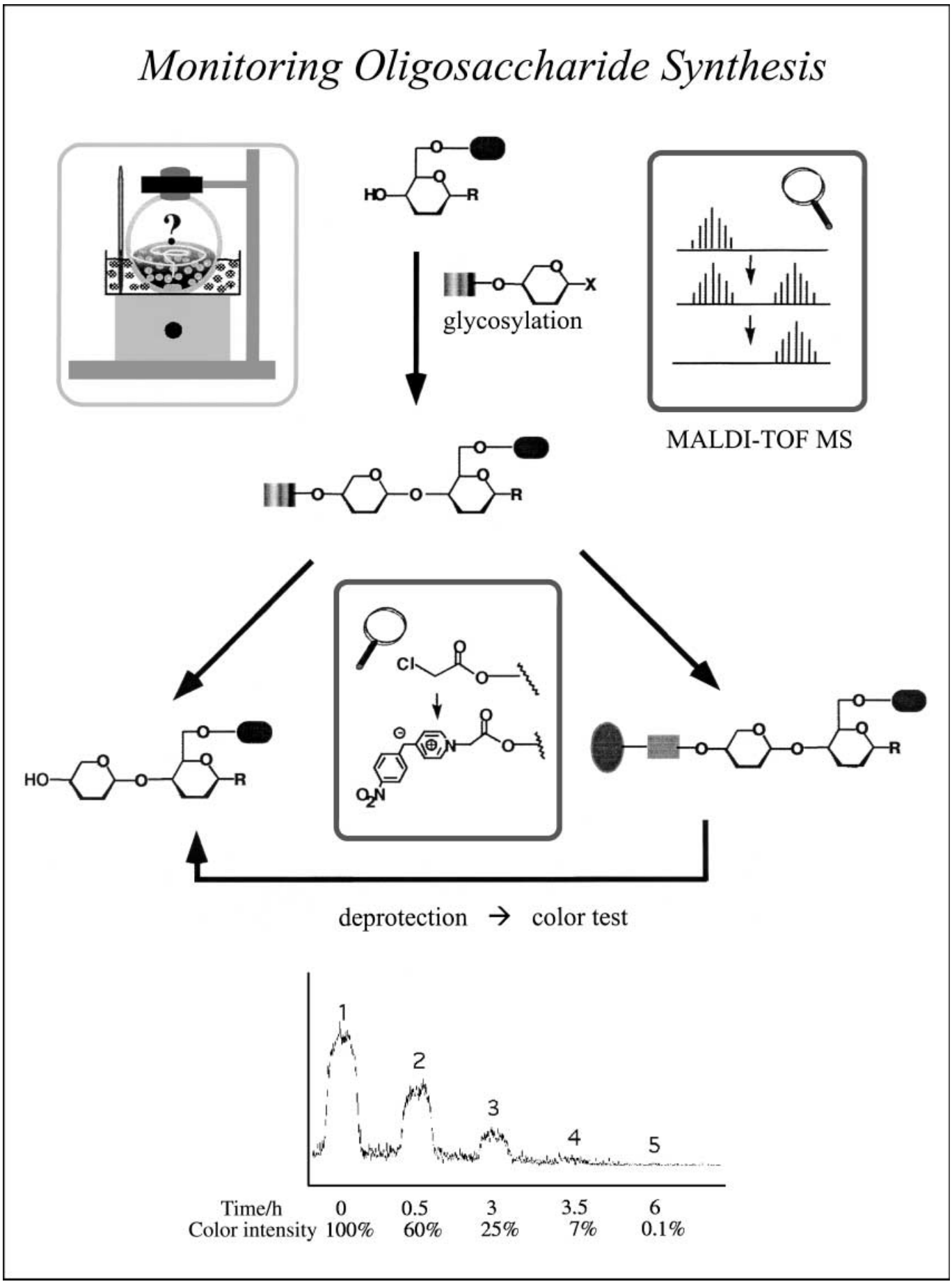

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Tag-Reporter and Resin Capture – Release Strategy in Oligosaccharide Synthesis

Yukishige Ito* and Shino Manabe[a]

Abstract: Low molecular weight poly(ethylene glycol) (LWPEG) has been found to be useful as a "tag" in oligosaccharide synthesis due to its high polarity. Real-time reaction monitoring was achieved by use of MALDI-TOF MS in the glycosylation reactions and color tests in the deprotection of the chloroacetyl group. Further, a cysteine-supported insoluble resin enables the purification of the chloroacetyl-bound compounds on soluble PEG.

Keywords: capture – release purification · oligosaccharides · polymer-supported synthesis · real-time monitoring · synthesis design

Introduction

The oligosaccharide portions of glycoconjugates are involved in various biological events, including cell adhesion, signal transduction, fertilization, protein trafficking, immune responses, malignant transformation, cell differentiation, and infection.[1] They also play important roles in protein stabilization, quality control, and degradation.[2] While the structure diversity of these molecules is extremely high, their availability from natural sources is limited, in terms of both quantity and variation. In order to pursue the detailed analysis of their functions, it is necessary to establish a methodology for synthesizing them.[3]

However, the preparation of oligosaccharides is quite often a labor-intensive task. To achieve it, multistep transformations including repetition of *O*-glycosylation and partial deprotection are required. Each sugar residue must be carefully designed so that positionally selective deprotection as well as stereoselective glycoside bond formation can be achieved. The preparation of such "tailor-made" building blocks from "raw" sugar needs a number of steps. Throughout the whole process, product isolation is usually achieved by chromatographic purification, which is very time consuming. This is particularly true whenever the glycosylation proceeds in low yield and a small amount of the glycosylated product must be separated from much larger amounts of unreacted acceptor and side product(s). In such cases, a large excess of glycosyl donor might be used to drive the reaction to completion, although the desired product inevitably becomes the minor component in the whole mixture. Since no all-encompassing method has been developed so far,[4] the identification of optimum glycosylation conditions requires extensive screening, which necessitates chromatographic separation after each trial. Any device that can remove the need for chromatographic separation would be highly useful in speeding up oligosaccharide synthesis.

