Traceless Photolabile Linker Expedites the Chemical Synthesis of Complex Oligosaccharides by Automated Glycan Assembly

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INTRODUCTION

The procurement of complex oligosaccharides in satisfactory quantities and quality as tools for glycobiology remains challenging. Harvesting from natural sources is untenable because the post-translational glycosylation pathway is heavily influenced by minute changes in the environment of the living cell, leading to multiple glycoforms. At present, chemical and enzymatic approaches, or combinations thereof, are often the only reliable methods to access pure glycans. Efforts to reduce the time and resources spent on traditional chemical syntheses have focused mainly on automated glycan assembly (AGA) and computer-assisted one-pot synthesis.

AGA relies on the delivery of building blocks to a suspension of a solid support that is equipped with a linker, followed by the addition of a suitable activating solution using either home-built instruments or the commercial Glyconeer 2.1 glycan synthesizer. Precise control of important reaction parameters such as temperature and concentration under an inert atmosphere allows for nearly quantitative coupling of building blocks to the resin. Repetitive cycles consisting of glycosylation, capping, and selective deprotection steps extend the time and resources spent on traditional chemical syntheses and the form of the glycan obtained at the end of it. Here, we describe a traceless photolabile linker used to prepare carbohydrates with a free reducing end. Modification of the o-nitrobenzyl scaffold of the linker is key to high yields and compatibility with the AGA workflow. The assembly of an asymmetrical biantennary N-glycan from oligosaccharide fragments prepared by AGA and linear as well as branched β-oligosaccharides is described to illustrate the power of the method. These substrates will serve as standards and biomarkers to examine the unique specificity of glycosyl hydrolases.

ABSTRACT: Automated glycan assembly (AGA) aims at accelerating access to synthetic oligosaccharides to meet the demand for defined glycans as tools for molecular glycobiology. The linkers used to connect the growing glycan chain to the solid support play a pivotal role in the synthesis strategy as they determine all chemical conditions used during the synthesis and the form of the glycan obtained at the end of it. Here, we describe a traceless photolabile linker used to prepare carbohydrates with a free reducing end. Modification of the o-nitrobenzyl scaffold of the linker is key to high yields and compatibility with the AGA workflow. The assembly of an asymmetrical biantennary N-glycan from oligosaccharide fragments prepared by AGA and linear as well as branched β-oligosaccharides is described to illustrate the power of the method. These substrates will serve as standards and biomarkers to examine the unique specificity of glycosyl hydrolases.
strategy to obtain an asymmetric biantennary N-glycan fragment. Additionally, several structurally defined linear and branched laminarin β-(1,3)-oligoglucans are prepared, which will be instrumental in elucidating the hydrolytic mechanism of various hydrolases from marine bacteria. Investigations into these highly specific processes are hampered by the lack of high-purity β-glucans from seawater that contain heterogeneous glycans released by different microbial species.

## RESULTS AND DISCUSSION

### Design and Synthesis of Photolabile Linkers.

One of the most frequently used photolabile groups to date is methyl-6-nitroveratryl (MeNV), which introduces two modifications to the o-nitrobenzyl (oNB) scaffold: the α-methyl group at the benzylic carbon and two methoxy groups on the aromatic ring. The methyl group was thought to improve the chemical yield by the release of a ketone byproduct instead of an aldehyde, which is less prone to side reactions. Meanwhile, the introduction of electron-donating substituents on the aromatic ring increases the absorption coefficient at longer wavelengths to match the peak emission at 366 nm from a mercury UV lamp. A design based on the MeNV motif should improve the photocleavage process. To provide an anchor point to the solid support, one of the methoxy groups in MeNV was substituted. Three different MeNV-type linkers (5, 6, and 7; Figure 2) were prepared starting from apocynin, an abundant plant extract. After photocleavage, the resin equipped with linker 5 will produce spacer-free reducible glycans while linker 6 returns glycans with the aminoalkyl spacer and linker 7 produces benzyl-protected glycans at the reducing end. For a direct comparison, oNB-type linkers 8 and 3 were prepared to provide the same products as linkers 5 and 6. Resin loading was determined for each batch of resin by the glycosylation of 50 mg of resin with 6-O-Fmoc mannose building block A on the synthesizer (Scheme 2). Cleavage of the Fmoc group present on the solid support was quantified by UV−vis spectrophotometry and revealed loading values ranging from 0.32 to 0.36 mmol/g.

