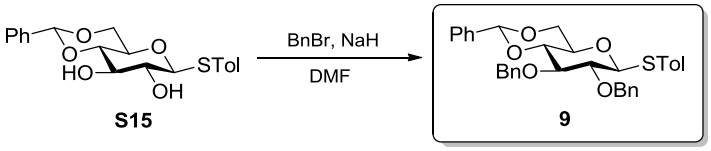



**S14** **S15**

To a solution of **S14** (1.19 g, 4.10 mmol) and $PhCH(OMe)_2$ (1.85 mL, 12.30 mmol) in $CH_3CN$ (30 mL), a catalytic amount of camphorsulfonic acid (pH = 2) was added. After 23 h the reaction mixture was neutralized with TEA and concentrated *in vacuo*. The crude residue was purified by flash chromatography (Hexane:AcOEt 1:1) to obtain **S15** (1.31 g, 85%).

The spectroscopic data of **S15** were in agreement with those previously reported.[17]

**4-Methylphenyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-1-thio-β-D-glucopyranoside (9)**




To a solution of **S15** (1.31 g, 3.49 mmol) and BnBr (1.04 mL, 8.72 mmol) in DMF (35 mL), NaH (0.18 g, 7.67 mmol) was slowly added. After 1 h the reaction mixture was carefully quenched by addition of MeOH (10 mL). The reaction mixture was concentrated *in vacuo*, then the crude residue was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 15 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo* and purified by flash chromatography (Hexane:EtOAc 9:1) to obtain thioglycoside **9** as a white solid (1.75 g, 95%).

The spectroscopic data of **9** were in agreement with those previously reported.[17]

## Glycosylation reactions

**Glycosylation with trichloroacetimidate donors under batch conditions (General procedure A)**

To a solution of the glycosyl acceptor (1 eq) and trichloroacetimidate (1.2 eq) in dry $CH_2Cl_2$ at room temperature and under Argon atmosphere, TMSOTf (0.1 eq) was added. After reaction completion (5 min), triethylamine was added to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Glycosylation with thioglycoside donors under batch conditions (General procedure B)**

To a solution of the glycosyl acceptor (1 eq), thioglycoside (1.2 eq) and *N*-iodosuccinimide (1.5 eq) in dry $CH_2Cl_2$ at room temperature and under Argon atmosphere, TMSOTf (0.1 eq) was added. After reaction completion (5 min), triethylamine was added to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Microfluidic glycosylation with trichloroacetimidate donors (General procedure C)**

A solution of the glycosyl acceptor (1 eq, 0.1 M) and trichloroacetimidate (1.2 eq, 0.12 M) in reagent grade $CH_2Cl_2$ (2 mL) was prepared (solution A). A solution of TMSOTf (0.1 eq, 0.01 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in a separate flask (solution B). Equal volumes (0.5 mL) of the two solutions were taken and injected into the microreactor (internal volume = 13 µL) via a double syringe pump, setting the desired flow rate (corresponding to the reaction time, see Table 1). The mixture flowed from the microreactor was dropped in a $CH_2Cl_2$ solution of triethylamine to

quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

**Microfluidic glycosylation with thioglycoside donors (General procedure D)**

A solution of the glycosyl acceptor (1 eq, 0.1 M), thioglycoside (1.2 eq, 0.12 M) and *N*-iodosuccinimide (1.5 eq, 0.15 M) in reagent grade $CH_2Cl_2$ (2 mL) was prepared (solution A). A solution of TMSOTf (0.1 eq, 0.01 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in separate flask (solution B). Equal volumes (0.5 mL) of the two solutions were taken and injected into the microreactor (internal volume = 13 µL) via a double syringe pump, setting the desired flow rate (corresponding to the reaction time, see Table 2). The mixture flowed from the microreactor was dropped in a $CH_2Cl_2$ solution of triethylamine to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt gradient).

## Glycosylations with Trichloroacetimidates

**Table 1, Entry 1**

Compounds **1** (50 mg, 0.108 mmol) and **4** (89 mg, 0.130 mmol) were treated as described in the *General Procedure A*. Product **10** was obtained in 66% yield (70 mg, 0.071 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

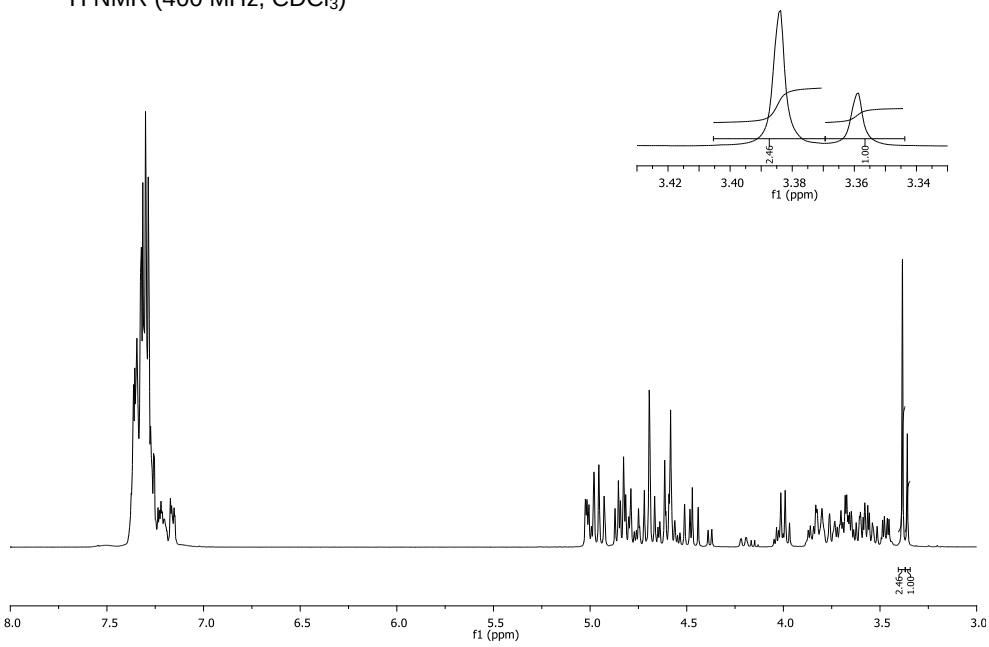


**Table 1, Entry 2**

Compounds **1** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 2.6 μL/min. Product **10** was obtained in 81% yield (40 mg, 0.041 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

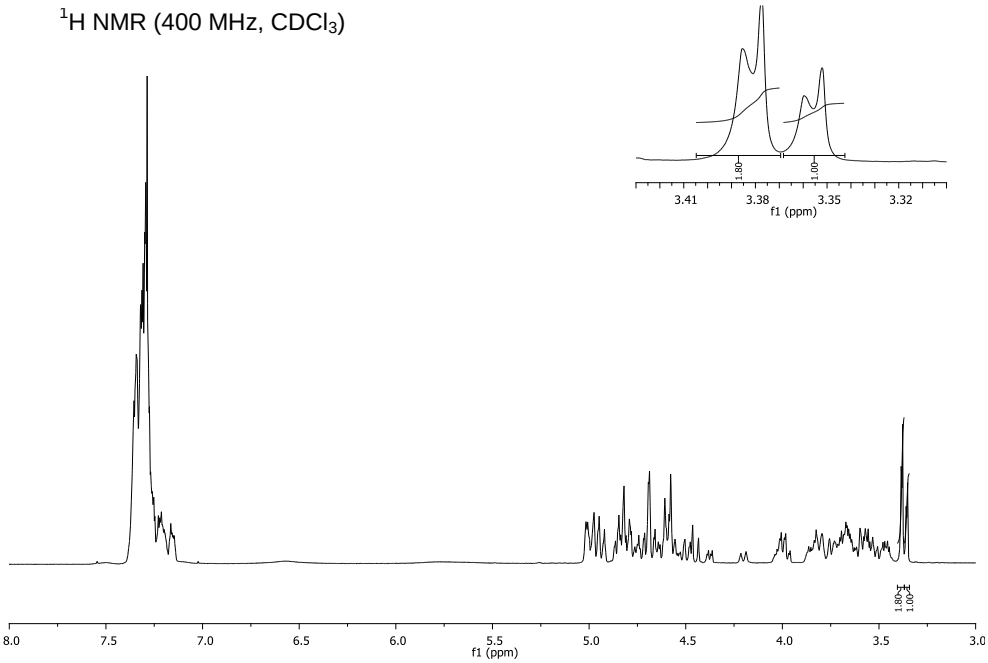


**Table 1, Entry 3**

Compounds **1** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min. Product **10** was obtained in 95% yield (47 mg, 0.048 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

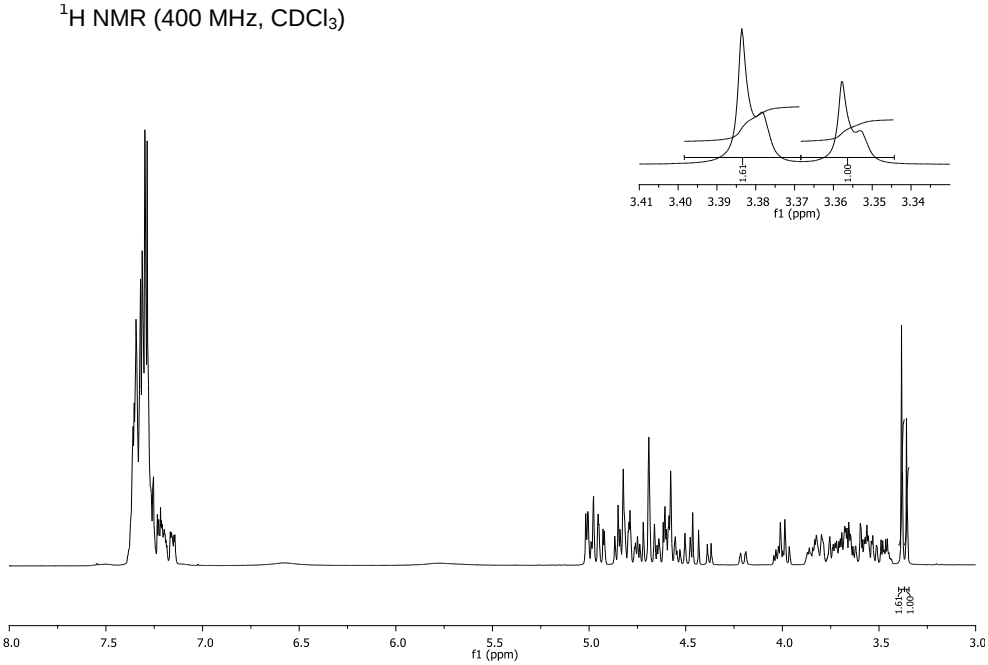


**Table 1, Entry 4**

Compounds **1** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min and the microreactor was putted in a cooled bath (0°C). Product **10** was obtained in 99% yield (49 mg, 0.049 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

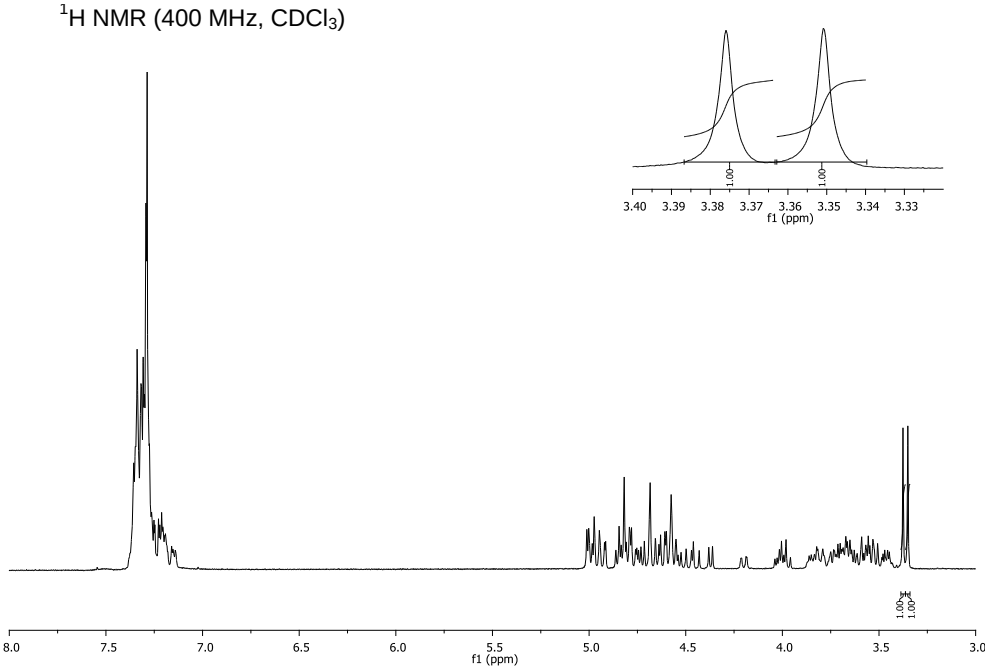


**Table 1, Entry 5**

Compounds **1** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min and the microreactor was cooled at -20°C in a cooling bath. Product **10** was obtained in 26% yield (13 mg, 0.013 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

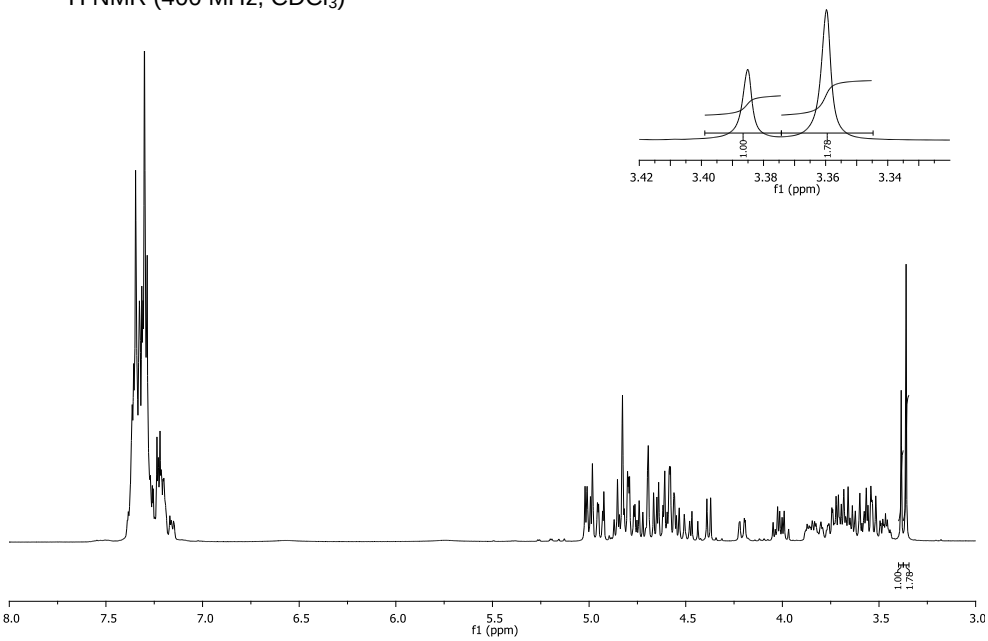


**Table 1, Entry 6**

Compounds **2** (50 mg, 0.108 mmol) and **4** (89 mg, 0.130 mmol) were treated as described in the *General Procedure A*. Product **11** was obtained in 28% yield (30 mg, 0.030 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

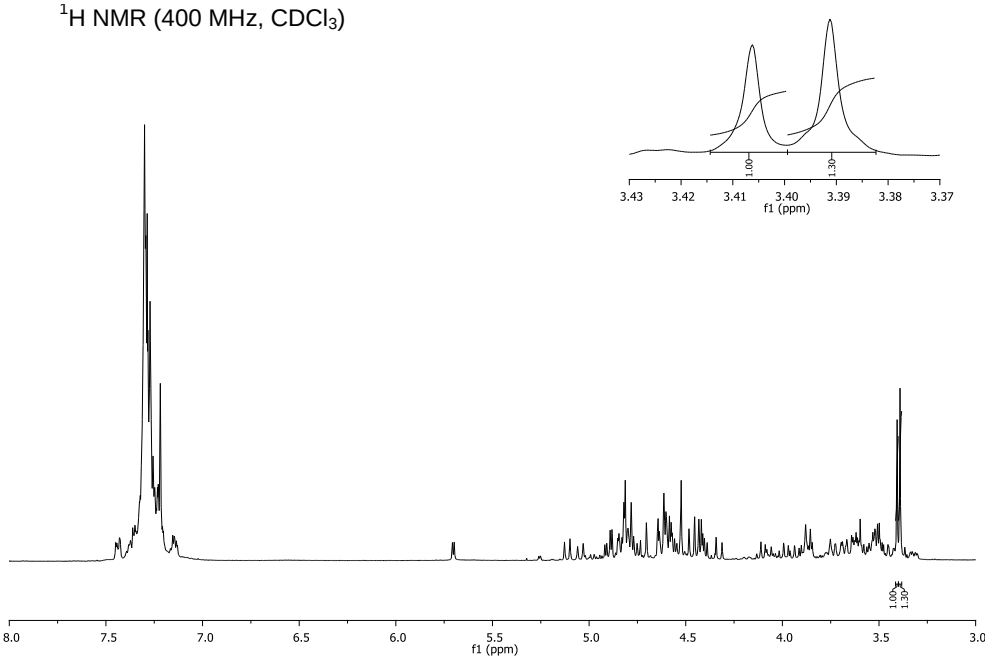


**Table 1, Entry 7**

Compounds **2** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 2.6 μL/min. Product **11** was obtained in 50% yield (25 mg, 0.025 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