Laboratory-scale preparation of oligopeptides[5] and oligonucleotides[6] can be readily conducted by an automated synthesizer. In these cases, polymer-support synthesis is adopted as the key technology. The advantage of polymer-support synthesis mainly stems from the ease of work-up of reactions. A large excess of reagents and/or coupling partners can be used to drive the reaction to completion, without complicating the product isolation. Due to the growing demand for glycoconjugate-derived oligosaccharides and their analogues as biochemical probes, polymer-support oligosaccharide synthesis is attracting a lot of attention.[7]

However, in order for polymer-support technology to be generally used, several important issues remain to be addressed. Firstly, substrates bound to polymers have an attenuated reactivity for entropic as well as steric reasons. This tends to be a serious concern, because *O*-glycosylation is generally less efficient than peptide-bond-forming reactions, and the result is often unpredictable even under conventional solution-phase conditions. Secondly, facile and sensitive methods for monitoring the progress of oligosaccharide-forming reactions on polymer supports are yet to be established. For peptide synthesis, detection of the residual amino group by ninhydrin test usually suffices. In contrast, in the case of oligosaccharide synthesis, estimation of coupling yields requires quantification of the hydroxy groups,[8] which is far more difficult. Recognizing this, several sophisticated approaches for observing the progress of the reaction, by

[a] Dr. Y. Ito, Dr. S. Manabe
RIKEN (The Institute of Physical and Chemical Research)
2-1 Hirosawa, Wako-shi, Saitama 351-0198 (Japan)
Fax: (+81) 48-462-4680
E-mail: yukito@postman.riken.go.jp

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using solid phase MAS ^{1}H NMR,[9] gated decoupling ^{13}C NMR,[10] and ^{19}F NMR,[11] have been investigated with substantial success. Alternatively, the use of photolabile linkers, allows facile post-cleavage analysis by MS and/or HPLC, proved to be useful.[12] However, any of these methods does not seem to be satisfactory for real-time monitoring.

Tag-Reporter Strategy

Besides solid-phase synthesis technology, tag-assisted solution-phase synthesis of oligosaccharide has been shown to be quite promising. For instance, Hindsgaul et al. demonstrated that hydrophobic tags are useful for facile separation of products after enzymatic glycosylation[13] or construction of glycoside libraries by chemical means.[14] On passing through a reverse-phase (C_{18}) cartridge, only oligosaccharides carrying hydrophobic tags are adsorbed. Subsequent washing with more hydrophobic solvent can retrieve them. Pozgay exploited lipophilic acyl-type protecting groups for the impressively facile synthesis of tetracosasaccharides.[15] Furthermore, oligosaccharide synthesis with soluble polymers has been investigated with substantial success.[16] In the latter case, however, room clearly remains for further improvements, particularly in terms of reaction monitoring.

Considering these circumstances together, our effort has been expended to develop a new method for real-time monitoring of the critical steps in polymer-supported oligosaccharide synthesis, namely chain elongation and chemoselective deprotection. The overview of the strategy, which is called "tag-reporter strategy" is depicted in Scheme 1.[17] Its key feature is the use of a low-molecular-weight poly(ethylene glycol) (LWPEG) supported acceptor (**1**) in combination with a glycosyl donor (**2**) carrying a monochloroacetyl (CAc) group for temporary OH protection. It allows sensitive monitoring of both the glycosylation and partial deprotection steps.

Chan et al. first reported the use of LWPEG as a support in oligosaccharide synthesis.[18] The facile product isolation takes advantage of the polar nature of LWPEG. Namely, the separation of supported material from the reaction mixture can be achieved by simple silica gel column chromatography. After initial elution with ethyl acetate (to wash out excess donor and all the side products), LWPEG-supported (tagged) material can be retrieved by elution with a more polar solvent, that is, methanol in ethyl acetate.

It was noticed that LWPEG has another important property that is useful as a "reporter"; it enables the real-time monitoring of glycosylation by MALDI-TOF MS. The spectra of LWPEG-bound materials have characteristic mountain-like shapes, because of the statistical distribution of the PEG chain length, which consists of about 8–20 ethylene glycol units. After successful coupling, the "mountain" migrates to the higher-molecular-weight region; this indicates the conversion of **1** to the coupled product **3** (Figure 1).