Linker 8 was prepared by the reduction of 5-hydroxy-2-nitrobenzaldehyde 9 and immobilization on Merrifield resin by heating with Cs₂CO₃ in the presence of catalytic amounts of TBAI (Scheme 1A). Linker 3 was prepared starting from the reductive condensation of 9 with 5-amino-pentan-1-ol to yield 11 in 85% yield. The addition of Cbz to the secondary amine was followed by immobilization onto the Merrifield resin to obtain linker-equipped resin 3 (Scheme 1B).

Syntheses of linkers containing the MeNV scaffold started from allyl-protected apocynin, which was selectively nitrated with KNO₃ in TFA. This reaction is very sensitive to the initial temperature because dinitrated products were formed when a slight excess of KNO₃ was introduced at 25 °C. Reactions initiated at 0 °C produced only trace amounts of 13. The reaction proceeded best when KNO₃ was added portionwise at exactly 10 °C, followed by heating at 60 °C for 4 h to give mononitro 13 selectively (59% conversion starting from 12). Next, the reduction of the ketone and cleavage of the allyl group employing Pd(PPh₃)₄ proceeded smoothly to provide precursor 15, which was ready for conjugation with the resin to give linker 5 (Scheme 1C).
benzylether-linked variations of linker 5 were prepared. The reductive amination of ketone 13 was known for its poor yield.\(^{17}\) Reasonable conversion was achieved by using \(\text{Ti}(\text{O} \cdot \text{Pr})_4\) in a one-pot transformation of 13 to Cbz-protected compound 16 (39% over three steps).\(^{18}\) Removal of the allyl group and on-resin functionalization afforded linker 6 (Scheme 1D). Double deallylation of 17 to linker 7 proceeded best when 1,3-dimethylbarbituric acid replaced \(\text{K}_2\text{CO}_3\) as a scavenger in which the latter failed to completely remove the protecting groups (Scheme 1E).

**Evaluation of Photolabile Linkers for AGA.** With the photocleavable linkers in hand, the evaluation of the support-bound photolytic process was based on the quantity of fully protected oligosaccharides released after cleavage (Scheme 2).

Recovery of the cleaved product is an important parameter that emphasizes the practical utility of each functionalized resin. The automated synthesis of tetra-mannose 22 proceeded as follows: functionalized resin 3 (40 mg = 0.013 mmol) was placed in the reaction vessel before acidic wash module I was executed (TMSOTf in \(\text{CH}_2\text{Cl}_2\) at \(-20^\circ\text{C} \) for 3 min) to remove any residual base and water from the resin. Next, module IIa delivered 6.5 equiv of mannose A and a solution of activator (NIS/TfOH in \(\text{CH}_2\text{Cl}_2\)/dioxane, \(-20^\circ\text{C} \) for 5 min and then \(0^\circ\text{C} \) for 20 min) to the reaction vessel. Capping module III (MeOH in \(\text{Ac}_2\text{O}/\text{CH}_2\text{Cl}_2\), 20 min) masked any unreacted hydroxyl groups, followed by cleavage of the Fmoc carbonate on the C-6 hydroxyl group with module IVa (20% piperidine in DMF, 5 min). Module sequence I-IIa-III-IVa was repeated four times to obtain resin-bound tetra-mannose 22. Cleavage of 22 from the solid support was achieved using a continuous flow photoreactor.\(^{19}\) Monomannose, tetra-\(\alpha\)-(1,6)-mannose, and hexa-\(\beta\)-(1,4)-glucose were assembled on resins equipped with different linkers. All linkers proved to be compatible with all standardized AGA protocols. No trace of deletion sequences was observed in any of the HPLC chromatograms of the crude products after photocleavage. Thus, differences in the isolated yield should correlate exclusively with the photolytic sensitivity of each linker. Linkers 5 and 8, for example, both delivered the free reducing end sugars but differed significantly in their photolytic efficiency. For \(\alpha\text{NB}-\text{type} \) linker 8, the highest conversion observed was monomer 18 at only 34%, with even lower yields for tetra-mannose (21, 22%), and hexa-glucose (24, 17%). In contrast, MeNV-type linker 5 consistently delivered the same products in 65–70% yield. \(O\)-linked resin 5 performed best in this study, surpassing both the \(N\)-linked resin 6 and \(O\)-Bn-linked resin 7.

