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# Preparation of oligosaccharides by homogenous enzymatic synthesis and solid phase extraction†‡

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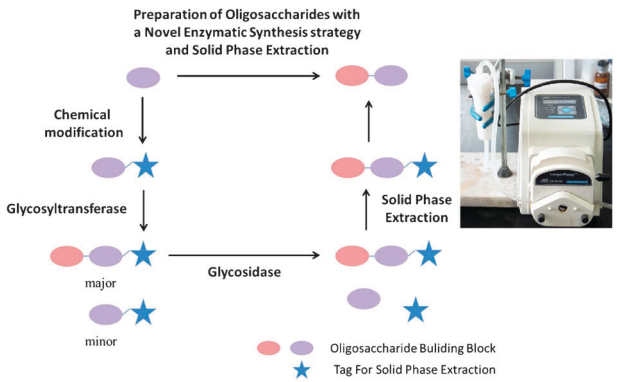
**This communication describes a method for enzymatic preparation of bioactive glycans, which integrated the high-efficiency of homogenous phase enzymatic reaction and fast separation of solid phase extraction.**

Preparation of bioactive glycans is of great significance for probing their biological functions and therapeutic applications. Although organic synthesis has been successfully applied in preparing grams of biomacromolecules such as polypeptides and nucleotides, when turning to glycans, it is much less efficient. A milestone work in this field was conducted by Seeberger and coworkers, utilizing a cross-linked polystyrene to synthesize a pentamannoside automatically.[1] To overcome the inherent deficiencies in solid phase glycans synthesis, a fluorinated tag was introduced by Pohl and coworkers to homogenous chemical glycans synthesis.[2] The target molecules can be efficiently purified by fluorous solid-phase extraction (FSPE) *via* fluorous–fluorous interactions. Compared to the traditional organic method, enzymatic synthesis works in a high stereo and regio selective manner avoiding multistep protection/deprotection and has been extensively used in glycan preparation.[3] By applying enzymatic synthesis on controlled-pore glass (CPG), Wong and coworkers prepared $SLe^x$ with a relatively low yield of 35% due to low reactivity, non-linear kinetics, stereochemical complexity and analytical difficulty.[4] A water-soluble polymer was later used instead of a solid phase to compensate the drawbacks,[5] whereas complicated chemical steps were required for ligating and releasing the glycans.

Herein, we report an improved in-solution enzymatic synthesis and purification strategy for the preparation of glycans. The strategy (Scheme 1) includes 5 steps: (1) chemical preparation of sugar acceptors with a cleavable hydrophobic tag; (2) enzymatic synthesis of glycans by glycosyltransferases in a homogeneous phase; (3) digestion of the sugar acceptors by special exo-glycosidases; (4) fast separation and concentrating of products *via* solid phase extraction (SPE); (5) releasing the hydrophobic tag and removing it by centrifugation or evaporation. Compared to the widely used size exclusive chromatography approach, which suffers from the dilution of desired compounds, SPE is able to enrich a tagged compound, thus exhibiting more delightful application potentials in large scale preparation of glycans. To ensure the purity, efficiency and recovery, glycosidases were introduced to assist with SPE.




**Scheme 1** Preparation of oligosaccharides with a novel enzymatic synthetic strategy and solid phase extraction.

A fluorenylmethyloxycarbonyl group and a phenylmethyloxycarbonyl group were chosen as hydrophobic tags for the following concerns: (1) they can be easily removed under basic conditions or by catalytic hydrogenation, respectively; (2) they are inert in glycosylation reactions (pH = 7.5). To facilitate the synthesis of the corresponding glyco-conjugates, an amine group was chosen as a "plug-in" group. Fluorenylmethyloxycarbonyl-(2-aminoethyl)-4-O-β-D-galactopyranosyl-β-D-glucopyranose (LacFmoc, compound **2**) and *N*′-phenylmethyloxycarbonyl-(2-aminoethyl)-2-(acetylamino)-2-deoxy-β-D-glucopyranose (GlcNAcCbz, compound **8**) were synthesized following the method as illustrated in Scheme 2. Only one step is required to transfer amide-containing acceptors to the corresponding hydrophobic-tag labeling ones.