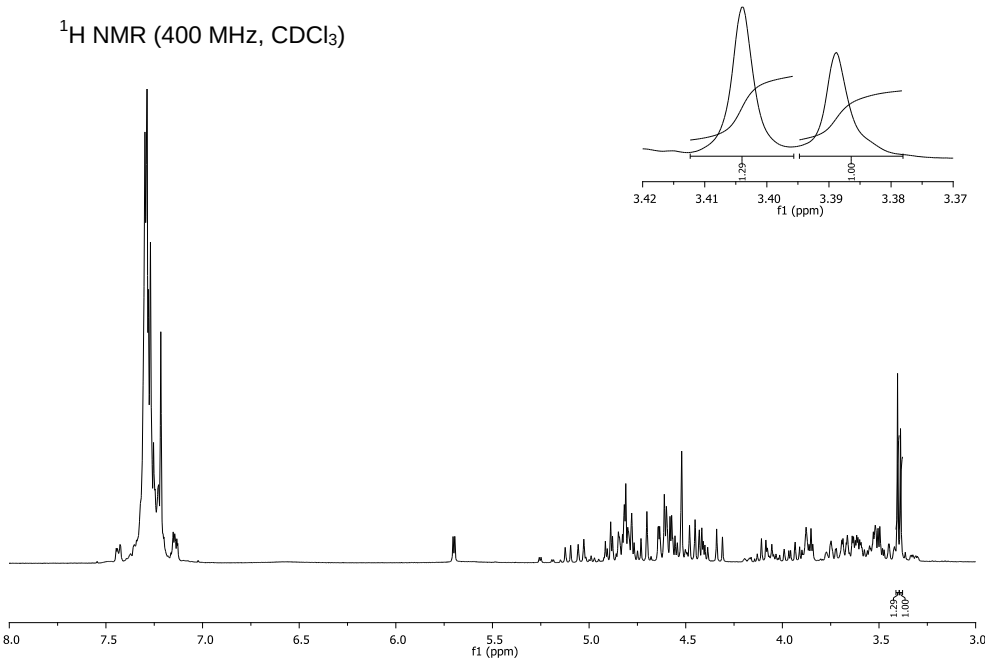


**Table 1, Entry 8**

Compounds **2** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min. Product **11** was obtained in 77% yield (38 mg, 0.039 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

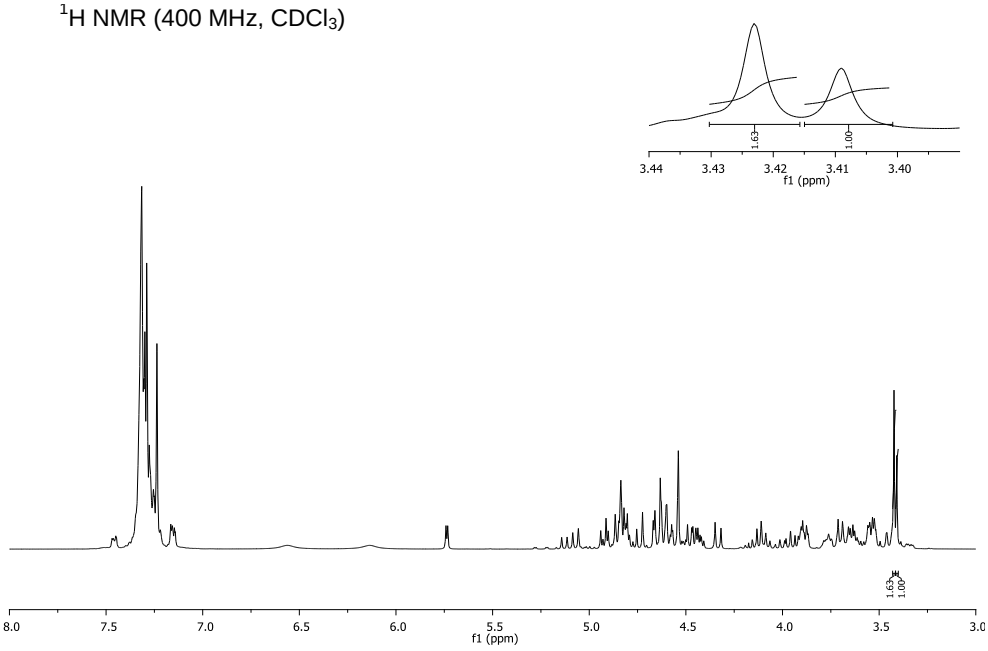


**Table 1, Entry 9**

Compounds **3** (50 mg, 0.134 mmol) and **4** (110 mg, 0.161 mmol) were treated as described in the *General Procedure A*. Product **12** was obtained in 58% yield (70 mg, 0.078 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

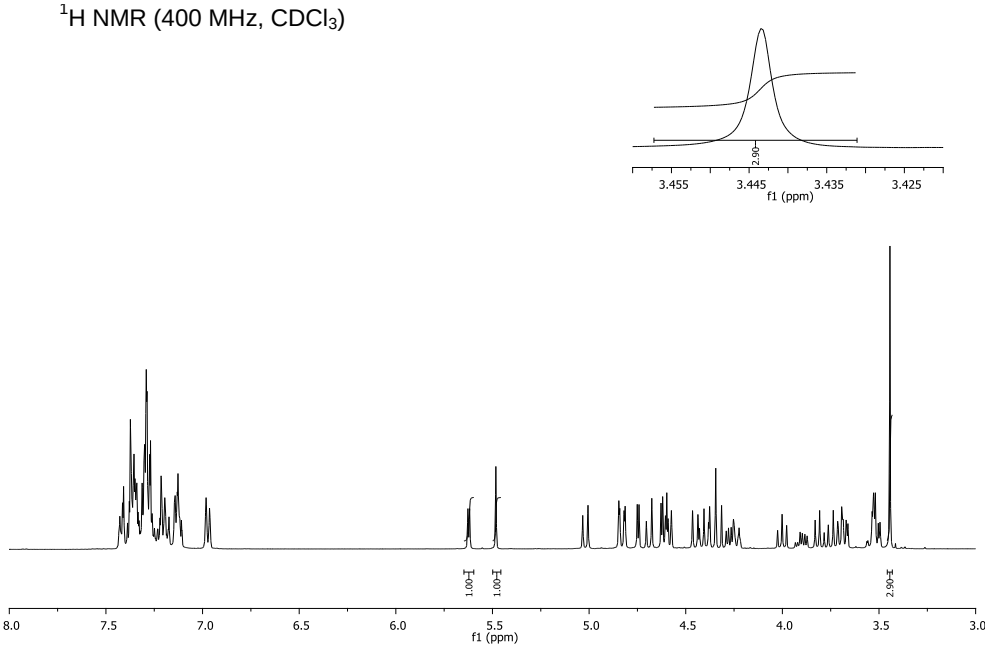


**Table 1, Entry 10**

Compounds **3** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 2.6 μL/min. Product **12** was obtained in 50% yield (22 mg, 0.025 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

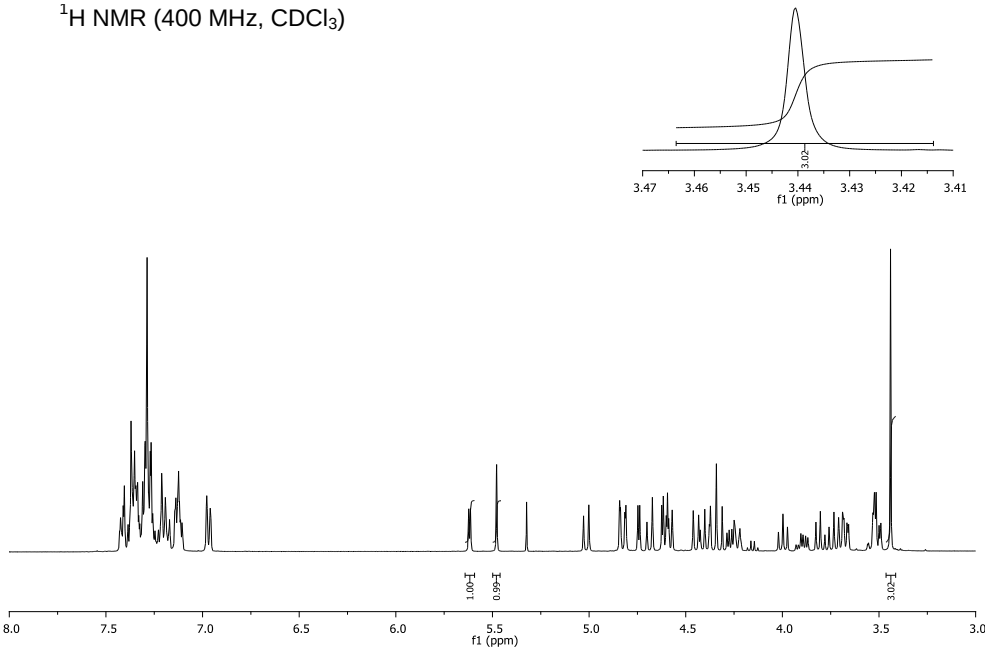


**Table 1, Entry 11**

Compounds **3** and **4** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min. Product **12** was obtained in 85% yield (38 mg, 0.043 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

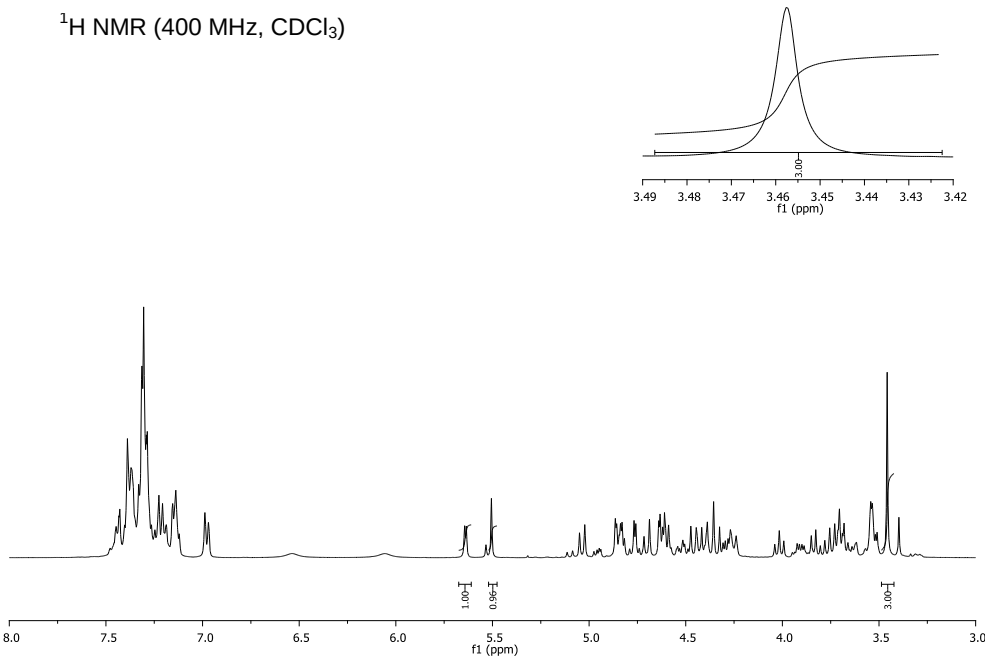


**Table 1, Entry 12**

Compounds **1** (50 mg, 0.108 mmol) and **5** (77 mg, 0.130 mmol) were treated as described in the *General Procedure A*. Product **13** was obtained in 62% yield (60 mg, 0.067 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

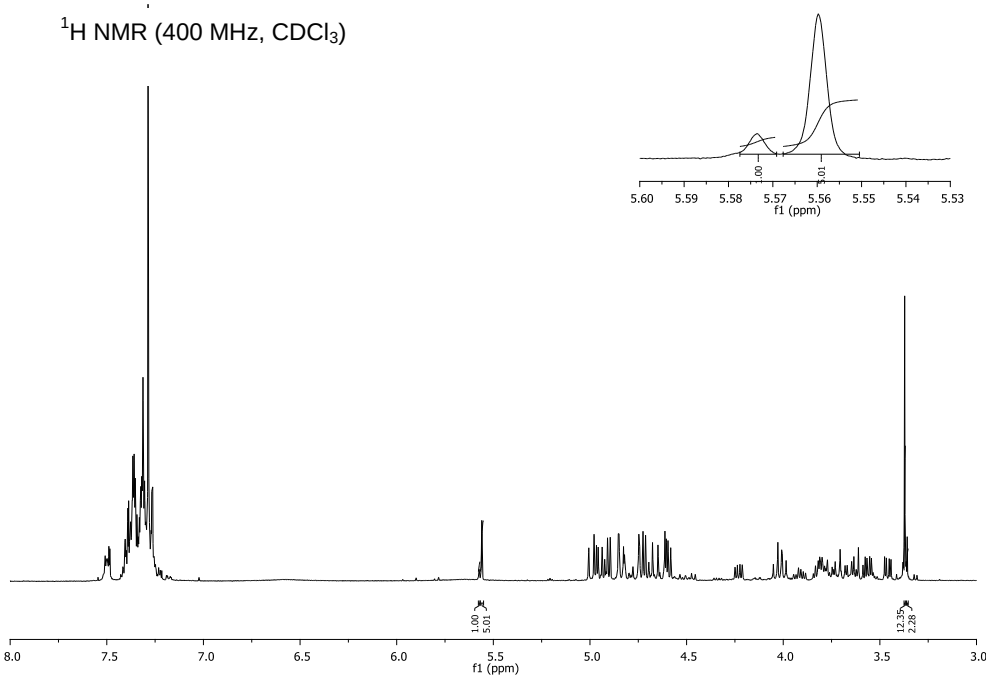


**Table 1, Entry 13**

Compounds **1** and **5** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min. Product **13** was obtained in 92% yield (41 mg, 0.046 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

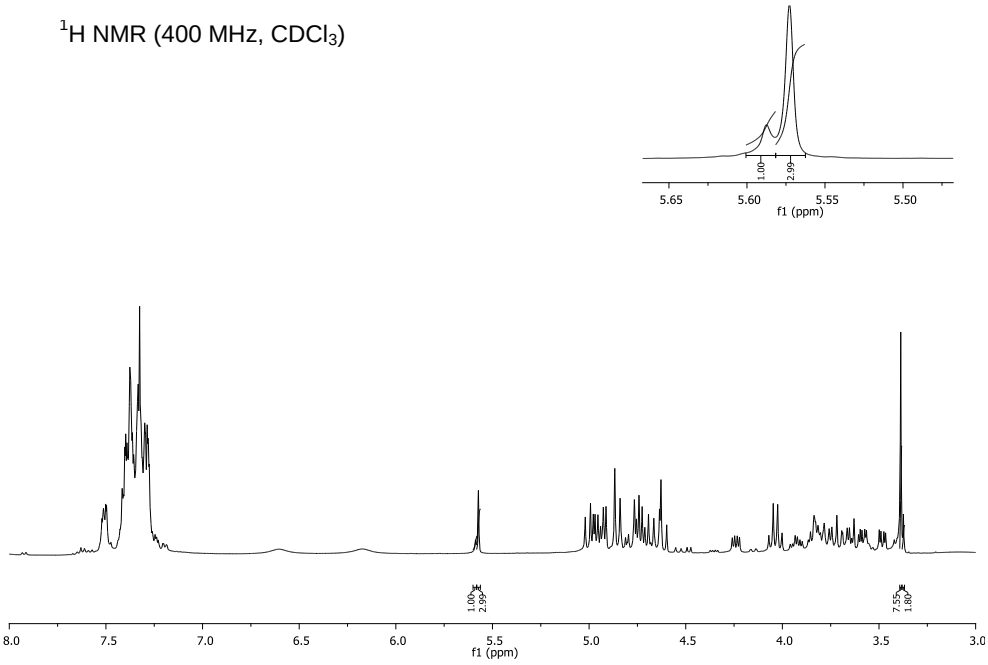


**Table 1, Entry 14**

Compounds **1** and **5** were treated as described in the *General Procedure C*. The flow rate was set to 26 μL/min. Product **13** was obtained in 93% yield (42 mg, 0.047 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