The resin equipped with \(N\)-linked \(\alpha\text{NB} \) 3 outperformed \(N\)-linked MeNV-type resin 6 in all experiments to procure 5-aminopentyl mannose 19, tetra-mannoside 22, and hexa-glucose 25. Still, the resin with linker 6 released more product than with linker 8. The photolytic sensitivity of \(O\)-Bn-linked resin 7 was found to be between that of the \(N\)-linked resins 3 and 6. The following order of photocleavage performance was observed: 5 > 3 > 7 > 6 > 8 (direct \(O\)-linked MeNV-type > \(N\)-linked \(\alpha\text{NB}\)-type > O-Bn-linked MeNV-type > \(N\)-linked MeNV-type > direct \(O\)-linked \(\alpha\text{NB}\)-type). This seemingly counterintuitive series highlights one of the caveats when picking a suitable photocleavable scavenger (Figure 3).

**Assembly of an Asymmetric \(N\)-Glycan Fragment.** AGA of asymmetrically branched octasaccharide 27, present in complex-type \(N\)-glycans,\(^{21,22}\) served to illustrate the utility of the new linker (Figure 3). The complexity of \(N\)-glycans and their biological importance have rendered them attractive targets for testing chemical\(^{25,28}\) and chemoenzymatic synthesis methods.\(^{29,32}\) Following the convergence strategy employed by most solution-phase syntheses of biantennary \(N\)-glycans, we embarked on a \([5+3]\) glycosylation strategy. Target oligosaccharide 27 was constructed by the union of trisaccharide donor 28 and pentasaccharide acceptor 29, which were both prepared by AGA (Figure 3). Resins with
photocleavable linkers 5 and 3 and six commercially available building blocks (C−H) were employed in the automated syntheses.

The automated synthesis of donor 28 commenced with glucosamine C (Figure 4), and the orthogonal removal of levulinoyl ester at the C-3 position was achieved by executing module IVb (N$_2$H$_4$·HOAc in pyridine). Stereoselective α-fucosylation was secured by running glycosylation module IIa twice for fucose D at lower temperature (−40 °C for 5 min and then at −20 °C for 20 min). The Fmoc group at the C-4 position of C was removed with module IVa (20% piperidine in DMF). Next, modules I-IIa-III-IVa were executed to

**Figure 4.** Automated synthesis of trisaccharide donor 28. A typical glycosylation cycle included module sequence I-IIa-III-IVa, represented by the letter of the building block being used (e.g., C). Changes in the sequence or parameters are indicated with a broken line and a box.
introduce galactose F as the third sugar. By capping after the
deprotection module, the Fmoc group at C-6 of galactose E
was converted to the more resilient acetate group. At any point
during the automated process, a small amount of resin could
be extracted and irradiated using a portable UV lamp (6 W, 366 nm) for 10 min to release the glycans from the solid
support. This convenient real-time monitoring of the reaction
progress via HPLC and MALDI analysis helped to optimize
the reaction conditions and identify potential problems.