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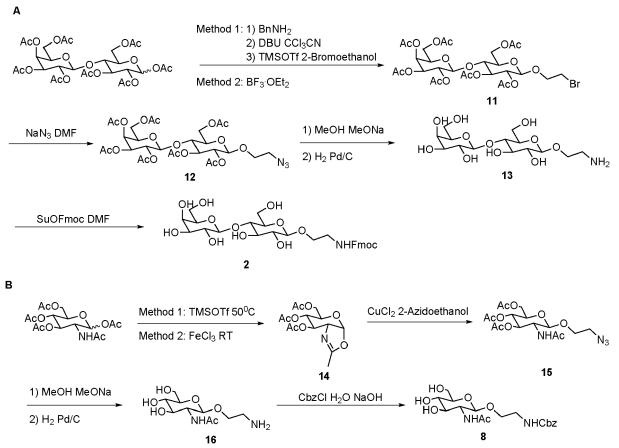
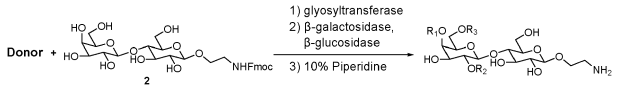


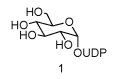
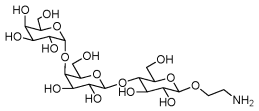
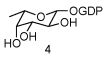
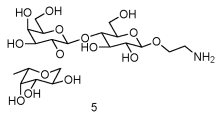
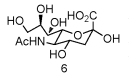
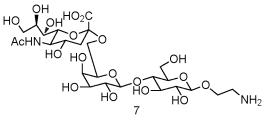
**Scheme 2** The synthesis of sugar primers with a hydrophobic tag.

In proof-of-concept experiments, Gb3 (compound **3**), type V human blood group H-antigen (compound **5**) and 6′-sialyllactose (compound **7**) were prepared using three glycosyltransferases (α1,4-galactosyltransferase (Lgt C),[6] α1,2-fucosyltransferase (WbsJ)[7] and α2,6-sialyltransferase (Pd2,6SiaT)[8]) respectively. In the preparation of **3**, UDP-Glc C4-epimerase (GalE) was employed to convert uridine 5′-diphosphoglucose (UDP-Glc, compound **1**) to precious uridine 5′-diphosphogalactose (UDP-Gal).[9] And in the preparation of **7**, cytidine 5′-monophosphate sialic acid synthase (NmCSS) was introduced to generate cytidine 5′-monophosphate sialic acid (CMP-Sia) from sialic acid and cytidine triphosphate *in situ*.[8]

Gb3 (compound **3**) was synthesized in a system containing acceptor **2**, UDP-Glc, GalE and LgtC. The reaction was allowed to proceed at 37 °C (pH = 7.5) with brief agitation and was monitored by TLC and $^{13}C$ DEPT135. After quenched by boiling for 5 min, β-galactosidase and β-glucosidase were added to a digest intact acceptor. After brief centrifugation, the supernatant was directly loaded on the SPE (C18 as a stationary phase). The Fmoc tagged Gb3 was enriched on the column, whereas the byproducts, including UDP, UDP-Glc, glucose, galactose and buffer salts, flowed through the column. After being eluted with 20% methanol, the Fmoc tag was released by adding 10% piperidine in water. Released pure Gb3 was collected from the supernatant after brief centrifugation. The yield of this reaction is 85% (Table 1, entry a). Furthermore, compound **3** can be coupled to BSA under the EDC/sulfo-NHS system to form a hapten-carrier protein complex

**Table 1** Enzymatic synthesis and purification of trisaccharides

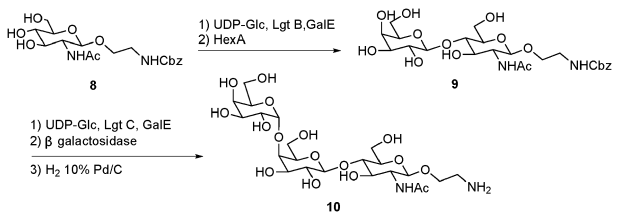


| Entry | Donor | Enzymes | Product | Yield[a] (%) |
|---|---|---|---|---|
| a |  | Lgt C, GalE |  | 85 |
| b |  | WbsJ |  | 70 |
| c |  | NmCSS, Pd2, 6SiaT |  | 80 |

[a] Isolated yield from SPE.