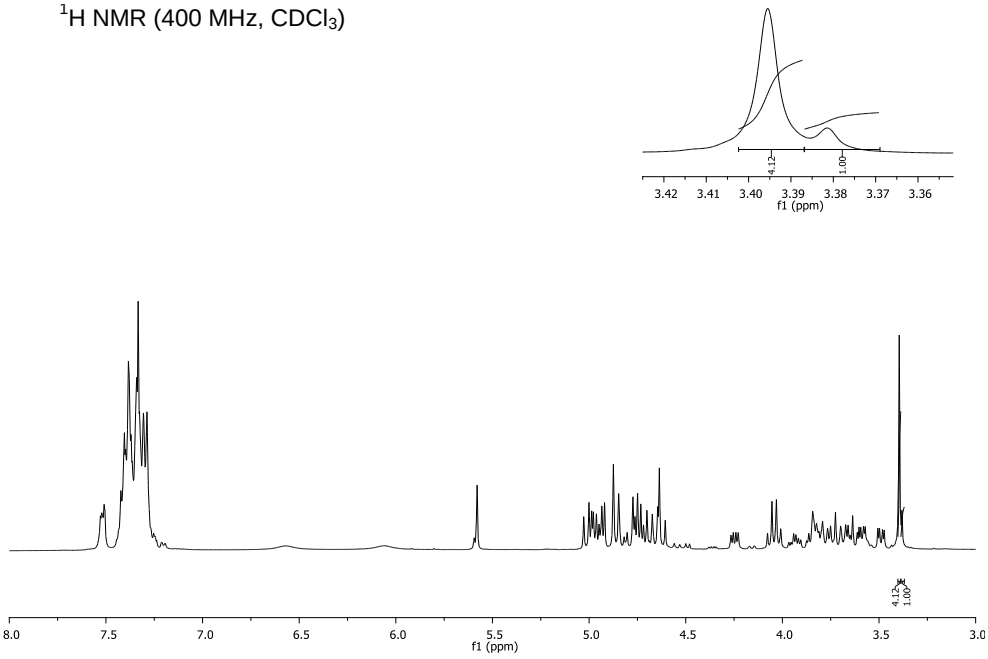


**Table 1, Entry 15**

Compounds **2** (50 mg, 0.108 mmol) and **5** (77 mg, 0.130 mmol) were treated as described in the *General Procedure A*. Product **14** was obtained in 71% yield (69 mg, 0.077 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

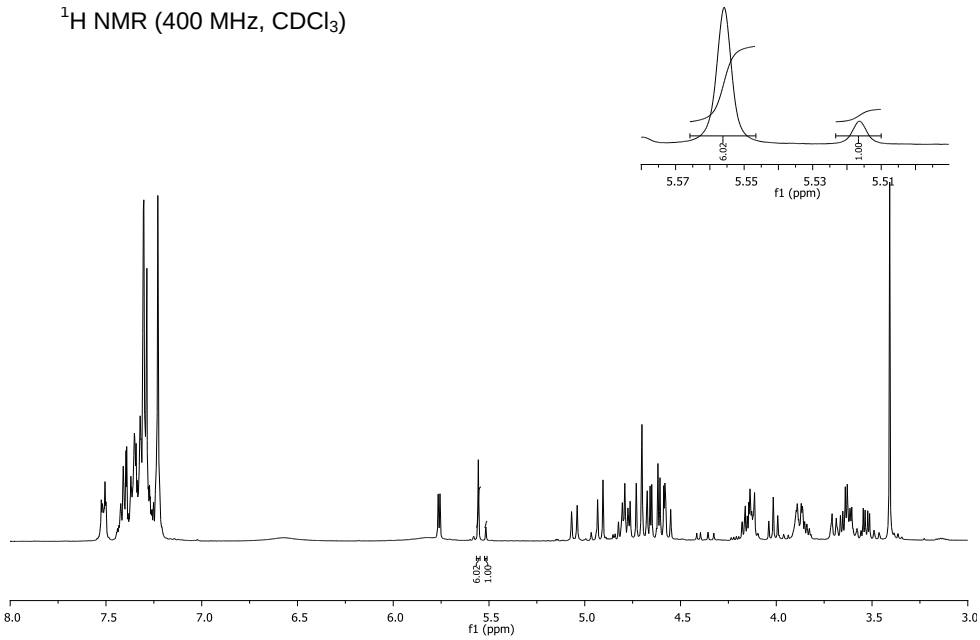


**Table 1, Entry 16**

Compounds **2** and **5** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min. Product **14** was obtained in 62% yield (28 mg, 0.031 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

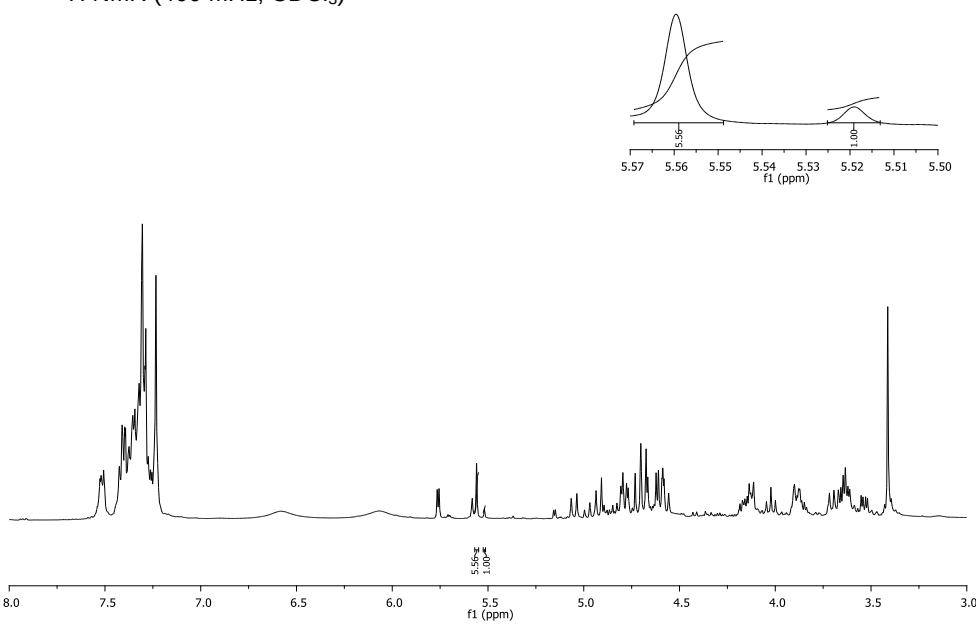


**Table 1, Entry 17**

Compounds **2** and **5** were treated as described in the *General Procedure C*. The flow rate was set to 26 μL/min. Product **14** was obtained in 65% yield (29 mg, 0.033 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

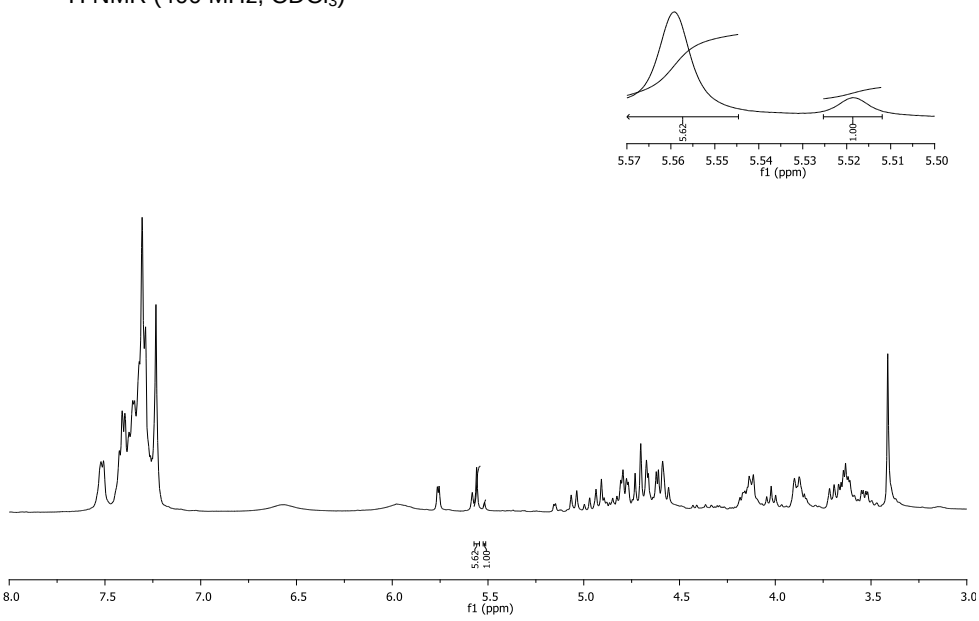


**Table 1, Entry 18**

Compounds **3** (50 mg, 0.134 mmol) and **5** (95 mg, 0.161 mmol) were treated as described in the *General Procedure A*. Product **15** was obtained in 56% yield (60 mg, 0.075 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)

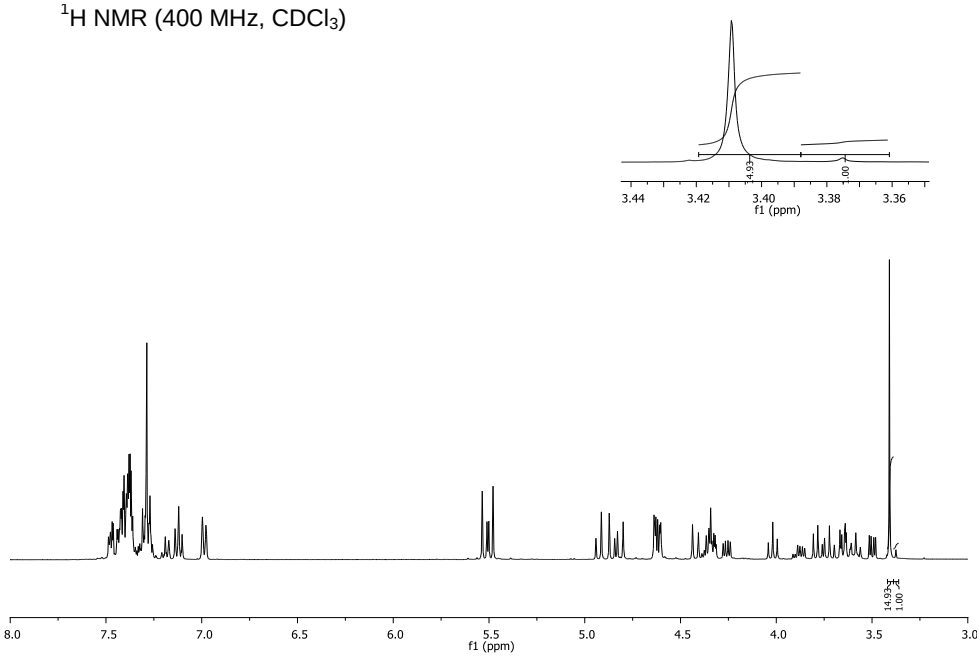
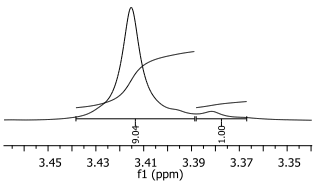


**Table 1, Entry 19**

Compounds **3** and **5** were treated as described in the *General Procedure C*. The flow rate was set to 13 μL/min. Product **15** was obtained in 60% yield (24 mg, 0.030 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)



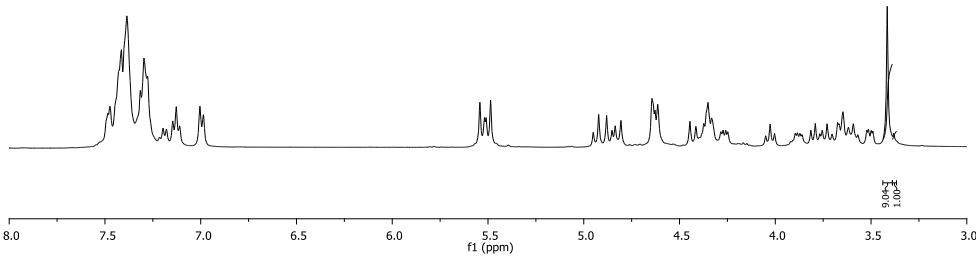
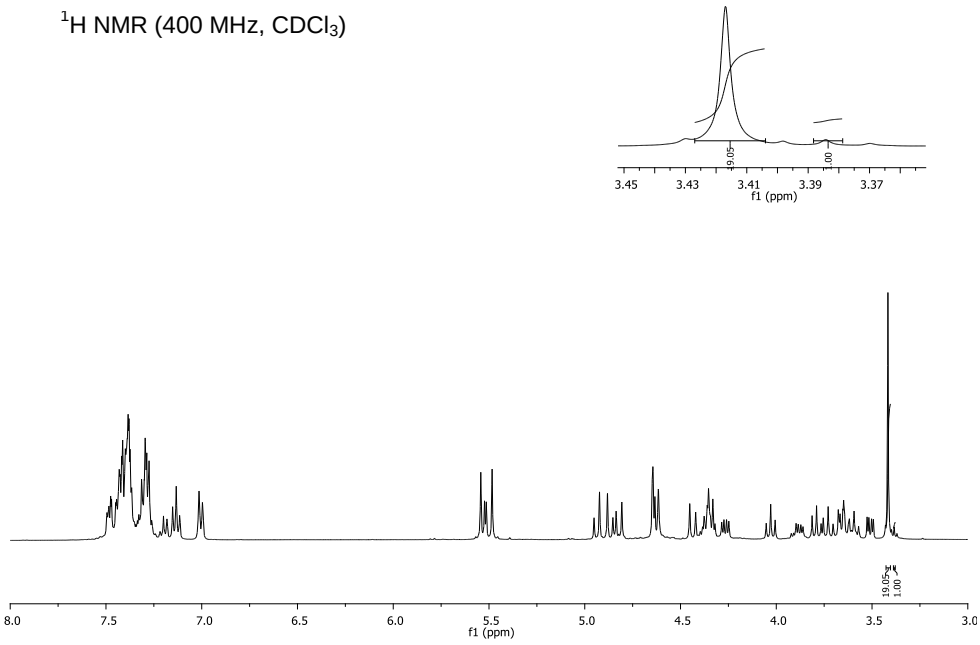



**Table 1, Entry 20**

Compounds **3** and **5** were treated as described in the *General Procedure C*. The flow rate was set to 26 μL/min. Product **15** was obtained in 57% yield (23 mg, 0.029 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)



## Glycosylations with Thioglycosides

**Table 2, Entry 1**

Compounds **1** (50 mg, 0.108 mmol) and **6** (76 mg, 0.130 mmol) were treated as described in the *General Procedure B*. Product **10** was obtained in 71% yield (76 mg, 0.077 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

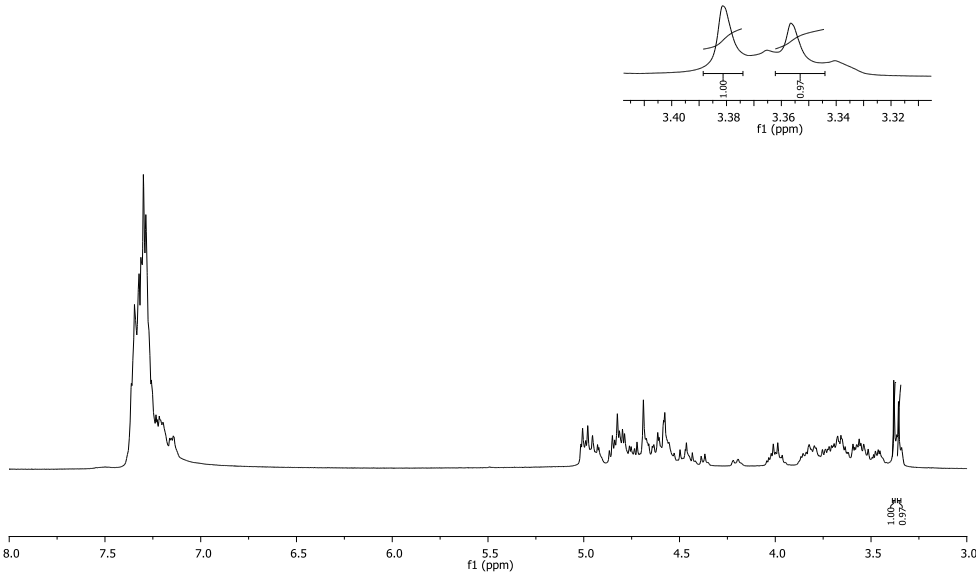


**Table 2, Entry 2**

Compounds **1** and **6** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **10** was obtained in 81% yield (40 mg, 0.041 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

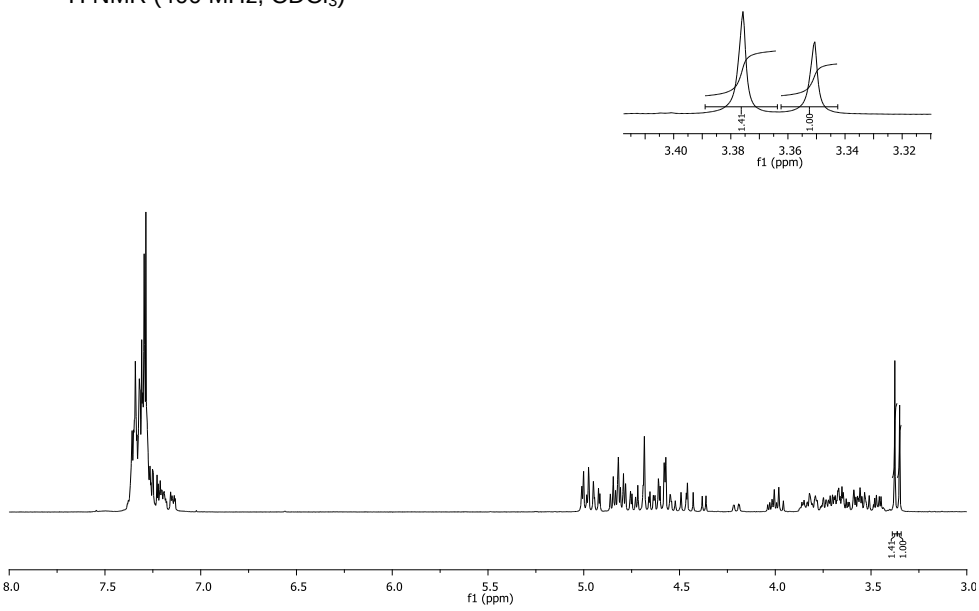


**Table 2, Entry 3**

Compounds **2** (50 mg, 0.108 mmol) and **6** (76 mg, 0.130 mmol) were treated as described in the *General Procedure B*. Product **11** was obtained in 69% yield (74 mg, 0.075 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

