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## Chemoenzymatic assembly of mammalian O-mannose glycans

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### Abstract

O-Mannose glycans, a family of highly heterogeneous complex glycans account up to 30% of total O-glycans in brain. Previous synthesis and functional studies only focused on the Core M3 O-mannose glycans of  $\alpha$ -dystroglycan which are a causative factor for various muscular diseases. In this study, a highly efficient chemoenzymatic strategy was developed that enabled the first collective synthesis of 63 Core M1 and Core M2 O-mannose glycans. This chemoenzymatic strategy features the gram-scale chemical synthesis of 5 judiciously designed core structures, and the diversity-oriented modification of the core structures with 3 enzyme modules to provide 58 complex O-mannose glycans in a linear sequence that does not exceed 4 steps. Of note, 55 of these O-mannose glycans are synthesized for the first time. Using the printed O-mannose glycan array, the human brain protein CD33, a key factor in the modulation of Alzheimer's disease was identified as strongly binding to sialylated Core M1 and Core M2.

### Keywords

O-mannose glycan; chemoenzymatic synthesis; glycosylation; dystroglycan; glycosyltransferase

$\alpha$ -Dystroglycan ( $\alpha$ -DG) is a highly glycosylated membrane-associated protein with more than half of the mass contributed by glycosylation. In addition to N-linked and conventional mucin-type O-linked *N*-acetylgalactosamine (O-GalNAc) glycans, the O-mannose glycans have been identified as the third most common glycosylation modification on  $\alpha$ -DG. The O-mannose glycans on  $\alpha$ -DG are known to play essential roles in muscle structure and functions. Defects in O-mannosylation lead to hypoglycosylation of  $\alpha$ -DG, which are a causative factor for multiple forms of congenital muscular dystrophy (CMD). The O-mannose glycans of  $\alpha$ -DG are also demonstrated to be involved in mesenchymal stem cell

differentiation, lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus (LFV) infection, and cancer metastasis.

The O-mannose glycan structures of  $\alpha$ -DG are highly heterogeneous with various branching structures linked to three O-mannose Cores (Core M1–3 in Scheme 1). The Core M3 can be further elaborated to a glycosaminoglycan-like polysaccharide-containing structure (Scheme 1). The Core M3 structures have been exclusively identified on  $\alpha$ -DG and have been confirmed as the only O-mannose Core structures capable of directly binding to extracellular matrix (ECM) components. The Core M1 and Core M2 structures are far more abundant and diverse than the Core M3 structures on  $\alpha$ -DG, and these highly heterogeneous and complex structures have also been characterized in many other brain glycoproteins. However, the biosynthetic pathway and the functional roles of the branching structures remain poorly understood. The Lewis x ( $Le^x$ , **V**), sialyl Lewis x ( $sLe^x$ , **VI** and **VII**) with one of the two sialic acid forms *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc), and their substructures **I–IV** are the most common branching structures for Core M1 and Core M2 glycans (GlcNAc-A or GlcNAc-B in Scheme 1b). The sulfated **I–VII** and a sulfated trisaccharide human natural killer-1 epitope (HNK-1) have also been identified as the branching structures for Core M1 and Core M2. So far, only over 20 Core M1 and Core M2 structures have been well characterized. However, according to the known branching structures **I–VII** and their high abundance (up to 30% of total O-glycans) in brain glycoproteins, there are at least 56 putative non-sulfated Core M1 and Core M2 structures with appendant branching structures **I–VII** potentially existing in various O-mannosylated proteins, including 7 Core M1 structures (Scheme 4b), 7 symmetrical Core M2 structures (Scheme 4c) and 42 asymmetrical Core M2 structures (Scheme 5)

To decipher the puzzling biosynthetic pathway, and disclose the functional roles of these highly heterogeneous O-mannose glycans in many brain glycoproteins, a collection of all putative Core M1 and Core M2 structures in sufficient amounts is highly desirable. Unfortunately, the collective synthesis of these complex glycans, especially the asymmetrical Core M2 structures is very challenging. Previous chemical or chemoenzymatic synthesis mainly focused on the simple Core M1 glycans with branching structures **I–III**, and the phosphorylated Core M3 trisaccharide. Except for a symmetrical Core M2 trisaccharide core structure, all other Core M2 symmetrical and asymmetrical structures have not been synthesized. We describe herein the first collective synthesis of 63 putative nonsulfated Core M1 and Core M2 structures of O-mannose glycans.