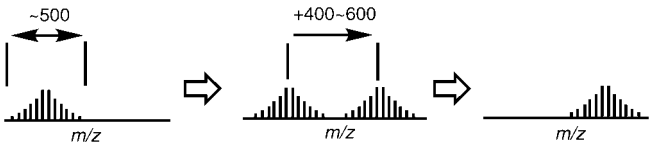
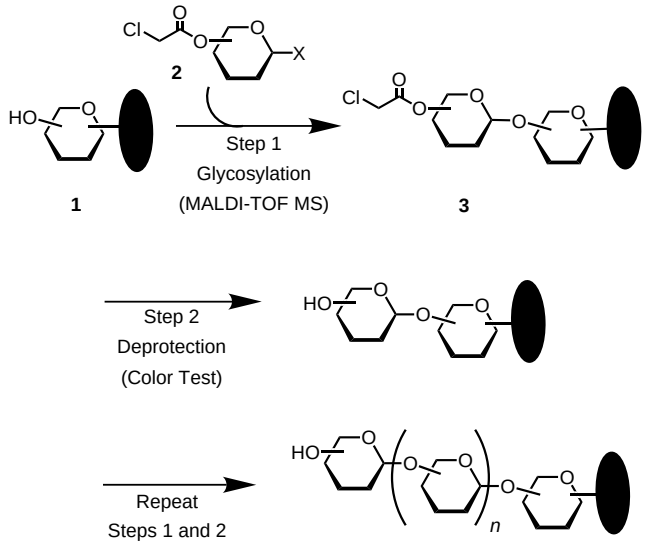
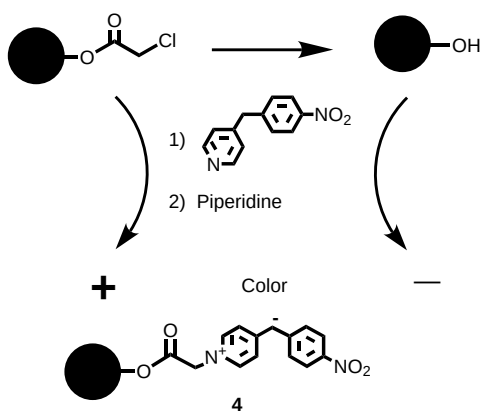


Figure 1. Monitoring the glycosylation **1** + **2** → **3** by MALDI-TOF MS.

The judicious choice of OH protecting groups is critical for any oligosaccharide synthesis to be successful. Temporary protecting groups are required that can be removed without affecting other functional groups, but that are stable under various glycosylation conditions. For the purpose of monitoring, additional requirements must be met; the protecting group's presence (or absence) should be detectable with high specificity and precision. CAc was selected to fulfill these multiple demands. For the real-time monitoring of CAc deprotection, a color test reported by Riguera[19] came to our attention. It was originally developed for the detection of alcohols and consists of three step operations; 1) conversion to tosylate, 2) treatment with *p*-nitrobenzylpyridine (PNBP), and 3) deprotonation of the pyridinium salt with piperidine. We expected that the CAc group would be detectable by PNBP/piperidine treatment, which generates the strongly colored zwitterionic salt **4** (Scheme 2).



Scheme 1. Real-time monitoring: tag-reporter strategy.



Scheme 2. Monitoring deprotection of chloroacetyl group by color test.

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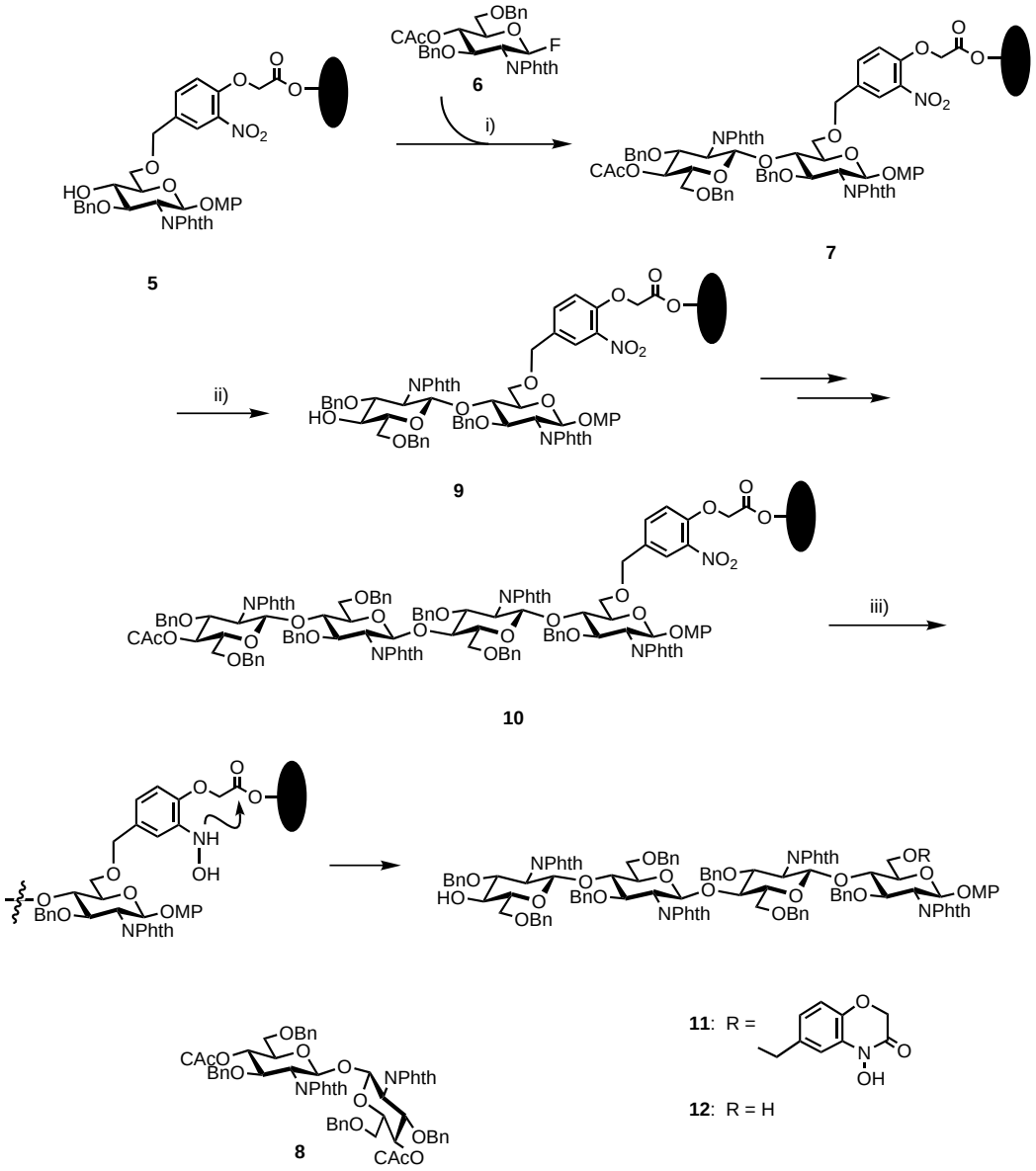
The feasibility of the strategy was tested as depicted in Scheme 3. The synthesis of the tetrasaccharide was conducted starting from the LWPEG-bound monosaccharide **5**. Since earlier investigations had revealed that conventional Wang resin-type linkers had limited stability, a novel nitro-modified linker was developed.[20] It proved to be stable under a variety of Lewis acidic *O*-glycosylation conditions and could be cleaved chemoselectively by reductive cyclorelease.