Photocleavage from the solid support afforded trisaccharide
30 as a mixture of α/β-anomers in 52% yield. Fluorination of
30 by Deoxo-Fluor yielded glycosyl fluoride 28 in 89% yield
after HPLC purification. Glycosyl fluorides are glycosyl donors
for both chemical 33 and enzymatic 34 trans-glycosylation
reactions.

With glycosyl donor 28 in hand, the automated synthesis of
glycosyl acceptor 29 was initiated (Figure 5). Branching at the
C-6 position of mannose F was achieved via the stepwise
introduction of mannose G, glucosamine H, and galactose E
with the on-resin capping of C-6 as the acetyl ester (module
IVA and then III). The Lev ester on mannose F was cleared to
reveal the C-3 hydroxyl group, ready for branching with
mannose building block G. Fmoc removal on G concluded the
automated sequence before photolytic release furnished
acceptor 29 (Figure 5A). However, building block sequence
FGHEG was not crowned by success. An analysis of the crude
mixture by HPLC and MALDI revealed the presence of several
deletion sequences and regioisomeric products (Figure 5B).
Deletion sequences resulting from incomplete couplings were
avoided by using the glycosylation modules twice on
unreactive building blocks such as F and H. Fmoc cleavage
using piperidine was previously found to be accompanied by
the partial migration of Lev to the liberated hydroxyl group.35
This unwanted byproduct was not formed when Et3N was used
as a base. Thus, we introduced deprotection module IVc (20% Et3N in DMF) to replace module IVA. Glycosylation module
IIa was used twice for building blocks F and H. The coupling
time needed to introduce building blocks H and E was
increased from 20 to 40 min. Gratifyingly, refined building block
sequence F,G,H,E,G significantly improved the synthesis,
evident from the HPLC traces of the crude product showing
only two peaks (Figure 5C). The structural analysis of each
compound revealed that the main glycan was acceptor 29 and
the minor was its diastereoisomer having an aberrant Man-β-
(1,3)-Man glycosidic bond with a 1JCH value of 160 Hz at 95.8
ppm (Figure 5A, circled in red). To support this assignment,
AGA of glycan sequence F,G,H,E proceeded smoothly to
afford the congener tetrasaccharide as the sole product (SI,
compound S6). The poor stereoselectivity (α/β = 3:2) of the
last glycosylation cycle leading to 29 was unexpected because
the preliminary screening of all building blocks showed
excellent selectivity. The “double stereodifferentiation” effect,
also referred to as the “matched and mismatched” principle,
may account for the stereochemical scrambling in the synthesis of β-(1,3)-glucans.37 A similar mechanism could be responsible
for the substantial ratio of β-isomer of 29.

With both glycosyl donor 28 and glycosyl acceptor 29 in
hand, the glycosylation reaction promoted by AgOTf/Cp3HCl was
further optimized in 21% yield (Scheme 3). An aliquot taken from the reaction vial after 4 h was analyzed by analytical HPLC and showed the nearly complete disappearance of donor 20. Product 27 was found to elute in close proximity to glycosyl acceptor 29. The fraction containing only glycan 27 was collected, and 1H-, 13C-, and coupled HSQC-NMR experiments confirmed the homogeneity of the sample, indicating a successful β-glucosaminidation. The synthesis of asymmetrical N-glycan octasaccharide 27 from glycan fragments prepared by AGA highlights the utility of
traceless photolabile linker S for the convergent synthesis of
complex oligosaccharides.

**Automated Synthesis of Oligo-β-glucans.** Marine algae are major carbon sinks that convert carbon dioxide
into carbohydrate materials such as laminarin, an oligosaccharide
comprising β-(1,3)-linked glucose with variable degrees of β-
(1,4)- and β-(1,6)-glucose residues in the backbone and the
branches. Studies concerning the laminarin hydrolyase mecha-
nism require homogeneous β-(1,3)-glucans. Traceless resin 5
expedites the automated synthesis of oligosaccharides that
serve as analytical standards and substrates for investigating
the ecological roles of laminarin.