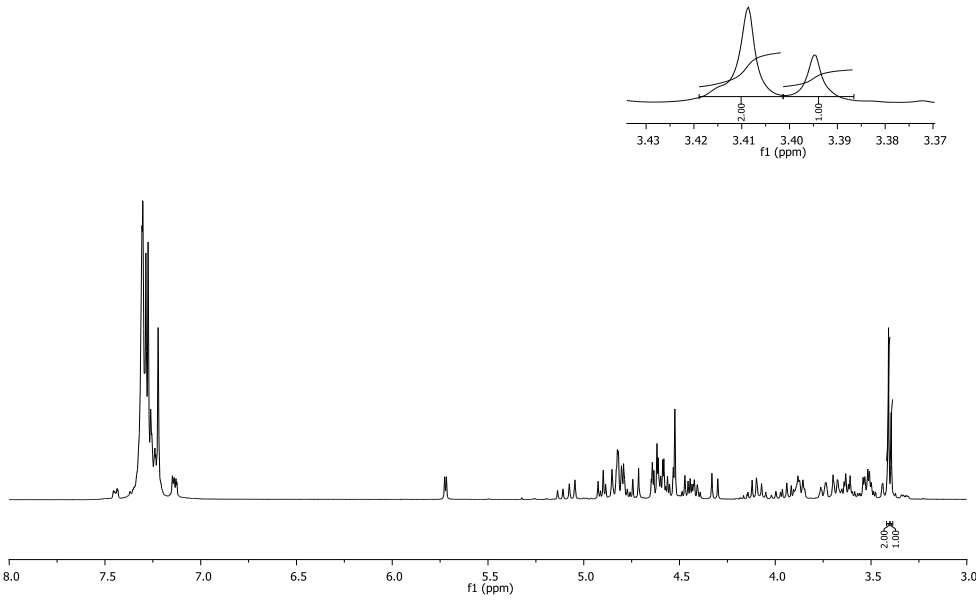


**Table 2, Entry 4**

Compounds **2** and **6** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **11** was obtained in 81% yield (34 mg, 0.034 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

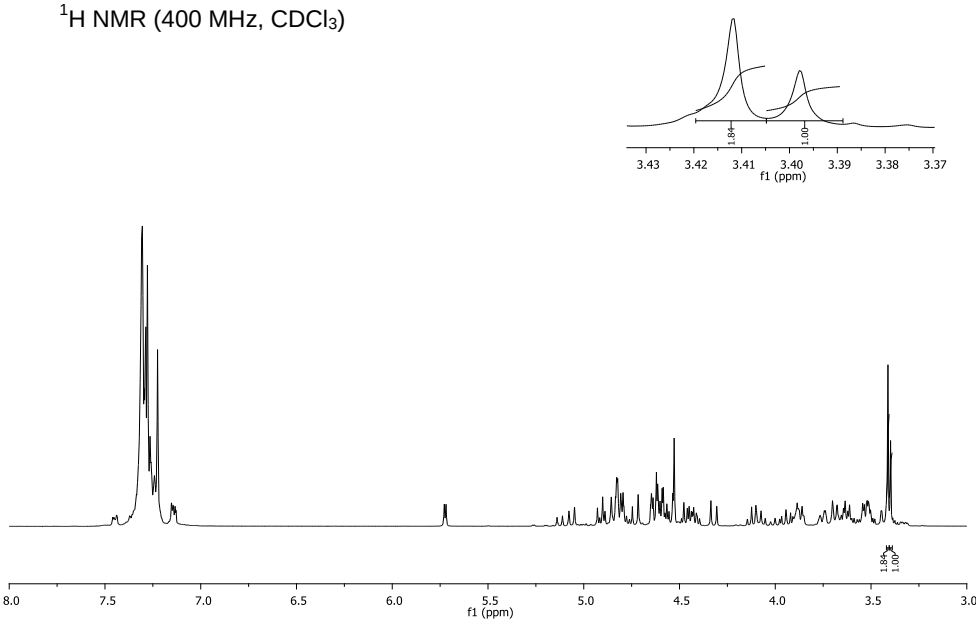


**Table 2, Entry 5**

Compounds **3** (50 mg, 0.134 mmol) and **6** (94 mg, 0.161 mmol) were treated as described in the *General Procedure B*. Product **12** was obtained in 90% yield (87 mg, 0.097 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

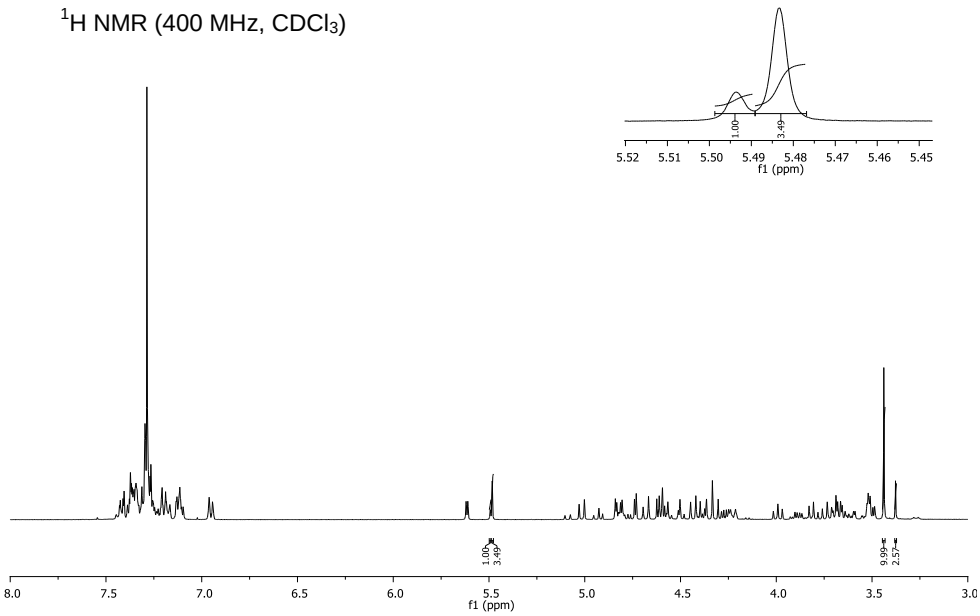


**Table 2, Entry 6**

Compounds **3** and **6** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **12** was obtained in 76% yield (34 mg, 0.038 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

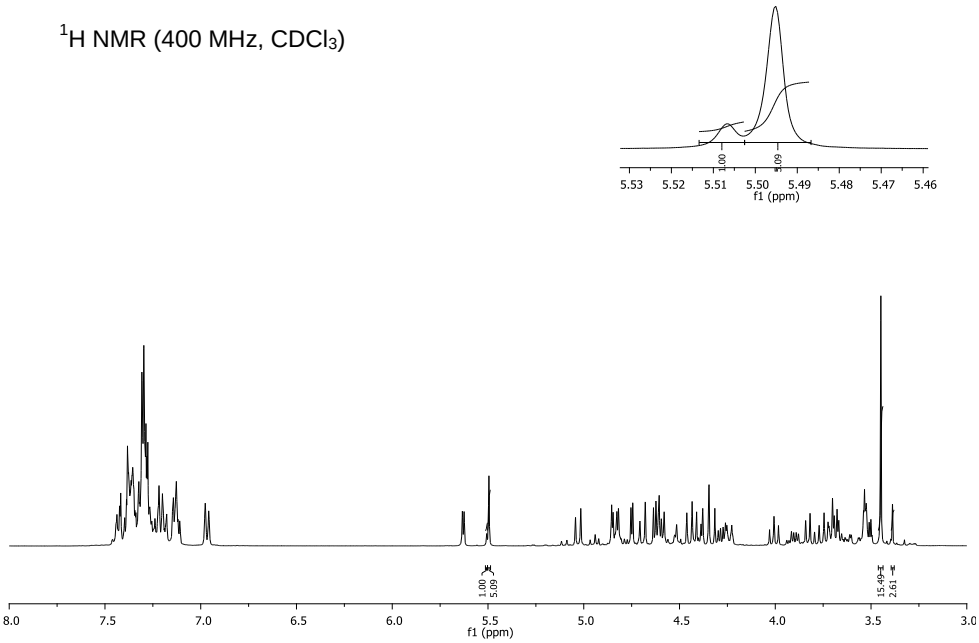
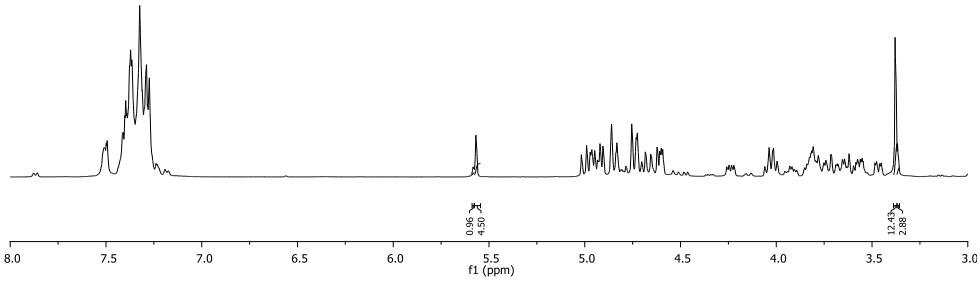


**Table 2, Entry 7**

Compounds **1** (50 mg, 0.108 mmol) and **8** (64 mg, 0.130 mmol) were treated as described in the *General Procedure B*. Product **13** was obtained in 80% yield (77 mg, 0.086 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)



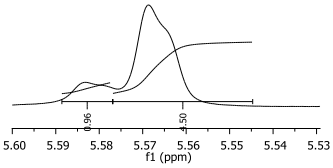



**Table 2, Entry 8**

Compounds **1** and **8** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **13** was obtained in 70% yield (31 mg, 0.035 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

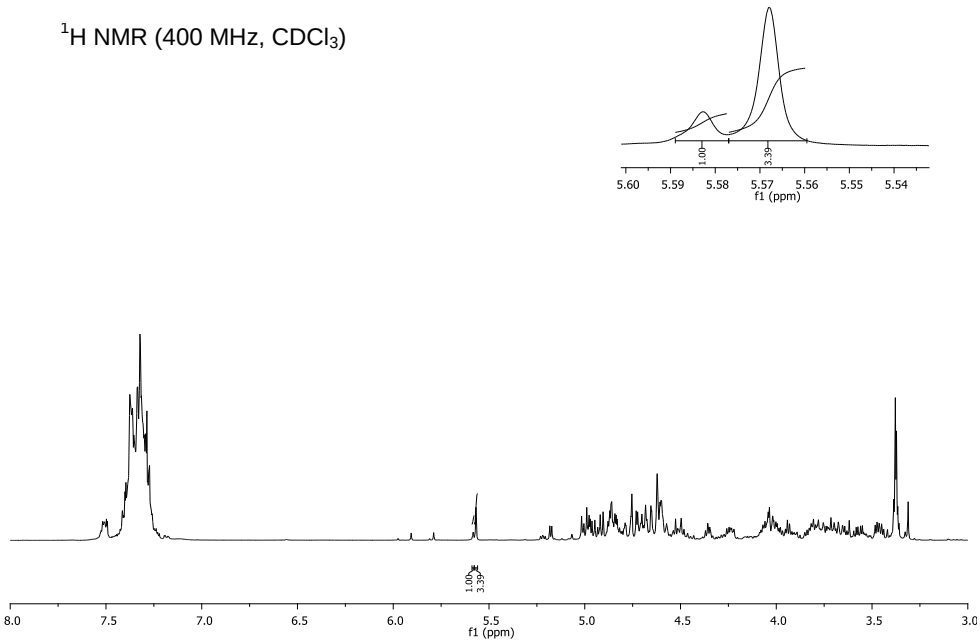


**Table 2, Entry 9**

Compounds **2** (50 mg, 0.108 mmol) and 8 (64 mg, 0.130 mmol) were treated as described in the *General Procedure B*. Product **14** was obtained in 55% yield (53 mg, 0.059 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

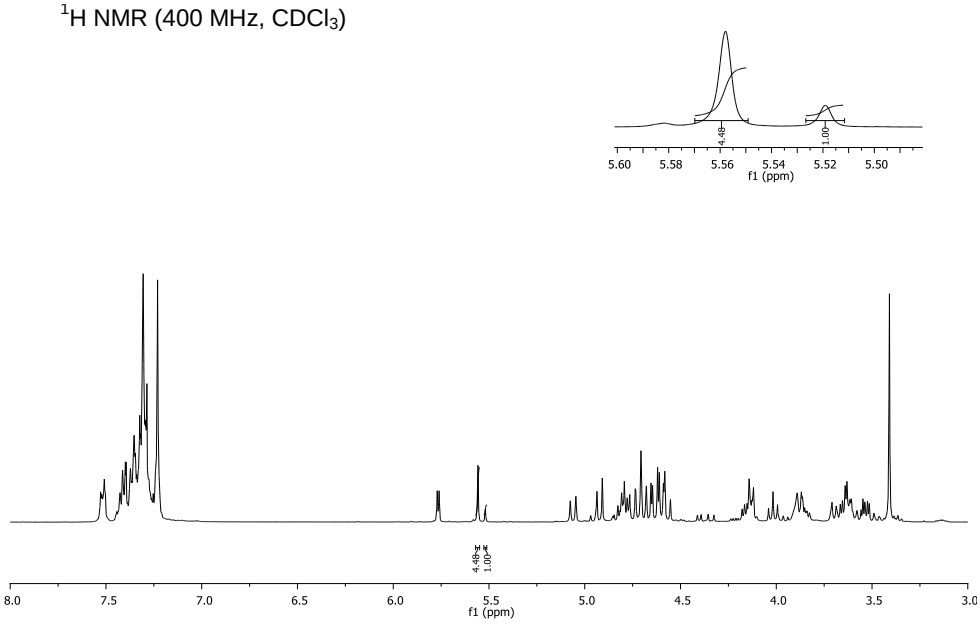


**Table 2, Entry 10**

Compounds **2** and **8** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **14** was obtained in 56% yield (25 mg, 0.028 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

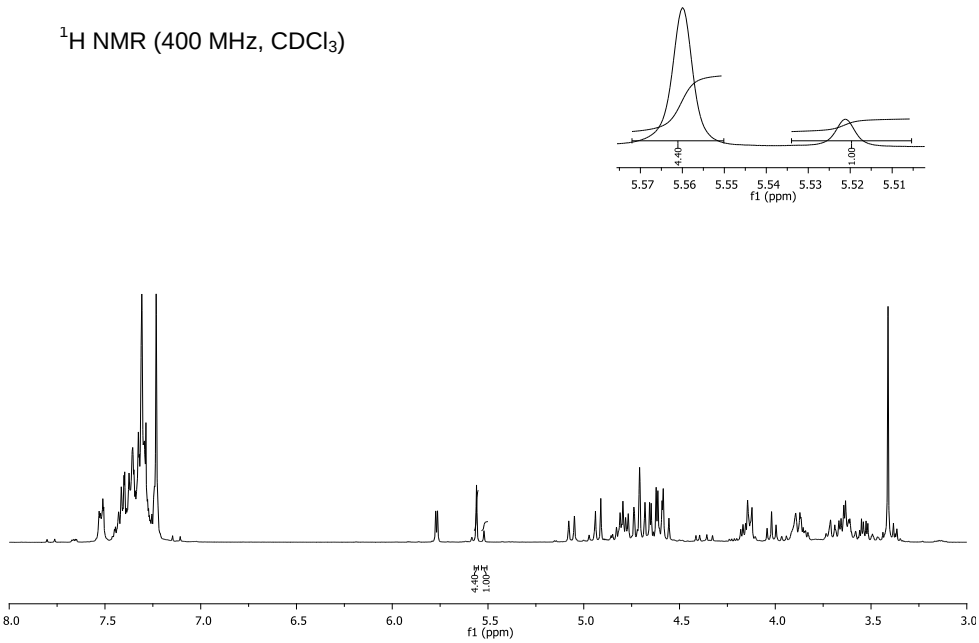


**Table 2, Entry 11**

Compounds **3** (50 mg, 0.134 mmol) and **8** (79 mg, 0.161 mmol) were treated as described in the *General Procedure B*. Product **15** was obtained in 88% yield (95 mg, 0.118 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)