Chain elongation was performed with fluoride **6** (3 equiv) (in the presence of Cp_2HfCl_2-AgOTf[21] and CH_2Cl_2). As shown in Figure 2, the progress of the reaction was clearly monitored by MALDI-TOF MS. Complete consumption of the acceptor as well as nearly quantitative formation of the disaccharide **7** was observed as the "migration of the mountain". Concomitantly, a signal that emerged at $m/z \approx$ 1140 (asterisked) may well indicate the formation of the trehalose-like dimer **8** as a major side product. Clean formation of disaccharide **7** was also confirmed by ^{1}H NMR spectroscopy, from which the coupling yield was estimated to be $>98\,\%$.

Removal of the CAc group to give **9** was achieved by treatment with aqueous pyridine (pH 8–9) containing 4-dimethylaminopyridine (DMAP). The reaction was monitored by a TLC color test, which was carried out according to Riguera's protocol (Figure 3). Color density was quantified by the NIH Image program[22] with the aid of a scanner devise. After 6 h, nearly complete disappearance of the color was observed, and deprotection was judged to be complete. Quantitative deprotection was supported by the ^{1}H NMR spectra of the resultant **9**, which revealed the complete disappearance of the low-field H4 signal that originally appeared at $\delta = 5.25$.

Disaccharide **9** was subjected to further cycles of coupling–dechloroacetylation, which were again monitored by MALDI-TOF MS and the color test, to afford tetrasaccharide **10**



Scheme 3. Synthesis of disaccharides on LWPEG. i) AgOTf-Cp_2HfCl_2, CH_2Cl_2, -45 to $-35\,^\circ$C, $>98\,\%$; ii) DMAP, aq. pyridine; iii) $Sn(SPh)_2$, PhSH, Et_3N, benzene.

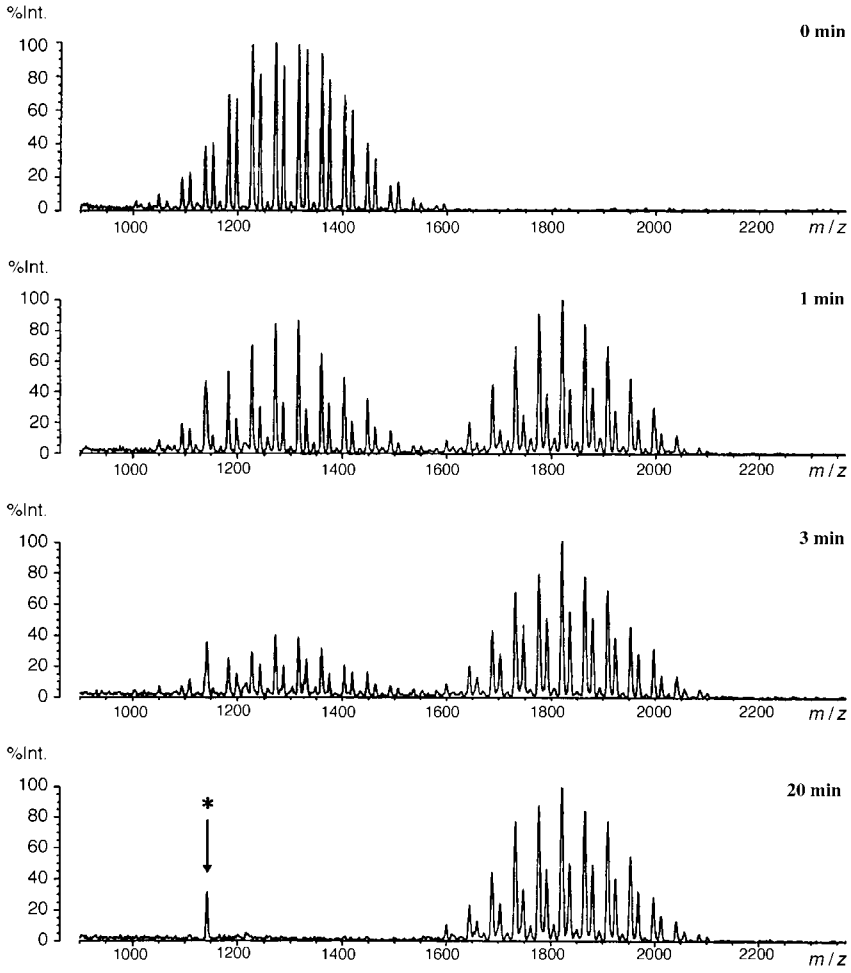


Figure 2. Monitoring the glycosylation **5** + **6** → **7** by MALDI-TOF MS.