(detailed procedure and results are described in ESI†). The usage of glycosidases is essential for the purity of the products. The products obtained are 97% pure (Fig. 1B) when glycosidase was included, but only 85% pure without glycosidase. These results revealed the efficiency of enzymatic synthesis and purification.

The conversions from compound **2** to compounds **5** and **7** were catalyzed by WbsJ or Pd2,6-SiaT, respectively. Following the same procedure mentioned above, compound **5** was obtained with a total yield of 70% (Table 1, entry b). As to the highly hydrophilic compound, such as compound **7**, NaCl was added to the reaction mixture to a final concentration of



**Scheme 3** Enzymatic synthesis and purification of P1 trisaccharide in tandem.th.

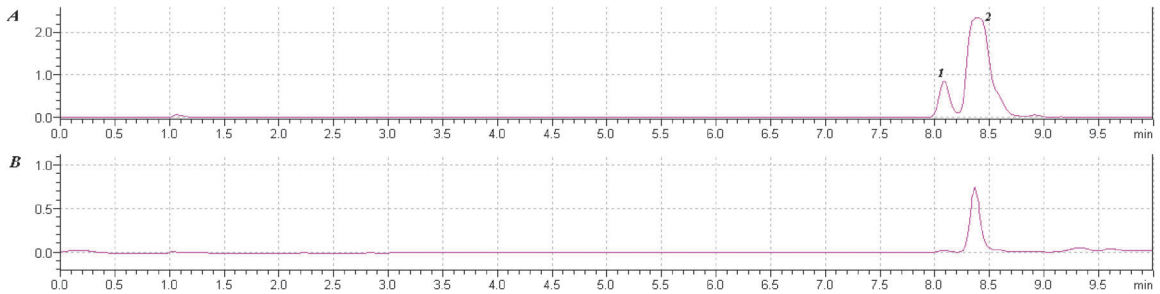


**Fig. 1** (A) HPLC profile of GB3-Fmoc purified without glycosidases, peak 1 is Lactose-Fmoc, and peak 2 is Gb3-Fmoc. Two peaks were confirmed by LC-MS; (B) HPLC profile of GB3-Fmoc purified with glycosidases.

200 mM before loading to the SPE column. After deprotection, compound **7** can be afforded with a yield of 80% (Table 1, entry c).

P1 trisaccharide (Gal-α(1,4)-Gal-β(1,4)-GlcNAc, compound **10**) was also prepared *via* a cascade enzymatic catalyzed reaction[10] starting from GlcNAcCbz (compound **8**) and UDP-Glc catalyzed by a β-1,4-galactosyltransferase (Lgt B),[11] GalE and Lgt C (Scheme 3). β-Galactosidase was subsequently added to digest the unreacted compound **9** and β-*N*-acetylglucosaminidase (HexA) was added to remove unreacted compound **8**. The final product was purified following the same procedure for preparing compound **3**. The Cbz tagged P1 trisaccharide was separated by SPE as compound **3**, and P1 trisaccharide was released by catalytic hydrogenation. The total yield of the reaction was 45%.

In summary, we reported a facile strategy for glycan preparation. With the aid of the combined glycosyltransferases-glycosidases strategy, this method utilizes a simple hydrophobic tag and SPE separation to get purified glycans. In this report, 8 enzymes with various functions cooperated to synthesize 4 bioactive glycans with a total yield of up to 85%, demonstrating that this method integrates the high-efficiency of homogenous phase enzymatic reactions and fast separation of solid phase technology. Further studies on the large scale preparation of more complicated glycans *via* the strategy are undergoing.

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