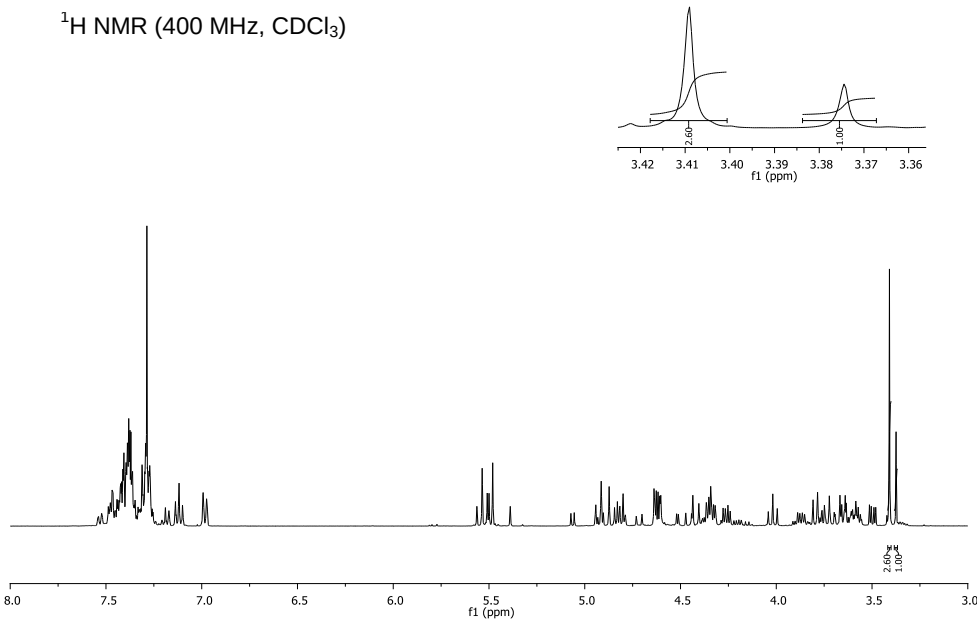


**Table 2, Entry 12**

Compounds **3** and **8** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **15** was obtained in 60% yield (24 mg, 0.030 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)

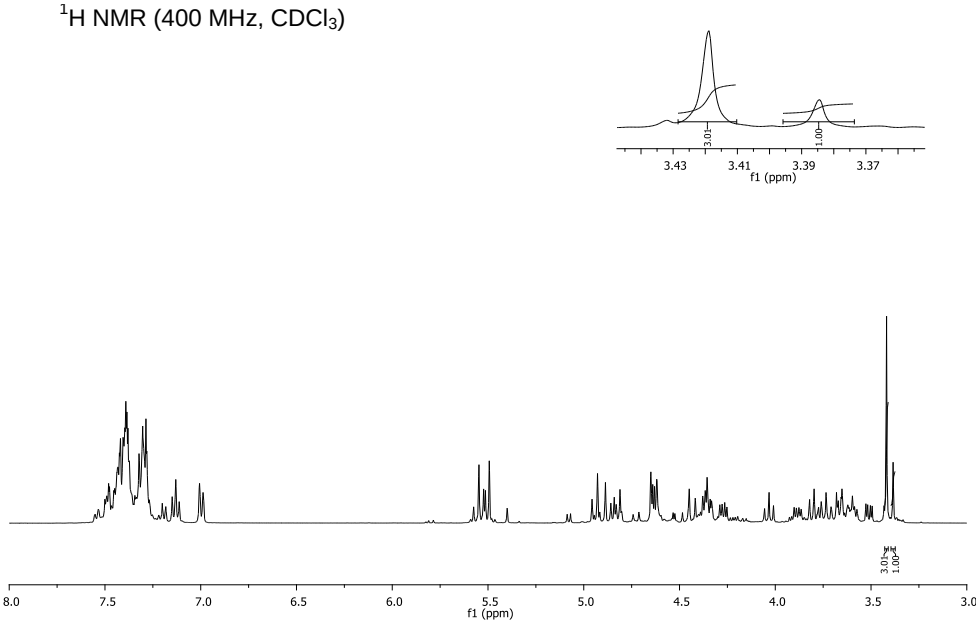


**Table 2, Entry 13**

Compounds **1** (50 mg, 0.108 mmol) and **7** (84 mg, 0.130 mmol) were treated as described in the *General Procedure B*. Product **10** was obtained in 68% yield (72 mg, 0.073 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

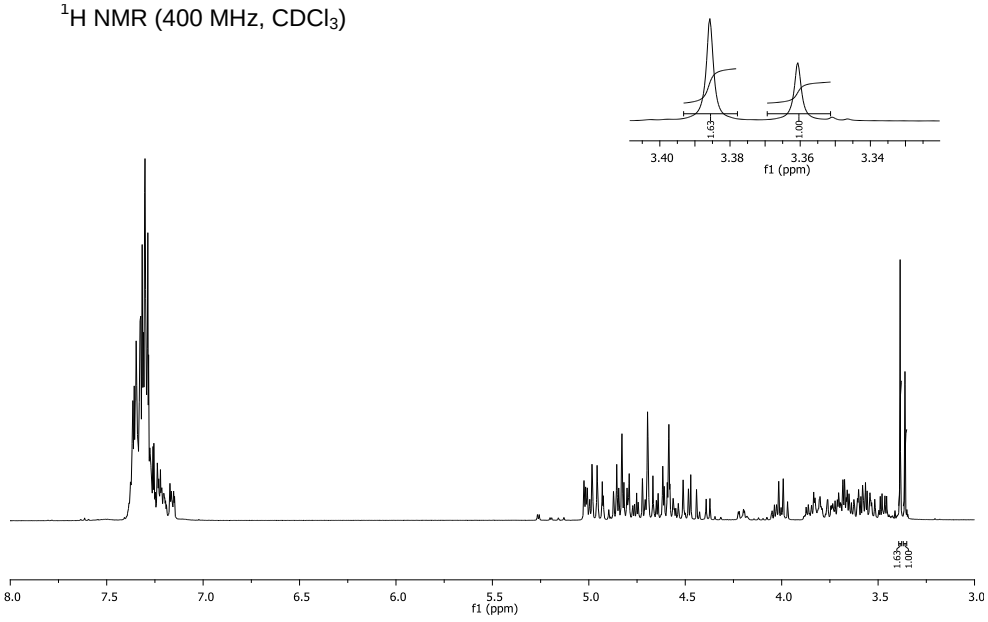


**Table 2, Entry 14**

Compounds **1** and **7** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **10** was obtained in 81% yield (40 mg, 0.041 mmol).

The spectroscopic data were in agreement with those reported in the literature.[18]

$^1$H NMR (400 MHz, $CDCl_3$)

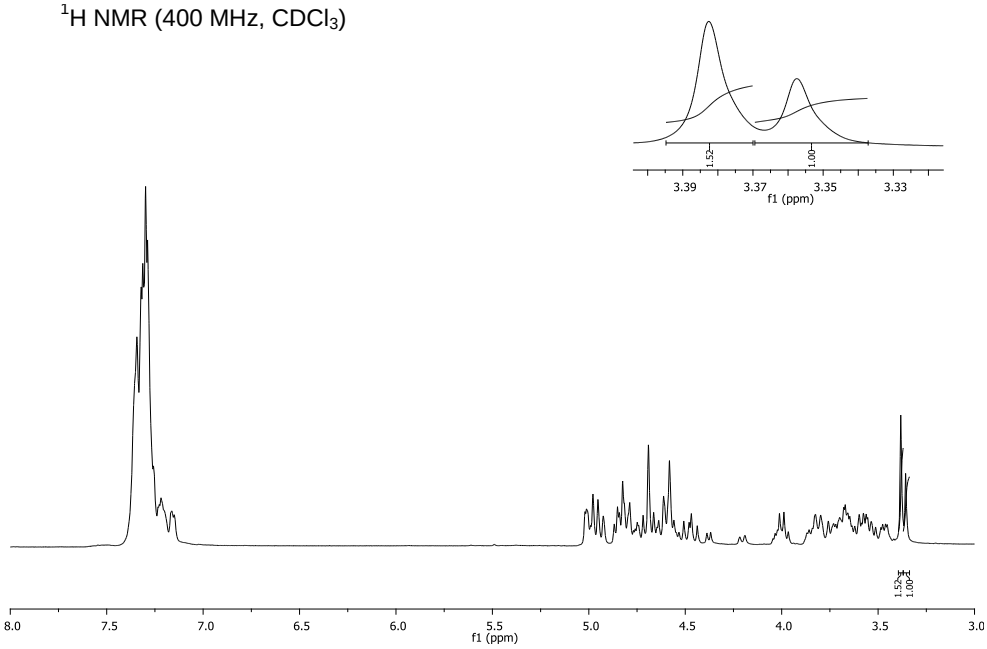


**Table 2, Entry 15**

Compounds **3** (50 mg, 0.134 mmol) and **7** (104 mg, 0.161 mmol) were treated as described in the *General Procedure B*. Product **12** was obtained in 86% yield (103 mg, 0.115 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

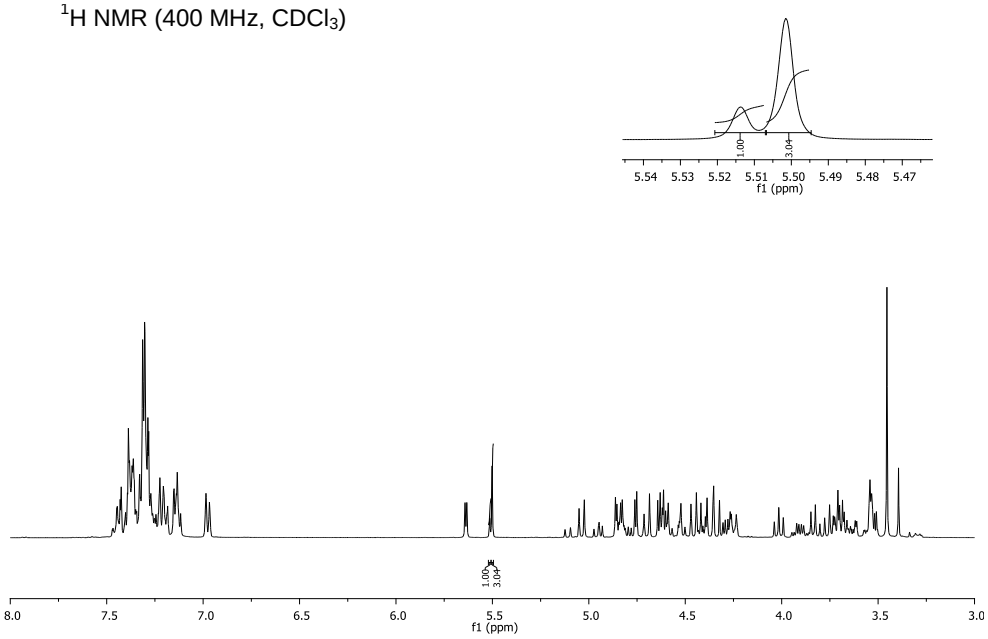


**Table 2, Entry 16**

Compounds **3** and **7** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **12** was obtained in 69% yield (31 mg, 0.035 mmol).

The spectroscopic data were in agreement with those reported in the literature.[19]

$^1$H NMR (400 MHz, $CDCl_3$)

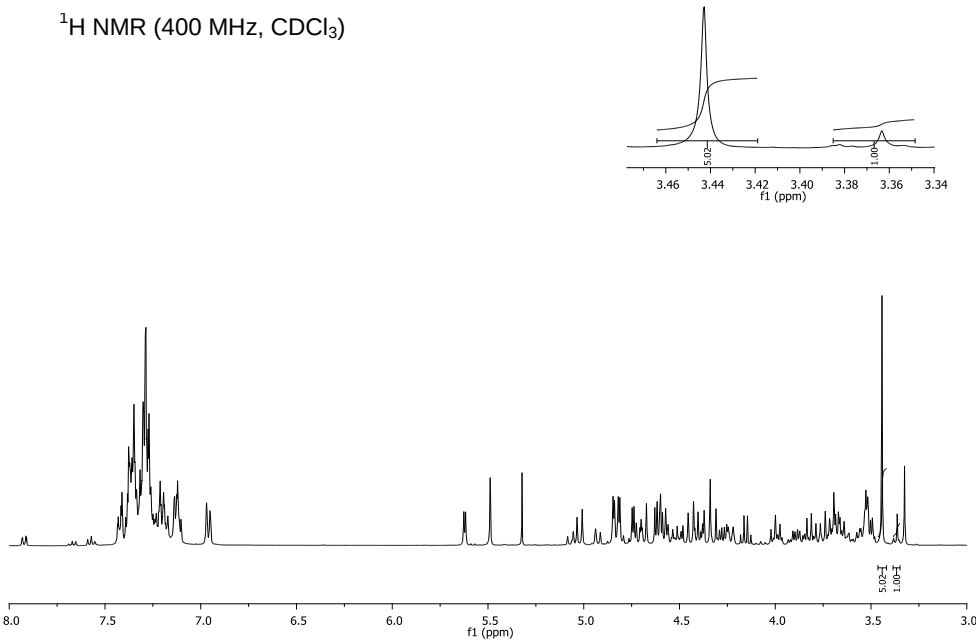


**Table 2, Entry 17**

Compounds **1** (50 mg, 0.108 mmol) and **9** (72 mg, 0.130 mmol) were treated as described in the *General Procedure B*. Product **13** was obtained in 65% yield (63 mg, 0.070 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

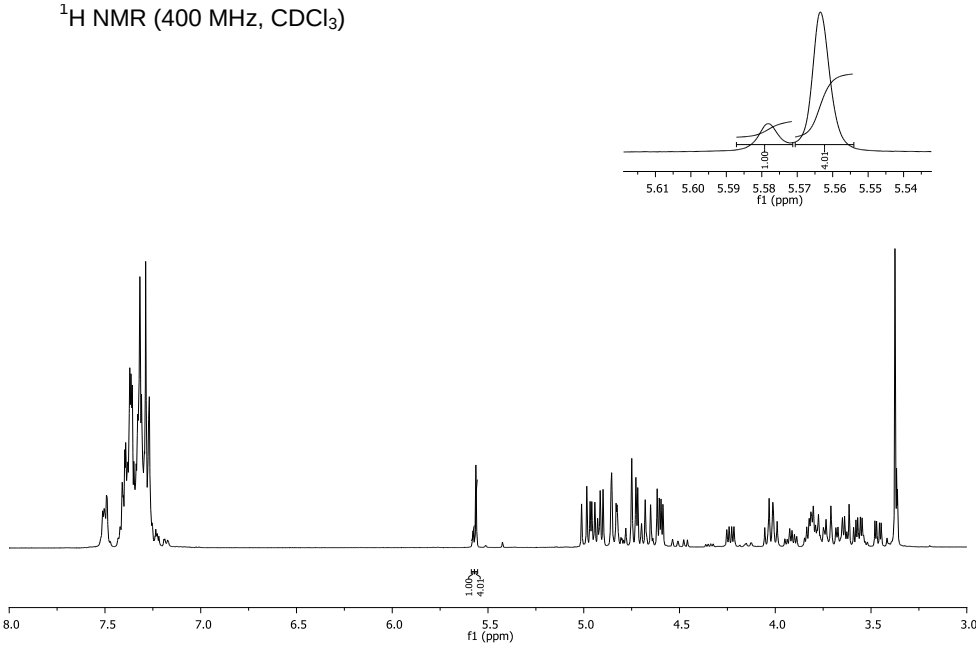


**Table 2, Entry 18**

Compounds **1** and **9** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **13** was obtained in 80% yield (36 mg, 0.040 mmol).