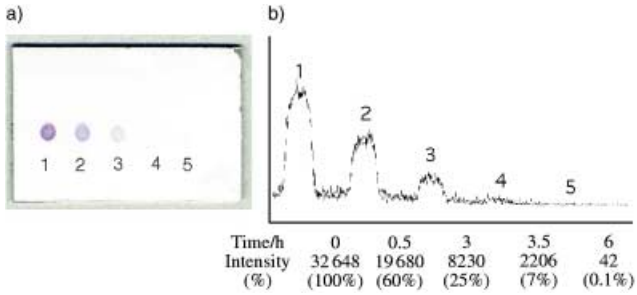


Figure 3. Monitoring dechloroacetylation by color test. a) Color test on a silica gel TLC plate after 0 h, 0.5 h, 3 h, 3.5 h, and 6 h (from left to right). b) Spot intensities were quantified by NIH image 1.62.

(Scheme 3). Liberation of the tetrasaccharide was performed under reductive cyclorelease conditions. Thus, treatment with $Sn(SPh)_2$, PhSH, and Et_3N smoothly afforded cyclic hydroxamate **11**, presumably via a hydroxylamine. In spite of its *p*-alkoxybenzyl-like structure, the hydroxamate moiety was unexpectedly resistant to oxidative (2,3-dichloro-5,6-dicyano-1,4-benzoquinone—DDQ, cerium(IV) ammonium nitrate—CAN) as well as hydrogenolytic (H_2, Pd/C) conditions. Fortunately, its removal could be achieved under mild acidic conditions (CSA/MeOH, 45 °C) to give **12** (59% overall yield from **5**).

Solid-Phase Capture–Release Strategy

Even if all reactions can be monitored, the success of polymer-support synthesis still relies on the presumption that coupling reactions can be driven to completion. Otherwise, repeated failure to attain a high degree of conversion results in the accumulation of side products, which make the isolation of correctly assembled products difficult. Especially when the reaction site is sterically hindered, it is quite common that a substantial portion of the acceptor remains unreacted, even if excess donor is used.[23]

It was inferred that the solution to this problem can be found by using additional tagging. For facile peptide synthesis, an affinity purification protocol was developed by Ramage et al.[24] Namely, by terminating the solid-phase synthesis with a tetrabenzofluorenyl-17-methoxycarbonyl (Tbfmoc) capped *N*-terminal residue, product isolation was achieved in a greatly simplified manner with graphatized carbon. Raines developed a dual affinity fusion system that enables facile isolation of expressed protein by using a polyhistidine tag in combination with an S peptide derived from RNase A.[25] The success of these approaches stems from the use of two independent tags.

Inspired by these works, a refined version of the LWPEG-supported oligosaccharide synthesis strategy was designed (Scheme 4),[26] based on the resin capture–release concept.[27] It employed a PEG-tagged glycosyl acceptor ($\mathbf{A}_1$) in combination with a CAc-carrying donor (**B**), as described before. After coupling and removal of excess donor, the PEG-tagged component consists of a coupled product **C**, together with unreacted $\mathbf{A}_1$ if the reaction is not complete. In order to discriminate between **C** and $\mathbf{A}_1$, the specific reactivity of the CAc group was used. For this purpose, resin-bound cysteine (**D**) was employed. It has a strongly nucleophilic thiol, which captures CAc-carrying molecules in the solid phase. Liberation into solution-phase can be effected by removal of the Fmoc group. The exposed amine cyclizes spontaneously to release disaccharide $\mathbf{A}_2$, which has a free OH and can be used for the next coupling. Repetition of this cycle (glycosylation–capture–release) provides the target oligosaccharide ($\mathbf{A}_n$), with a minimum need for purification.