Convergent solution-phase syntheses of β-(1,3)-glucans
were hampered by low yields and aberrant α-linkage formation
due to double-stereodifferentiation effects as well as limitations
in the chain length, position, and degree of branching. AGA of
linear and branched β-(1,3)-glucans carrying an aminoalkyl
spacer at the reducing end was achieved earlier using glycosyl

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**Figure 5.** (A) Automated synthesis of pentasaccharide acceptor 29 using building blocks E-H. (B) Standard AGA condition leading to complicated HPLC traces of crude products. (C) Optimized condition revealing glycan 29 as a mixture of diastereoisomers.
phosphate building blocks and photolabile linker. 3.38
Unprotected, spacer-free laminarin derivatives 31–34 were
chosen as model substrates for comparing the linker perform-
ce to that in the earlier study (Scheme 4). In addition to
linear β-(1,3)-heptaglucose 31, different branched β-glucans
such as penta-glucose 32 as well as octa-glucoses 33 and 34
were prepared (Scheme 4, circled in red). More reactive
phosphate building blocks K and L required 4.0 equiv for the
AGA cycle. Extension of the linear β-(1,3)-glucose backbone
employed building block K bearing an Fmoc group at the C-3
position. For structures with a β-(1,6)-branch, the linear
backbone was first built up using the predefined sequence of
glycosyl phosphates K and L. Building block L contains a C-3
Fmoc-protected hydroxyl group and a Lev ester on the C-6
hydroxyl. Selective removal of the levulinyl ester allowed for
the extension of the β-(1,6)-branch. Module IVa (20% piperidine in DMF) was used to remove Fmoc, except when
Lev was present, in which case module IVc (20% Et3N in
DMF) was employed. Further optimization 36 resulted in a 20%
reduction in building block consumption. The methanalysis of
base-labile protecting groups directly on the solid support was
more effective than in solution. The partially protected
oligosaccharides were photocleaved from the resin and
immediately debenzylated via hydrogenation using the Pd/C
catalyst (60 psi in <1 h, module VII). With this protocol, only
a single purification with reverse-phase HPLC was needed to
yield oligosaccharides 31 and 32 in 30 and 22% yields,
respectively. Branched octasaccharides 33 and 34 were isolated
in lower quantities because these glycans unexpectedly
fragmented during hydrogenation to create mixtures of
truncated glycans.

CONCLUSIONS
Traceless photolabile linkers for automated glycan assembly
were developed to prepare complex oligosaccharides with a
free reducing end. For oligosaccharides with an aminoalkyl
spacer at the reducing end, nNB-type linker 3 offers the best
photocleavage efficiency, whereas for spacer-free hemiacetal
glycans, MeNV-type linker 5 is the best choice. The new linker
enabled the convergent synthesis of an asymmetrically
branched N-glycan octasaccharide and pure laminarins.
Levulinoyl ester migration during Fmoc cleavage is suppressed

Scheme 4. Automated Synthesis of Oligo-β-glucans
by using Et\textsubscript{3}N as a base. AGA of four β-(1,3)-glucans was achieved using 20\% fewer building blocks than previously. A new protocol for the global deprotection of oligosaccharides with a free reducing end was developed. AGA using the new linkers enables access to glycans that can be converted to glycosylating agents to be used on block couplings. Glycans with a free reducing end are valuable standards for mass spectrometry and biological assays.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.9b03769.

Synthesis procedures, AGA modules, and characterization data of glycans, including HPLC chromatograms and NMR spectra (PDF)

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We thank the Max-Planck Society, EU ITN Marie-Curie Program (IMMUNOSHAPE grant no. 642870), Minerva-Fast-Track Program, and MPG-FiFo Cooperation Project Glyco3-Display for generous financial support. We thank Eva Settels and Olaf Niemeyer for technical support.

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