The spectroscopic data were in agreement with those reported in the literature.[20]

$^1$H NMR (400 MHz, $CDCl_3$)

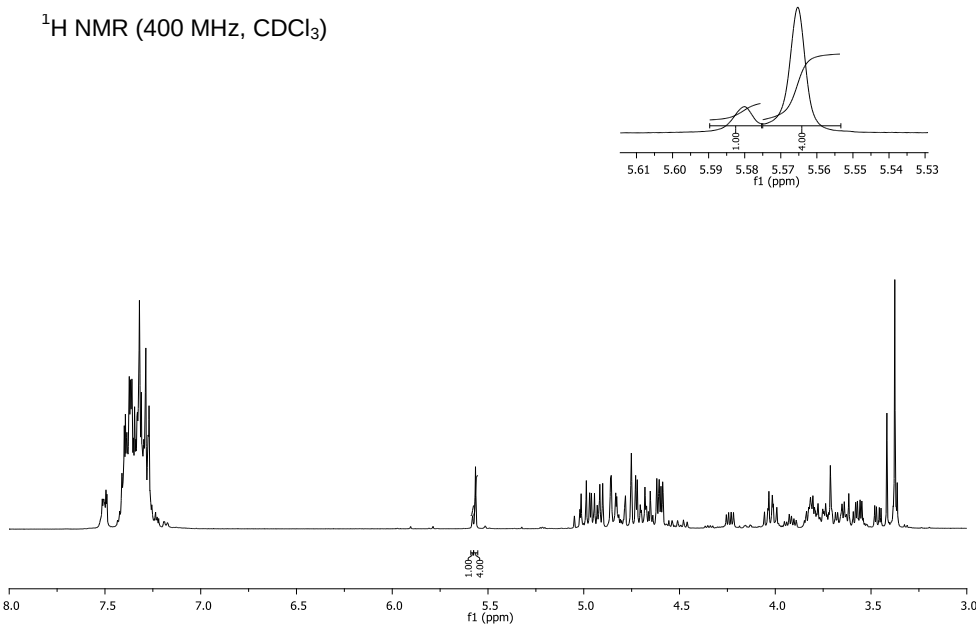


**Table 2, Entry 19**

Compounds **3** (50 mg, 0.134 mmol) and **9** (89 mg, 0.161 mmol) were treated as described in the *General Procedure B*. Product **15** was obtained in 88% yield (95 mg, 0.118 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)

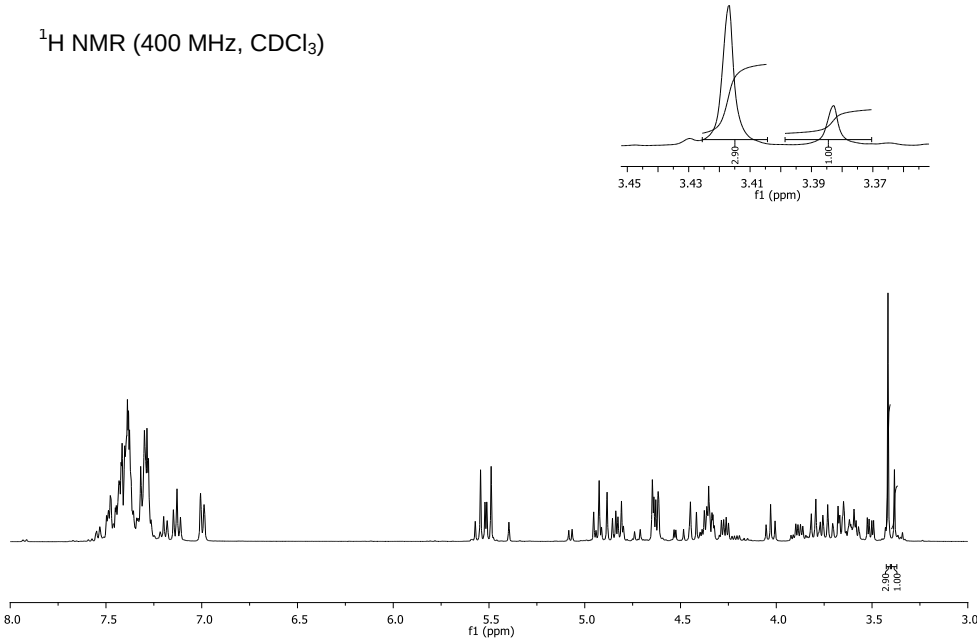
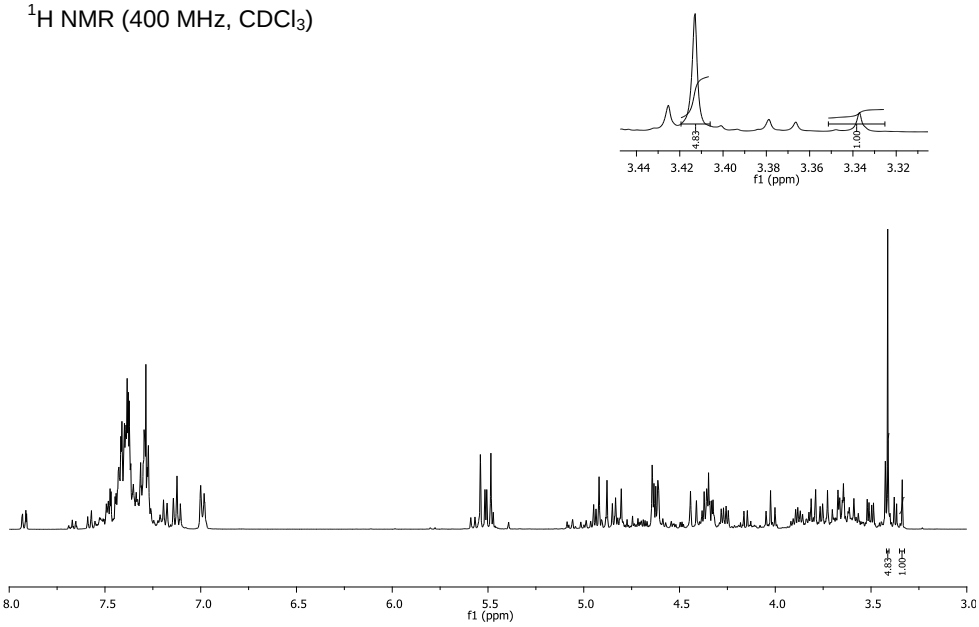


**Table 2, Entry 20**

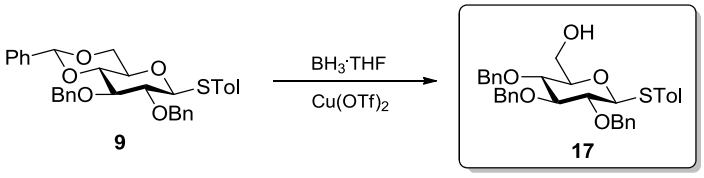
Compounds **3** and **9** were treated as described in the *General Procedure D*. The flow rate was set to 13 μL/min. Product **15** was obtained in 75% yield (31 mg, 0.038 mmol).

The spectroscopic data were in agreement with those reported in the literature.[21]

$^1$H NMR (400 MHz, $CDCl_3$)



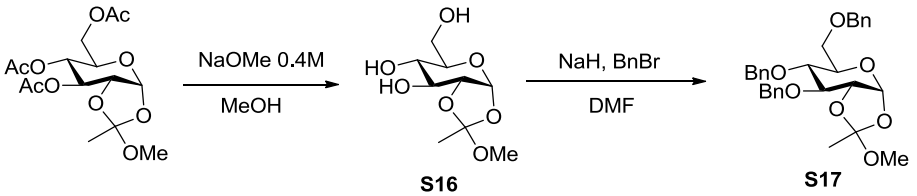
**4-Methylphenyl 2,3,4-tri-*O*-benzyl-1-thio-β-D-glucopyranoside (17)**




To a solution of **9** (0.50 g, 0.90 mmol) in $BH_3$·THF complex (1M in THF, 4.50 mL, 4.50 mmol), $Cu(OTf)_2$ (0.016 g, 0.045 mmol) was added. After 6 h the reaction was cooled to 0°C and neutralized with TEA (0.13 mL, 0.90 mmol) and MeOH (1.6 mL). The reaction mixture was concentrated *in vacuo* and purified by flash chromatography (Hexane:AcOEt 8:2) to obtain compound **17** as a white solid (0.48 g, 96%).

The spectroscopic data of **17** were in agreement with those previously reported.[22]

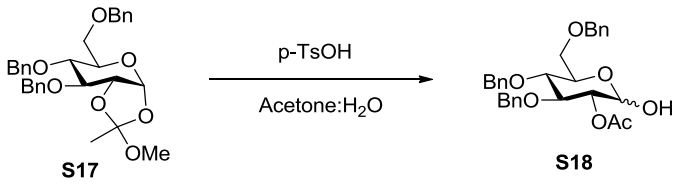
**3,4,6-tri-*O*-benzyl-1,2-*O*-(1-methoxyethylidene)-α-D-glucopyranoside (S17)**




To a solution of 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-α-D-glucopyranoside (2.00 g, 5.52 mmol) in dry MeOH (50 mL), NaOMe 0.4M in MeOH (2.07 mL, 0.83 mmol) was added. After 30 min the reaction mixture was diluted with MeOH and neutralized with AMBERLITE® resin ($H^+$ form). The mixture was filtered and the solvent was removed *in vacuo*, obtaining compound **S16** as a white solid (1.30 g, quant). To a solution of **S16** (1.30 g, 5.52 mmol) and BnBr (3.28 mL, 27.6 mmol) in DMF (50 mL), NaH (0.66 g, 27.6 mmol) was slowly added. After 3 h the reaction mixture was carefully quenched by addition of MeOH (10 mL). The reaction mixture was concentrated *in vacuo*, then the crude residue was diluted with $H_2O$ and extracted with $CH_2Cl_2$ (3 x 20 mL). The combined organic layers were washed with brine, dried over $Na_2SO_4$, concentrated *in vacuo*, and purified by flash chromatography (Hexane:EtOAc 8:2→7:3 + 1% TEA) to obtain the **S17** as a colourless oil (2.23 g, 82%).

The spectroscopic data of **S17** were in agreement with those previously reported.[23]

**2-*O*-Acetyl-3,4,6-tri-*O*-benzyl-D-glucopyranose (S18)**

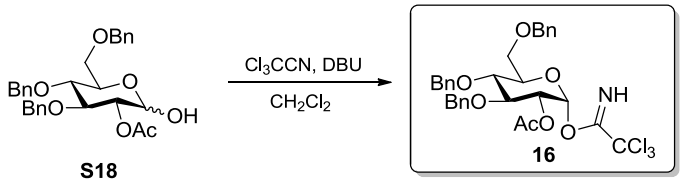



**S17** **S18**

To a cooled solution (0°C) of **S17** (0.66 g, 1.30 mmol) in acetone:$H_2O$ 7:3 (10 mL), *p*-TsOH (0.034 g, 0.39 mmol) was added. After 2 h the reaction mixture was neutralized with TEA and concentrated *in vacuo*. The crude residue was purified by flash chromatography (Hexane:AcOEt 7:3) to obtain **S18** (0.54 g, 85%).

The spectroscopic data of **S18** were in agreement with those previously reported.[24]

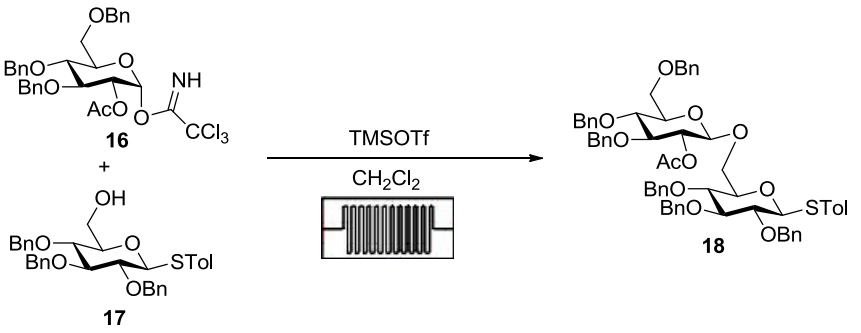
**2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-glucopyranosyl trichloroacetimidate (16)**




To a solution of **S18** (0.54 g, 1.10 mmol) and $Cl_3CCN$ (1.10 mL, 11.0 mmol) in dry $CH_2Cl_2$ (11 mL), a catalytic amount of DBU was added. After 1 h, the reaction mixture was concentrated *in vacuo* and purified by flash chromatography (Hexane:EtOAc 8:2 + 1% TEA) to obtain donor **16** as a colourless oil (0.64 g, 91%, only $\alpha$).

The spectroscopic data of donor **16** were in agreement with those previously reported.[25]

**Microfluidic glycosylation between 16 and 17**



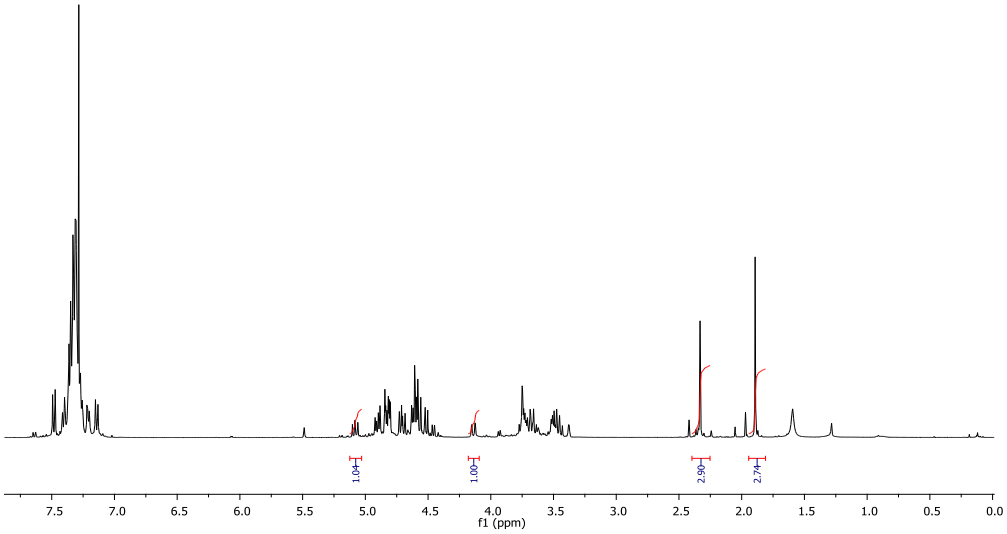

A solution of the glycosyl acceptor **17** (56 mg, 0.1 M) and trichloroacetimidate **16** (76 mg, 0.12 M) in reagent grade $CH_2Cl_2$ (1 mL) was prepared (solution A). A solution of TMSOTf (18 μL, 0.02 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in a separate flask (solution B). Equal volumes (0.5 mL) of the two solutions were taken and injected into the microreactor (internal volume = 13 μL) via a double syringe pump, setting the flow rate at 3.25 μL/min (total flow rate = 6.5 μL/min, corresponding to a residence time = 2 min). The mixture flowed from the microreactor was dropped in a $CH_2Cl_2$ solution of triethylamine to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt 9:1→8:2) to obtain the disaccharide **18** as white solid (46 mg, 90%).

$^1$H-NMR (400 MHz, $CDCl_3$) δ 7.60-7.00 (34H, m, $H_{Ar}$), 5.06 (1H, t, $J_{2,3}$ 8.0 Hz, H2'), 4.93-4.54 (13H, m, $CH_2$Ph, H1), 4.51 (1H, d, $J_{1,2}$ 8.1 Hz, H1'), 4.14 (1H, d, H6), 3.76-3.61 (7H, m, 2H6', H6, H4', H4, H3', H3), 3.55-3.42 (3H, m, H5, H5', H2), 2.33 (3H, s, $CH_3$-Tol), 1.89 (3H, s, $CH_3$-Ac)
$^{13}$C-NMR (100 MHz, $CDCl_3$) δ 132.3, 131.7, 130.0, 129.8, 128.4, 128.4, 128.4, 128.2, 128.0, 127.9, 127.8, 127.8, 127.8, 127.7, 127.6, 100.9, 87.8,

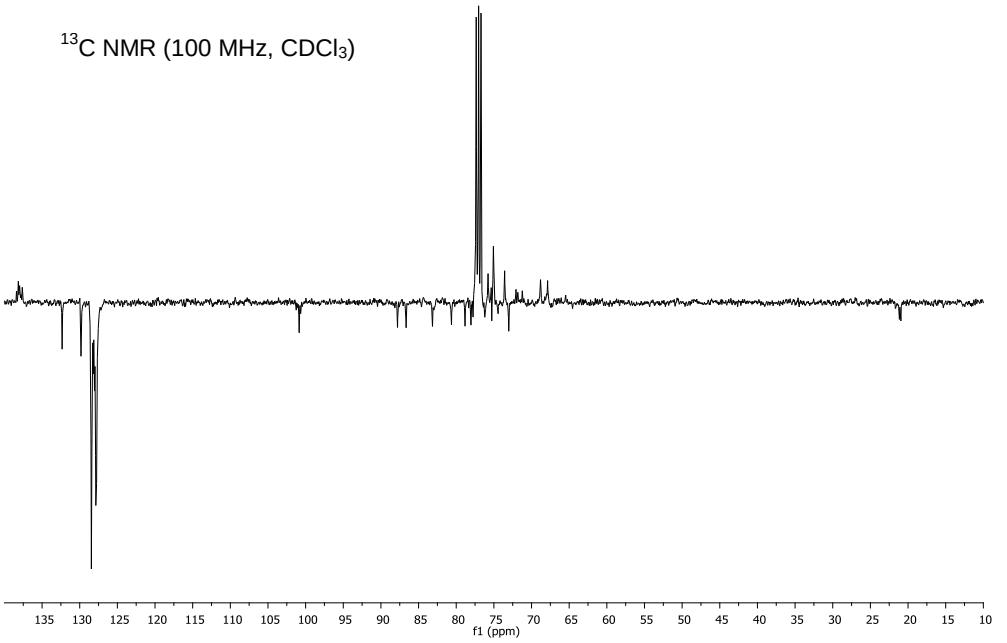
86.6, 83.2, 80.7, 78.8, 78.1, 77.8, 75.7, 75.5, 75.3, 75.3, 75.0, 74.9, 73.6, 73.1, 68.8, 67.9

HRMS (Q-Tof) $[M+Na]^+$ *m/z* calcd for $C_{63}H_{66}O_{11}SNa$ 1053.4223, found 1053.4486

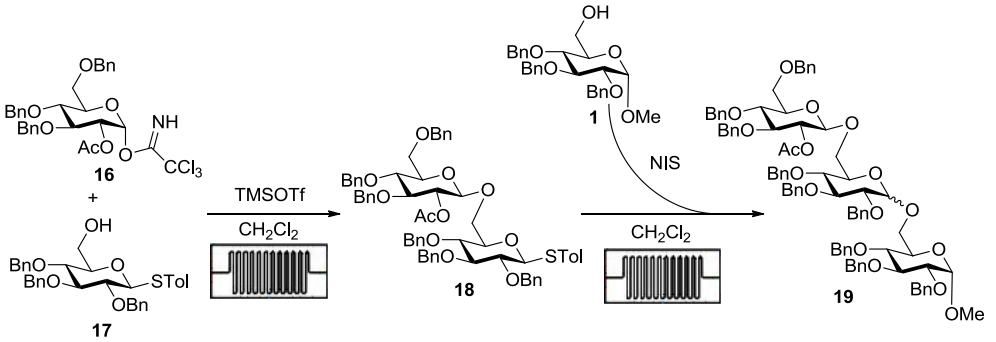
$^{1}$H NMR (400 MHz, $CDCl_3$)