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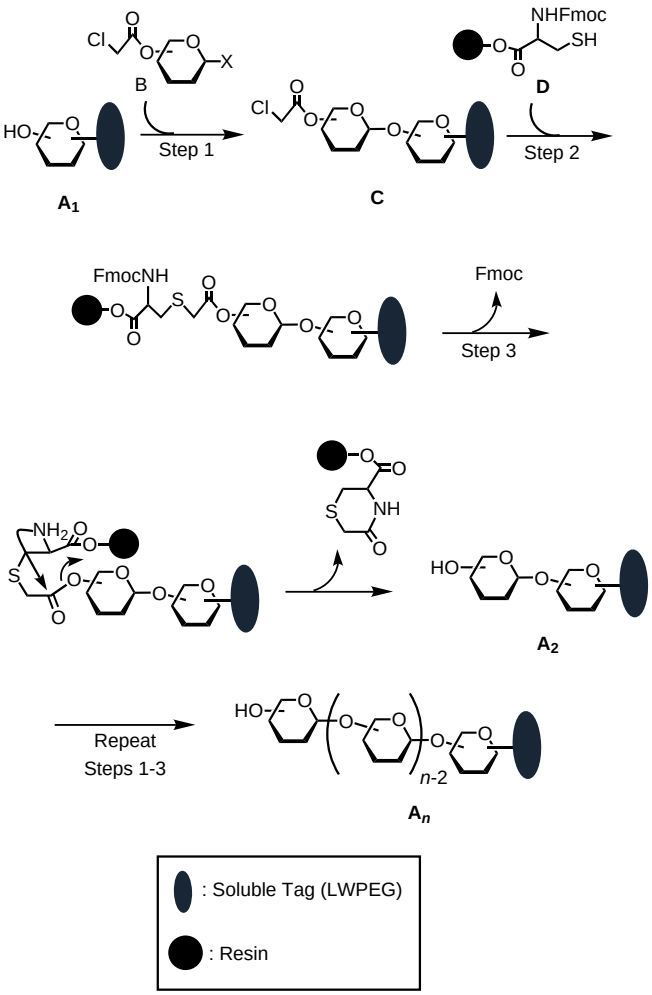
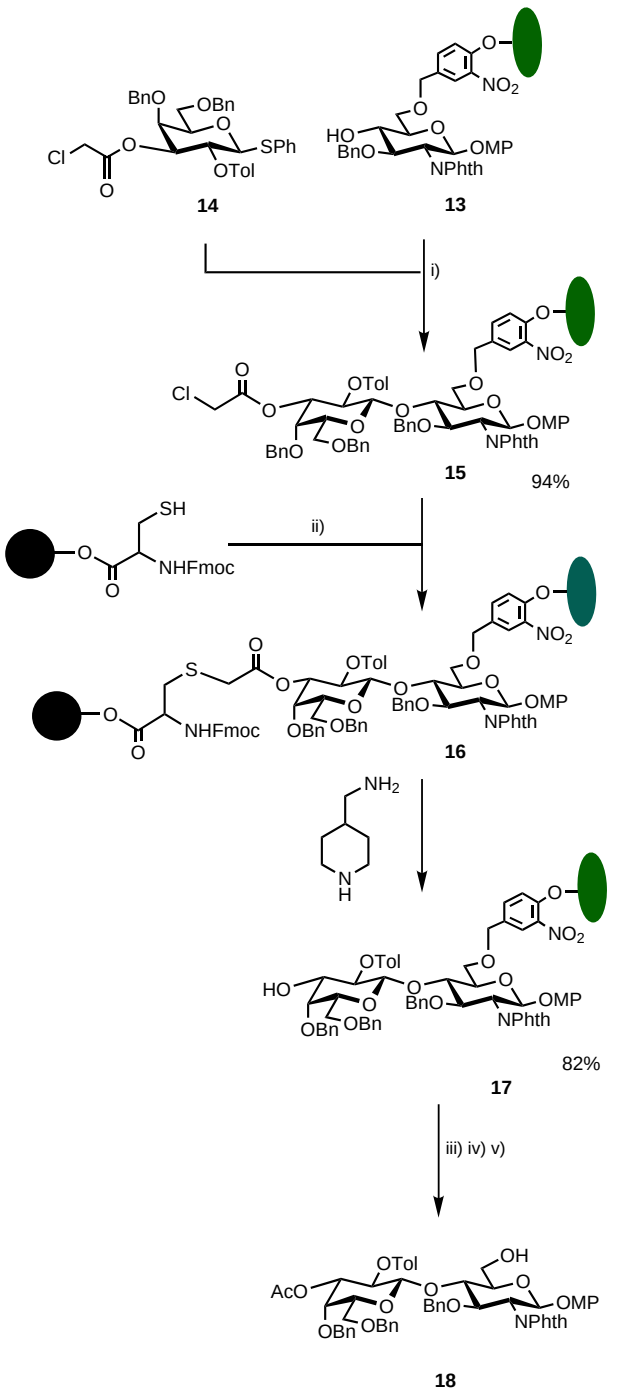


Scheme 4. Solid-phase capture–release strategy.

As a test case, a lactosamine repetition sequence was selected as a target. It is well known that polylactosamine $[(Gal\beta1\rightarrow4GlcNAc\beta1\rightarrow3)_n]$ is an important structure motif of both glycoproteins[22] and glycospingolipids.[22]

To start with, monosaccharide **13**, which has a nitro carrying safety catch linker, was prepared. Thioglycoside **14** was employed as a galactosyl donor, which was activated by dimethyl(methylthio)sulfonium triflate (DMTST)[30] in CH_2Cl_2 (Scheme 5). In order to critically evaluate the efficacy of the capture–release, the reaction was performed with a substoichiometric amount (0.9 equiv) of **14**. ^{1}H NMR as well as MALDI-TOF MS analyses of the crude product **15** revealed contamination by about 30% of unreacted acceptor **13** (Figure 4). Compound **15** was then captured with resin-supported cysteine and released by treatment with 4-(aminomethyl)piperidine to give **17**, presumably via **16**. As shown in Figure 4, acceptor **13** was completely removed. The quality of **17** can be compared with chromatographically purified disaccharide **18**, which was obtained by cleavage with Zn-Cu, Ac_2O and DDQ (86% overall).

Further chain elongation to give a tetrasaccharide was performed by repetition of glycosylation capture–release with **19** and **14** as glycosyl donors. The resultant **20** was cleaved in threes steps to give **21** (Scheme 6).



Scheme 5. Solid-phase capture–release preparation of disaccharide. i) DMTST/CH_2Cl_2; ii) iPr$_2$EtN, CH_2Cl_2-MeCN; iii) Zn–Cu; iv) Ac_2O; v) DDQ.

In principle, the solid-phase capture–release strategy avoids contamination of any deletion product, irrespective of the efficiency of the coupling reactions. Since the capturing site is three atoms away from the hexopyranoside ring carbon, this process is likely to be insensitive to steric hindrance.