$^{13}$C NMR (100 MHz, $CDCl_3$)



**Two-steps synthesis of trisaccharide 19**



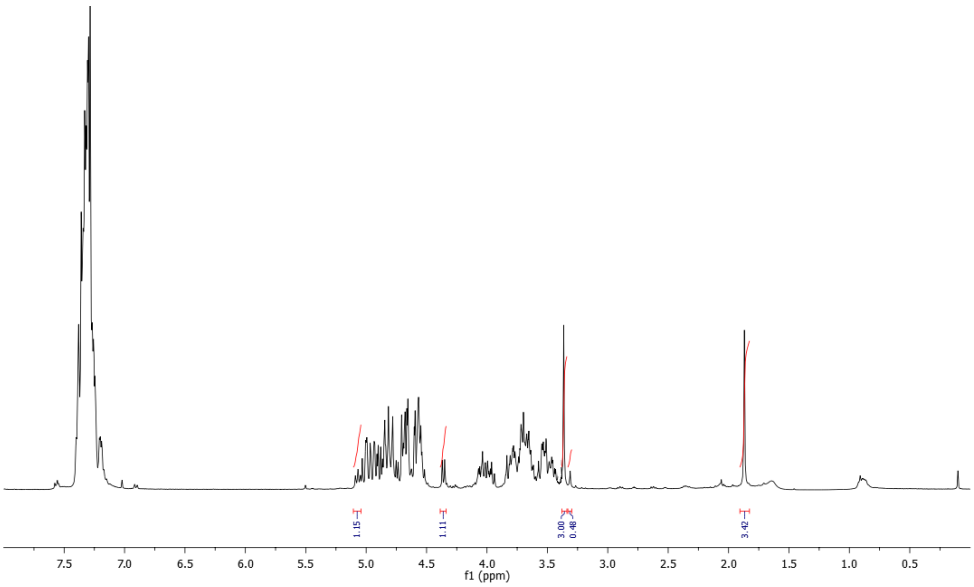

A solution of donor **16** (76 mg, 0.12 M) and thioglycoside acceptor **17** (56 mg, 0.1 M) in reagent grade $CH_2Cl_2$ (1 mL) was prepared (solution A, Scheme 3). A solution of TMSOTf (0.018 mL, 0.02 M) in reagent grade $CH_2Cl_2$ (5 mL) was prepared in a separate flask (solution B, Scheme 3). Equal volumes (0.5 mL) of the two solutions were taken and injected into the first microreactor (internal volume = 13 µL) via a double syringe pump, setting the flow rate at 3.25 µL/min (total flow rate = 6.5 µL/min, corresponding to a residence time = 2 min). The reaction mixture (containing the disaccharide donor **18**) flowed from the microreactor was pumped into the second microreactor together with a solution of acceptor **1** (47 mg, 0.05 M) and NIS (34 mg, 0.075 M) in reagent grade $CH_2Cl_2$ (2 mL). The flow rate of the second solution was set at 6.5 µL/min (total flow rate in the second microreactor = 13 µL/min), in order to have an overall residence time = 1 min. The reaction mixture flowed from the microreactor was eventually quenched in a $CH_2Cl_2$ solution of triethylamine The mixture was concentrated *in vacuo* and the crude was purified by flash chromatography (Hexane/AcOEt 8:2→7:3), furnishing trisaccharide **19** (35 mg, 51%, mixture of anomers at the newly formed glycosidic bond) as a colourless oil.

$^{1}$H-NMR (400 MHz, $CDCl_3$) δ 7.61-7.05 (45H, m, $H_{Ar}$), 5.07 (t, 1H, t, $J_{2,3}$ 8.8, H2”), 5.03-4.48 (20H, m, $CH_2$Ph, H1’, H1), 4.38 (1H, d, $J_{1,2}$ 8.0 Hz, H1”), 4.10-3.42 (17H, m, 2H6, 2H6’, 2H6”, H5, H5’, H5”, H4, H4’, H4”, H3, H3’, H3”, H2, H2’), 3.36 (3H, s, $OCH_3$-α), 3.31 (s, 3H, OCH3-β), 1.87 (s, 3H, $CH_3$-Ac)

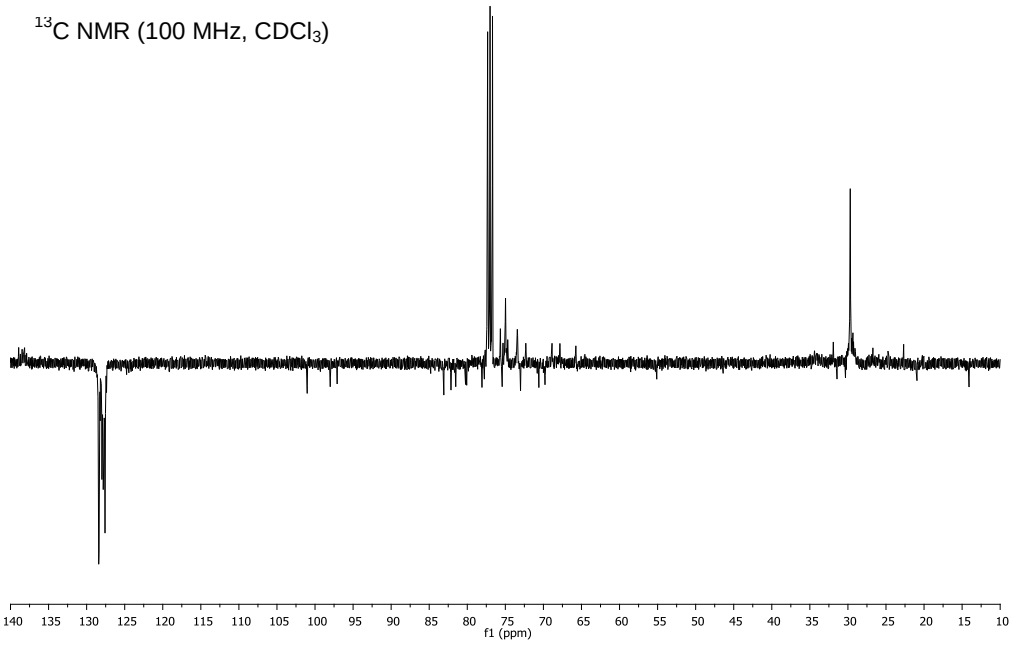
$^{13}$C-NMR (100 MHz, $CDCl_3$) δ 128.4-127.4, 101.0, 98.0, 97.1, 78.1, 75.7, 75.4, 75.3, 75.0, 74.7, 73.4, 73.0, 72.3, 70.6, 69.8, 68.9, 67.8, 65.7, 55.1, 20.9

HRMS (Q-Tof) $[M+Na]^+$ *m/z* calcd for $C_{84}H_{90}O_{17}Na$ 1393.6076, found 1393.6066

$^{1}$H NMR (400 MHz, $CDCl_3$)




$^{13}$C NMR (100 MHz, $CDCl_3$)



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# MRT and Glycosyl phosphodiesters

# 1 Phosphoglycans: structural diversity and chemical synthesis

The most common type of inter-monomeric linkage in carbohydrate-containing polymers is the glycosidic linkage between monosaccharides. However, a different type of intersaccharide linkage is also widespread: a phosphodiester linkage formed by a glycosyl phosphate unit and an hydroxyl group of another monosaccharide. This type of linkage is found in many polymers (commonly named “phosphoglycans”) of the cell wall or capsule of bacteria and yeasts. It is also present in glycan chain of some glycoproteins in animals. In many cases, the glycosyl phosphate units participating in the formation of phosphodiester linkages are constituents of the antigenic determinants defining the immunological specificity.

The importance of phosphoglycans as immunologically active components of the cell wall and/or capsule of numerous pathogenic microorganisms upholds the need to develop potential routes for the chemical preparation of these biopolymers. The synthesis of glycosyl phosphosaccharides (anomeric phosphodiesters in which phosphorus is linked to one monosaccharide through the hemiacetal hydroxyl group at C-1 and to another through an alcoholic hydroxyl group) becomes a primary task. Chemical formation of glycosyl phosphosaccharides is complicated as both the correct stereochemistry at C-1 and the lability of anomeric phosphodiester linkages must have to take into consideration.

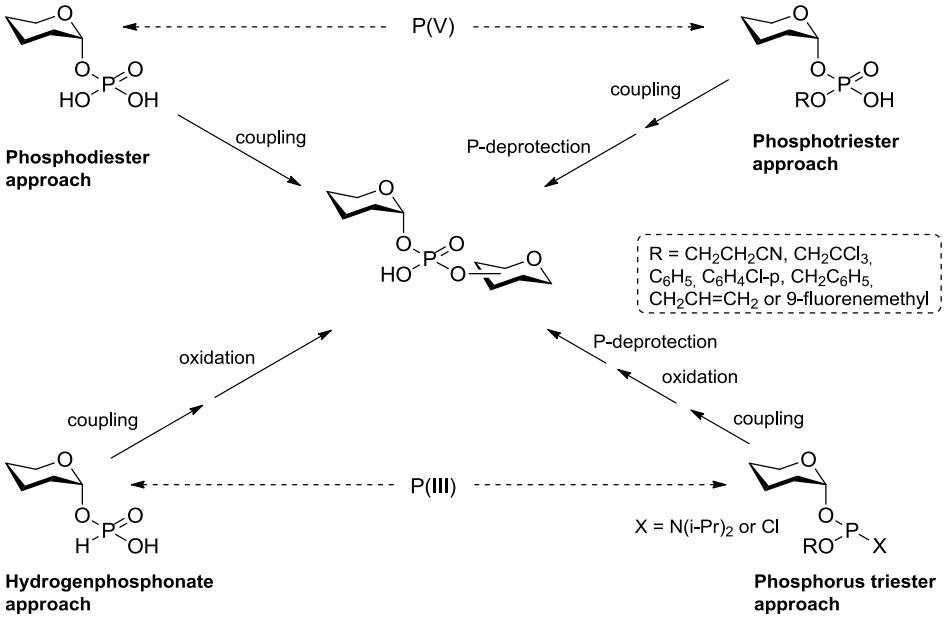
## 1.1 Chemical synthesis of glycosyl phosphosaccharides

For the preparation of glycosyl phosphosaccharides five basic synthetic methodologies have been reported:[1]

I. Phosphodiester approach;
II. Phosphotriester approach;
III. Phosphorus (or phosphate) triester (including phosphoramidite and phosphorichlorite version) approach;
IV. Hydrogenphosphonate (H-phosphonate) approach;
V. Approach based on glycosylation reaction.

The five methods mentioned are systematically represented in Scheme 16. Approaches I and II are based on P(V) chemistry, while approaches III and IV on P(III) chemistry.

**Scheme 16. Schematic representation of methods I-IV in phosphodiester synthesis**



The first four methods (Scheme 16) were originally designed for nucleotide chemistry: they involved the condensation of various P-containing glycosyl components (electrophiles) with alcohol monosaccharide derivatives (nucleophiles). The last one (approach V) is a solely carbohydrate method as it involves the reaction of a glycosyl donor (electrophile) with a P-containing glycosyl acceptor (nucleophile).

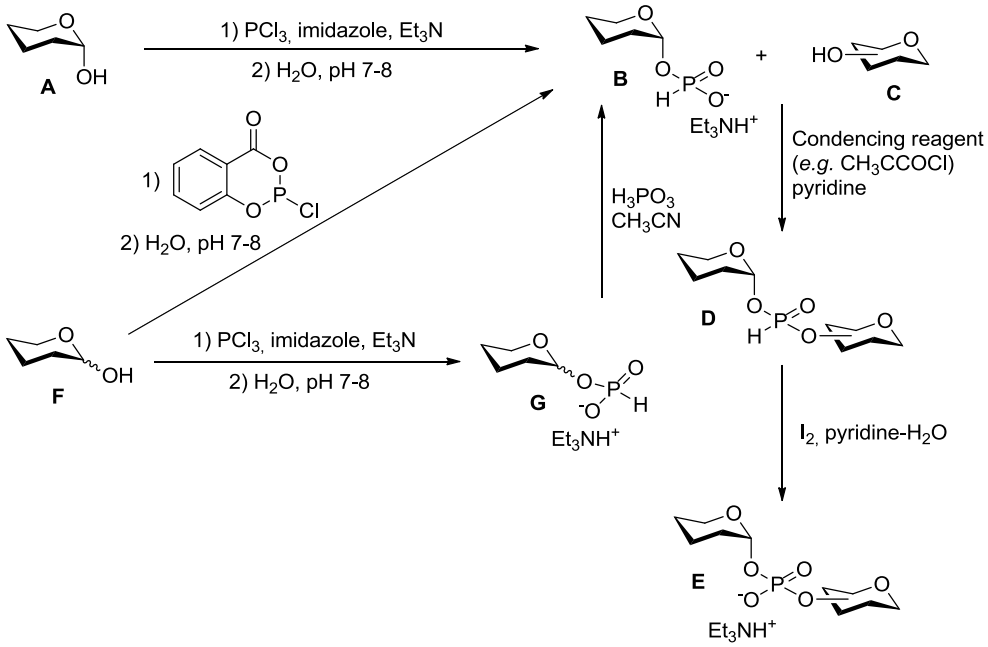
Among all these basic synthetic methodologies employed for the preparation of glycosyl phosphosaccharides, the approaches based on P(III) chemistry are currently the most widespread techniques: in particular, the Hydrogenphosphonate approach is the most used, also in our laboratory.

## 1.2 Hydrogenphosphonate methods

In the mid-1980s the first applications of H-phosphonate method in carbohydrate chemistry were described by van Boom's group[2] and by Nikolaev's group.[3]

In the glycosyl hydrogenphosphonate (H-phosphonate) method glycosyl H-phosphonate derivative **B** is coupled with a monohydroxyl component **C** in the presence of a condensing agent to produce a phosphorous (H-phosphonic) diester **D**. The intermediate **D** is then oxidized to form the targeted glycosyl phosphodiester **E** (Scheme 17).

**Scheme 17. H-phosphonate approach**




The most frequently used condensing reagent is trimethylacetyl chloride. The oxidation reaction is normally performed in situ using iodine in aqueos pyridine (in general at low temperature, *e.g.* -40°C).

Glycosyl H-phosphonate **B** can be effectively prepared from the corresponding α-hemiacetal derivative **A** using either tri-imidazolylphosphine (made from $PCl_3$, imidazole and $Et_3N$) or 2-chloro-1,2,3-benzodioxaphosphorin-4-one (commonly named salicyl chlorophosphite).

It has been reported that the $\alpha,\beta$-hemiacetal derivative **F** can be used to form pure $\alpha$-glycosyl H-phosphonate **B** on reaction with either salycilchlorophosphite in pyridine or $H_3PO_3$, suggesting that the reactivity of the α-hemiacetal constituent towards these reagents is markedly higher than that of the β-hemiacetal. Alternatively, α,β-glycosyl H-phosphonate **G**

can be converted into α-glycosyl H-phosphonate **B** on treatment with $H_3PO_3$ in acetonitrile.

The H-phosphonate method appears to be the most efficient for the preparation of glycosyl phosphosaccharides linked through either primary or secondary hydroxyl groups and containing monosaccharides of diverse composition. The main characteristics of this approach are high effectiveness and high reaction rate for all the three steps involved: glycosyl H-phosphonate preparation, condensation with a mono-hydroxyl component and final oxidation (the latter two steps are normally performed in situ). Another advantage is that it does not require a P-protecting group.

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# 1 Neisseria Meningitidis type X

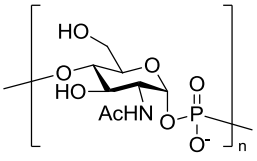
The Gram-negative organism Neisseria Meningitidis is the major cause of bacterial meningitis. This invasive infection affects mostly infants, children, and adolescents who do not possess specific antibodies.

Bacterial meningitis is an inflammation of the protective membranes covering the brain and spinal cord, known as the meninges.

Based on the chemical composition of the polysaccharide capsule, 13 different serogroups of *N. Meningitidis* have been defined so far, but only six serotypes (A, B, C, Y, W135 and recently X) are currently associated with significant pathogenic diseases.

In particular, MenX is responsible of rare cases of meningococcal diseases in North America, Europe, Australia, Africa and Republic of China. The capsular polysaccharide (CPS) of *Neisseria meningitidis* X (Men X) is a homopolymer of (1→4)-linked 2-acetamido-2-deoxy-α-D-glucosamine phosphate residues (Figure 11).

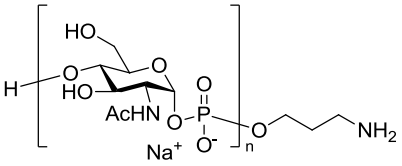
**Figure 11. Repeating unito f *MenX* CPS**




In 2006 WHO started to consider MenX as a substantial threat for public health, suggesting to include this serotype as antigenic component in anti meningococcal conjugate vaccines. Since vaccines currently available do not contain this component, we became interested in the synthesis of Men X CPS fragments.

In our laboratory, short chain MenX oligomers were synthesized using the hydrogen phosphonate approach (Figure 12).[1]

**Figure 12. Synthetic monomer, dimer and trimer of MenX CPS**




However the synthesis of these fragments showed some significant drawbacks, such as the low yield in the final step of azide reduction and the long reaction time (ca. 10 days) required for the synthesis of the α-pure anomeric H-phosphonate.

All these problems prompted us to investigate new routes for more efficient preparation of even longer Men X fragments. In particular, we decided to explore the use of microfluidic reactors for the synthesis of glycosyl phosphosaccharides.

# 2 Bibliography

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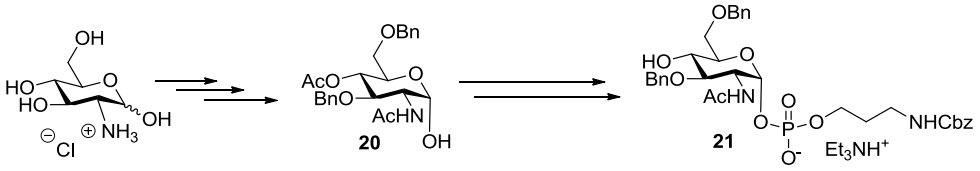
# 1 Results and discussions

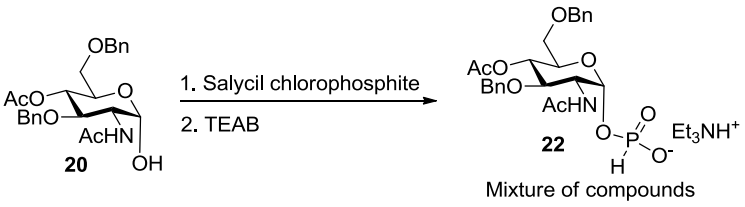
In a previous work carried out in our laboraty, the preparation of two distinct intermediates (**20** and **21**) for the synthesis of MenX oligomers was optimized using standard batch conditions starting from commercially available D-glucosamine hydrochloride (Scheme 18).[1]

**Scheme 18. Synthesis of intermediates 20 and 21**




The subsequent steps of H-phosphonate preparation showed different problems. In particular, during the synthesis of the anomeric H-phosphonate from the α-hemiacetal **20** with salicyl chlorophosphite in pyridine, the expected α-glycosyl H-phosphonate was obtained in very low yield (Scheme 19), and together with an unidentified compound also having α-configuration.

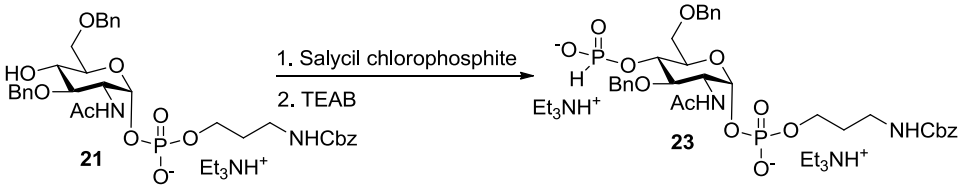
**Scheme 19. Synthesis of H-phosphonate 22**




In order to overcome this difficulty, the synthesis of the H-phosphonate at C-4 of **21** was tested. Any attempt to convert acceptor **21** into the H-

phosphonate **23** (Scheme 20) in batch conditions gave disappointing results.

**Scheme 20. Synthesis of H-phosphonate 23**



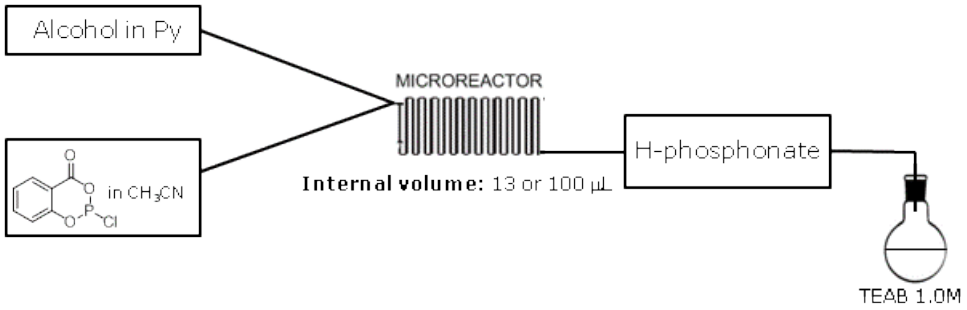

Although two different batch conditions were tested (Table 6), the isolation of H-phosphonate **23** resulted unfeasible.

**Table 6. Batch conditions tested for the synthesis of 23**

| entry | solvent | time (min) | products |
|---|---|---|---|
| **1** | Py | 60 | no product |
| **2** | Py:$CH_3CN$ (1:1) | 60 | no product |

As an alternative option, the synthesis of the H-phosphonate under microfluidic condition was explored. The reaction was performed pumping into the microreactor two distinct solutions, containing the sugar residue **21** in pyridine (0.1 M, Solution A) and salicyl chlorophosphite in $CH_3CN$ (0.2 M, Solution B). The device is completed by a reservoir connected to the outlet of the microreactor and containing a solution of Triethylammonium bicarbonate buffer (TEAB) 1.0 M to stabilize the H-phosphonate product (Figure 13).

**Figure 13. Diagramatic representation of continuous-flow H-phosphonate synthesis**




First, the synthesis of H-phosphonate **23** was tested under microfluidic conditions using a microreactor with an internal volume of 13 μL. The results of this exploration are reported in Table 7.

**Table 7. Synthesis of 23 under microfluidic conditions**

| entry | residence time (sec) | products (%yield) |
|---|---|---|
| **1** | 60 | **21** + **23** |
| **2** | 120 | **21** + **23** |
| **3** | 180 | **21** (trace) + **23** (54%) |
| **4** | 240 | **23** (58%) |
| **5** | 210 | **23** (59%) |

The first attempt was accomplished by setting the flow rate at 13 μL/min corresponding to 60 seconds of residence time (Table 7, entry 1): under these conditions only a portion of **21** was converted to **23** (the unreacted alcohol **21** was recovered after flash chromatography). Therefore the residence time was increased to 120, 180 and 240 seconds. With 180 seconds of residence time unreacted alcohol **21** was still present (Table 7, entry 3), but it was fully consumed at 240 second of residence time (Table 7, entry 4).

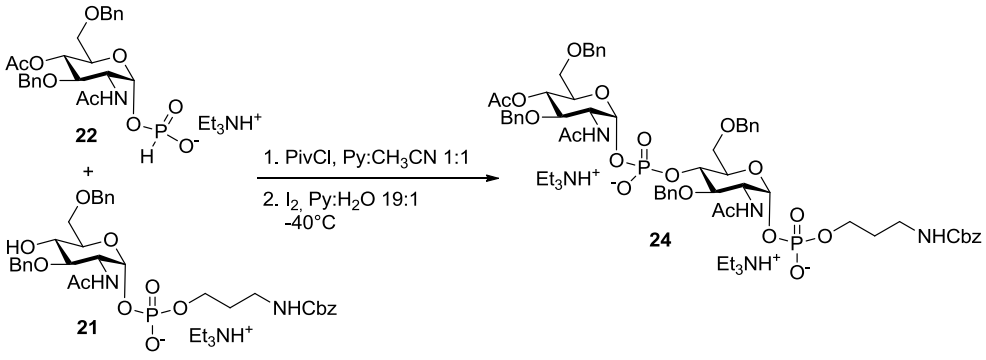
A additional attempt with residence time of 210 seconds (flow rate 3.7 mL/min, Table 7, entry 5) demonstrated that an increase of residence time up to 240 seconds did not enhance the yield.

Next, the synthesis of the anomeric H-phosphonate **22** under microfluidic conditions was investigated. For this purpose, the same instrumentation and procedure described in Figure 13 were used. In this case the reaction was carried out using a microreactor with an internal volume of 100 μL. When the residence time was set to 3 minutes the H-phosphonate **22** was obtained in good yield (76%) exclusively in α configuration.

To the best of our knowledge, these are the first examples of synthesis of H-phosphonate on saccharide substrates performed in a microreactor under continuous-flow conditions.

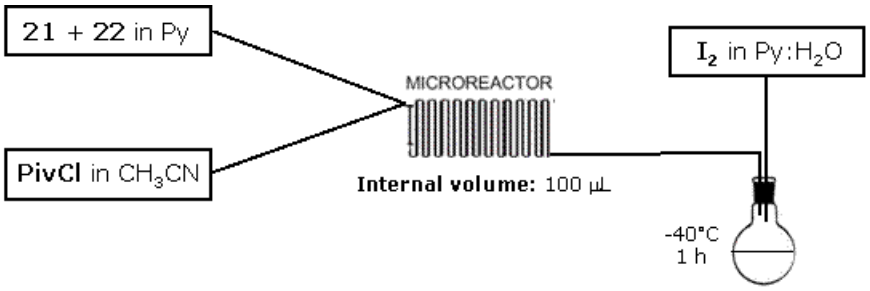
Finally, the oxidative coupling in order to obtain the dimer **24** was explored. The reaction consists of a first coupling step between the H-phosphonate **22** and the glycosyl acceptor **21**, followed by the oxidation of the H-phosphonate diester intermediate with iodine (Scheme 21).

**Scheme 21. Oxidative coupling between 22 and 21**



The microreactor was used to perform the coupling under microfluidic condition (Solution A: **21** + **22** in Py; Solution B: PivCl in $CH_3CN$), while the oxidation step was carried out in a standard round-bottomed flask under batch conditions, as depicted in Figure 14.

**Figure 14. Diagramatic representation of flow oxidative coupling**




The residence time into the microreactor was set to 3 minutes, then the mixture flowed out the reactor was collected and cooled to -40°C. At this point the oxidation was performed by a slow addition of $I_2$ solution in a mixture Py-water. After 1 hour the reaction was quenched and the mixture purified by flash chromatography. The dimer **24** was obtained in low yield (5%), confirmed by NMR analysis, and no degradation products were observed.

The synthesis of glycosyl phosphosaccharides associated to pathogenic microorganisms is a major goal for our laboratory.

Further investigations will be therefore undertaken in order to obtain a reliable continuous-flow protocol suitable for the synthesis of various glycosyl phosphodiester under microfluidic conditions.

# 2 Bibliography

[1] Synthesis and conjugation of *Neisseria Meningitidis* X capsular polysaccharide fragments, PhD thesis of L. Morelli

# 1 Experimental section

## 1.1 General procedures

The reactions performed under microfluidic conditions were carried out in a glass microreactor with an internal volume of 13 or 100 µL purchased by Micronit Microfluidics®.

All commercially available reagents including dry solvents were used as received. Nonvolatile materials were dried under high vacuum.

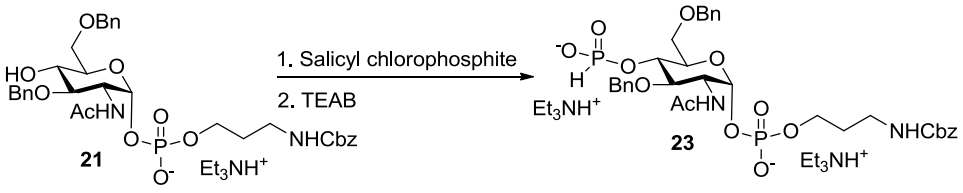
Reactions were monitored by thin-layer chromatography on pre-coated Merck silica gel 60 F254 plates and visualized by staining with a solution of cerium sulfate (1g) and ammonium heptamolybdate tetrahydrate (27 g) in water (469 mL) and concentrated sulfuric acid (31 mL).

Flash chromatography was performed on Fluka silica gel 60.

NMR spectra were recorded at 300 K on spectrometer operating at 400 MHz. Proton chemical shifts are reported in ppm (δ) with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard ($CDCl_3$ δ = 7.26 ppm). *J* values are given in Hz. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, δ = 77.0 ppm).

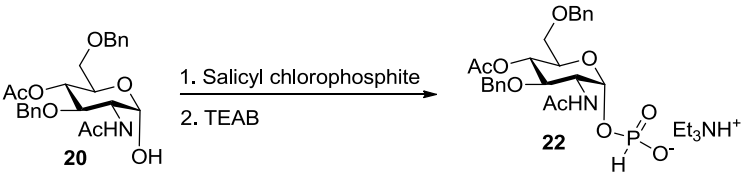
High resolution mass spectra (HRMS) were performed with micro hybrid quadrupole time of flight (Q-Tof) Mass Spectrometer (Waters) with Electronspray Ionization (ESI) source and *MassLinx NT* software.

## 1.2 3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-(2-acetamido-4-hydrogen-phosphonate-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate) (23), bis-triethylamonium salt



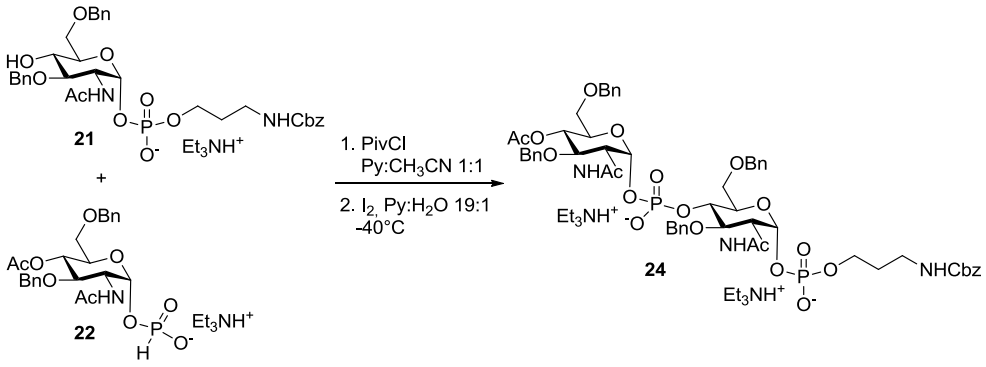

A solution of the glycosyl acceptor **21** (136 mg, 0.088 M) in pyridine (2 mL) was prepared (solution A). A solution of Salicyl chlorophosphite (71 mg, 0.176 M) in $CH_3CN$ (2 mL) was prepared in a separate flask (solution B). Equal volumes (1 mL) of the two solutions were taken and injected into the microreactor (internal volume = 13 µL) via a double syringe pump, setting the flow rate at 1.85 µL/min (total flow rate = 3.7 µL/min, corresponding to a residence time = 3.5 min). The mixture flowed from the microreactor was dropped in a TEAB 1.0M aqueous solution (1 mL) to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography ($CH_2Cl_2$/MeOH 9:1 + 1% TEA) to obtain the H-phosphonate **23** as colourless oil (49 mg, 59%).

## 1.3 2-Acetamido-4-*O*-acetyl-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl hydrogen-phosphonate (22), triethylamonium salt




A solution of the glycosyl acceptor **20** (89 mg, 0.1 M) in pyridine (2 mL) was prepared (solution A). A solution of Salicyl chlorophosphite (81 mg, 0.2 M) in $CH_3CN$ (2 mL) was prepared in a separate flask (solution B). Equal volumes (1 mL) of the two solutions were taken and injected into the microreactor (internal volume = 100 µL) via a double syringe pump, setting the flow rate at 16.5 µL/min (total flow rate = 33.3 µL/min, corresponding to a residence time = 3 min). The mixture flowed from the microreactor was dropped in a TEAB 1.0M aqueous solution (1 mL) to quench the reaction. The reaction mixture was concentrated *in vacuo* and the crude was purified by flash chromatography ($CH_2Cl_2$/MeOH 9:1 + 1% TEA) to obtain the H-phosphonate **22** as a white solid (46 mg, 59%).

## 1.4 3-(*N*-Carbobenzyloxy)aminopropyl 1-*O*-[2-Acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate 4-(4-*O*-Acetyl-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranosyl phosphate)] (24), bis-triethylammonium salt




A solution of H-phosphonate **22** (134 mg, 0.22 M) and glycosyl acceptor **21** (155 mg, 0.2 M) in pyridine (1 mL) was prepared (solution A). A solution of PivCl (72 mg, 0.3 M) in $CH_3CN$ (2 mL) was prepared in a separate flask (solution B). Equal volumes (0.5 mL) of the two solutions were taken and injected into the microreactor (internal volume = 100 µL) via a double syringe pump, setting the flow rate at 16.5 µL/min (total flow rate = 33 µL/min, corresponding to a residence time = 3 min). The reaction mixture flowed from the microreactor was collected in a separate flask. The solution was cooled to -40°C and a solution of $I_2$ (38 mg, 0.15 mmol) in Py:$H_2O$ 19:1 (1 mL) was slowly added. The temperature was raised up to 0°C, then a solution of $Na_2S_2O_3$ 0.5 M was added to quench the reaction. The mixture was extracted with $CHCl_3$ (3 x 5 mL). The combined organic layers were washed with TEAB 1.0M, dried over $Na_2SO_4$, and concentrated *in vacuo*, and purified by flash chromatography ($CH_2Cl_2$/MeOH 9:1 + 1% TEA).