Other approaches that share a similar advantage have emerged. For instance, Fukase and Kusumoto reported a clever approach[31] that utilizes 4-azido-3-chlorobenzyl[32] as protecting group. In their "catch and release" purification approach, resin-supported phosphine was used. The product,

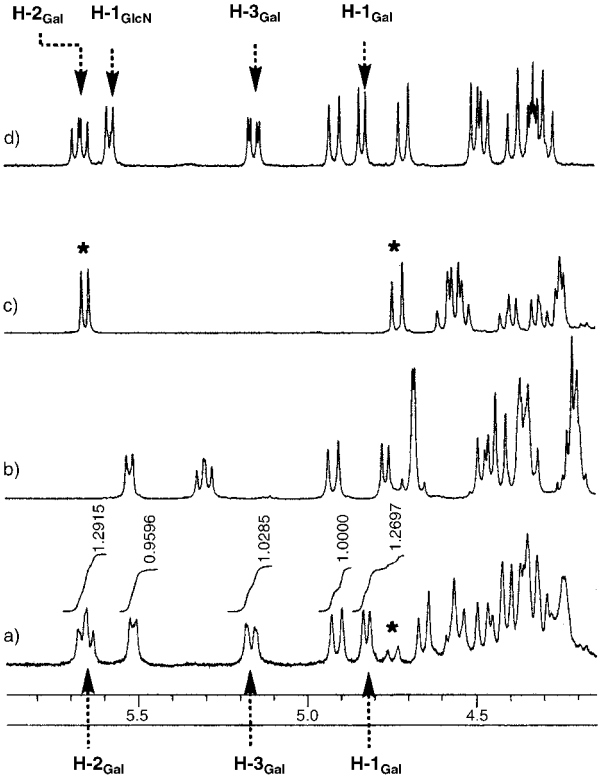


Figure 4. ^{1}H NMR spectra of a) crude **15**, b) **17** after capture–release, c) acceptor **13**, and d) purified disaccharide **18**.

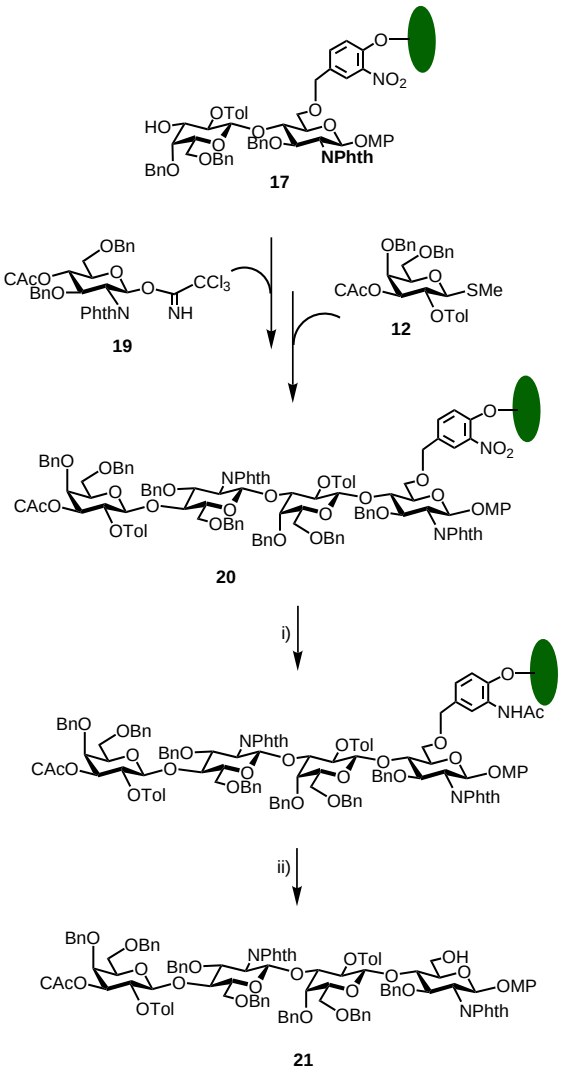
carrying a 4-azido-3-chlorobenzyl group, (**22**) was caught by a resin and released by treatment with DDQ (Scheme 7).

More recently, Seeberger reported a "cap–tag" approach for the facile isolation of oligosaccharides synthesized by solid-phase automated synthesis by using glycosyl phosphate **23** as a donor (Scheme 8).[33] In this case, unreacted acceptors were capped as either 2-azido-2-methyl propionate (**24A**) or perfluorinated silyl ether (**24F**). After completion of the chain elongation and cleavage from resin, any deletion products have these tags and can be removed either by scavenger resin (for **24A**) or perfluorinated reverse phase cartridge (for **24F**) to afford oligosaccharide **25** in a highly purified form.

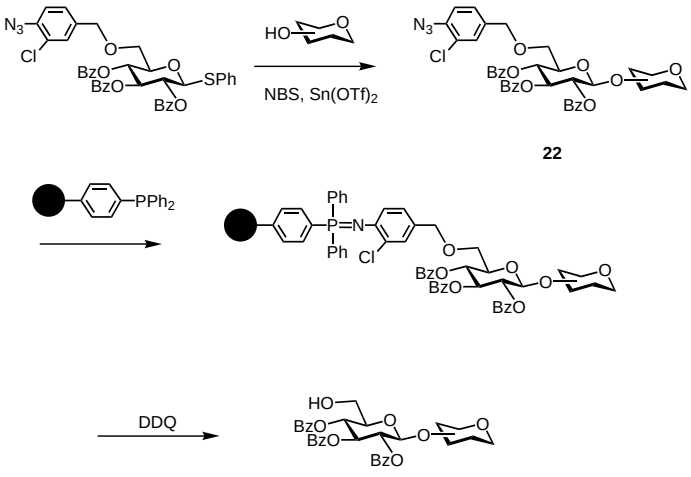
Inazu et al. reported their own approach on the "fluorous" synthesis of oligosaccharide by using Bfp (bisfluorous chain type propanoyl) as a protecting group.[34] The advantages of Bfp are easy purification by simple extraction by fluorous-organic solvent and characterization of the products by NMR, MS, and TLC.

Another interesting approach was developed by Fukase et al., in which the affinity purification method plays the key role. It is based on molecular recognition between the resin-bound bis(2,6-diamnopyridine)amide of isophthalic acid **26** and the barbituric acid derivatives **27**,[35] and was originally developed by Chang and Hamilton (Scheme 9).[36]

Stereochemical control is, of course, a fundamental problem in oligosaccharide synthesis, and is most serious in the case of polymer-support synthesis. It must be admitted that none of the strategies discussed in this account addresses this important issue. Oligosaccharides that solely consist of 1,2-*trans* glycosidic linkages were selected as an initial



Scheme 6. Assembly of tetrasaccharide. i) Zn–Cu, $CH_3COCH_2COCH_3$, then Ac_2O, ii) DDQ.



Scheme 7. Fukase–Kusumoto's catch and release purification approach.

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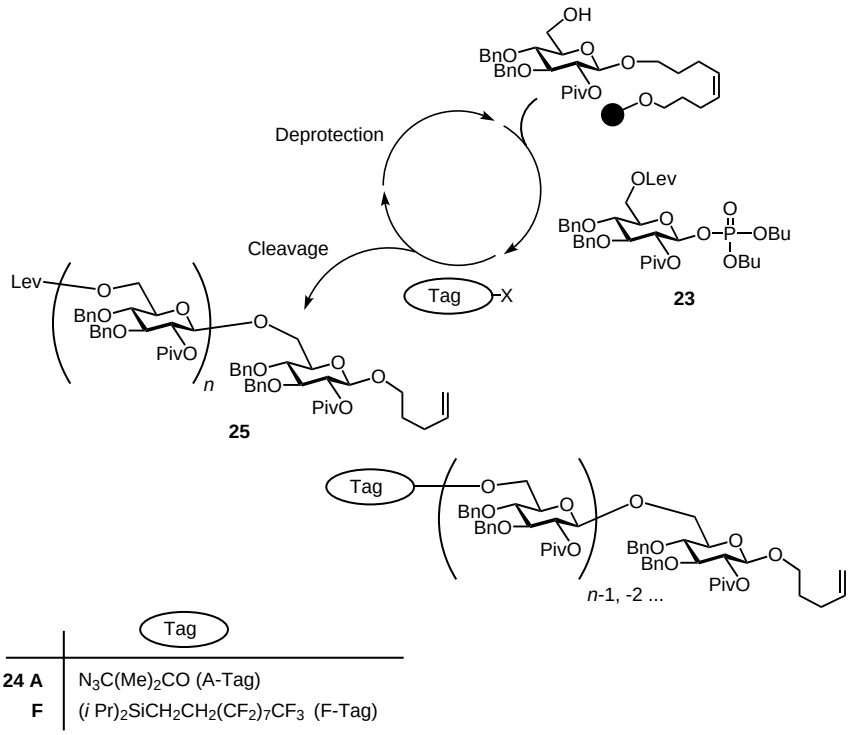
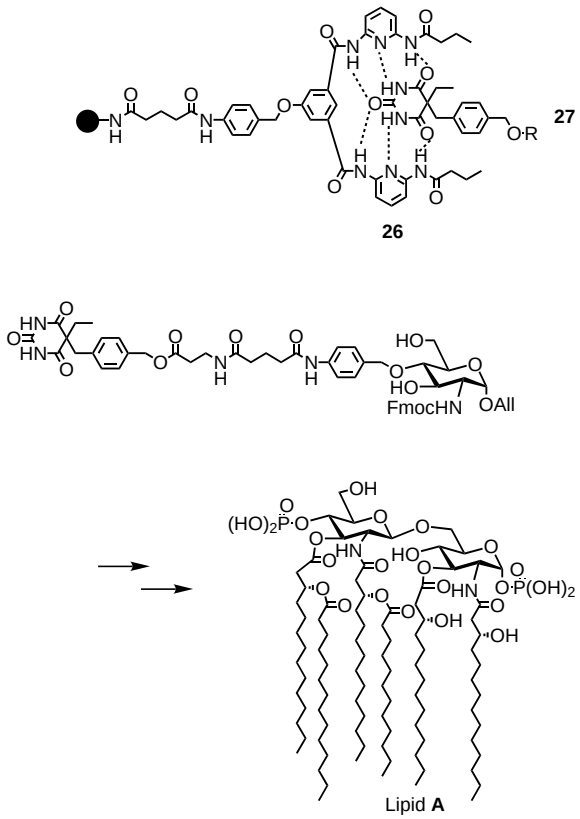


Scheme 8. Seeberger's Cap–tag approach.



Scheme 9. Fukase's Affinity purification based on molecular recognition.

target in order to avoid any stereochemical ambiguity. Continuous efforts to broaden the understanding of the factors that govern the stereochemistry of *O*-glycosylation would expand the generality of polymer-support oligosaccharide synthesis.

Acknowledgements

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