The overall chemoenzymatic synthetic plan is depicted in Scheme 2. To overcome the enzyme limitation, five chemically synthesized core structures **1–5** were designed as the key intermediates for further enzymatic diversification. The core structures **1** and **2** were designed for the enzymatic synthesis of linear Core M1 and C6-branched Core M1 isomers, respectively. The C6-branched Core M1 structures have not been identified from nature, while these structures can be used as controls and standards for future functional and enzymology studies. Trisaccharide **3** was designed to target the symmetrical Core M2 structures by synchronous enzymatic extension at both C2- and C6-branched GlcNAc, and the asymmetrical tetrasaccharides **4** and **5** were designed for the selective enzymatic extension to generate all remaining 40 putative asymmetrical Core M2 structures.

## Chemical synthesis of core structures 1–5.

To acquire core structures **1–5** at gram scales for further enzymatic diversification, a concise chemical approach was designed using 2,6-diol **7**, a known monosaccharide imidate **14**, and a disaccharide imidate **15** as the key building blocks (Scheme 3).

It was envisaged that the 2,6-diol **7** could serve as the only acceptor required for the synthesis of core structures **2–5** via regio- and stereoselective glycosylation (Scheme 3). The regioselective glycosylation at C6-OH or double glycosylation at 2,6-diol of **7** with imidate **14** provided C6-branched disaccharide **10** (81%) or C2 and C6-branched trisaccharide **11** (85%), respectively. Taking advantage of the difference in reactivity between the primary C6-OH and the secondary C2-OH of **7**, a one-pot sequential glycosylation approach was applied for the synthesis of asymmetrical tetrasaccharide isomers **12** and **13** by simply changing the glycosylation sequence of donor **14** and **15**. This operationally simple one-pot double glycosylation approach provided tetrasaccharides **12** (65% for two steps) and **13** (75% for two steps) in good yields. For the synthesis of fully protected Core M1 disaccharide **9**, the 2,6-diol **7** was selectively protected with *tert*-butyldimethylsilyl (TBS) at C6-OH. The resulting mannoside **8** was glycosylated with imidate **14**, yielding the desired disaccharide **9** in 70% yield for two steps. Next, a unified 3-step procedure was applied for the global deprotection of **9–13** to provide core structure **1–5**. In this manner, the gram-scale synthesis of all 5 designed core structures **1–5** was achieved, which makes it possible to explore the enzymatic diversification in the next stage.

## Enzymatic diversification of core structures 1–5 for the collective synthesis of Core M1 and Core M2 O-mannose glycans 16–71.

Previous, we developed three enzyme modules to target three different glycosidic linkages presenting in branching structures **II–VII** (Scheme 1) for the synthesis of serine-appended Core M1 O-mannose glycans. By taking advantage of the promiscuous substrate specificity of bacterial enzymes, we anticipate these enzyme modules could also be applied for the synthesis of sterically hindered Core M2 structures. As shown in Scheme 4a, the enzyme module 1 is a one-pot three-enzyme system that was designed for  $\beta$ 1–4-galactosylation to form the Gal $\beta$ 1–4GlcNAc (LacNAc) moiety. The enzyme module 2 is another one-pot three-enzyme system which is designed for the  $\alpha$ 2–3-sialylation to form both Neu5Ac $\alpha$ 2–3Gal and Neu5Gc $\alpha$ 2–3Gal units. The enzyme module 3 is a one-pot two-enzyme system which is applied for the  $\alpha$ 1–3-fucosylation to form the Fuca1–3GlcNAc linkage.

Enzymatic diversification of core structures **1–5** for the synthesis of diverse Core M1 and Core M2 structures was commenced with the Core M1 structures **16–21**. The synthesis of Core M1 structures **16–21** was achieved through stepwise enzymatic extension of disaccharide **1** following the general procedures of three enzyme modules (see Scheme S1 and Experimental section in Supporting Information for details). Accordingly, the disaccharide **1** was firstly converted to LacNAc-terminated trisaccharide **16** (200 mg, 90%) using enzyme module 1, then the purified trisaccharide **16** was used as the acceptor by enzyme module 2 for the parallel synthesis Neu5Ac $\alpha$ 2–3-linked sialoside **17** and

Neu5Gca2–3-linked sialoside **18** by using ManNAc or ManNGc as the sialic acid precursor, respectively. The isolated sialosides **17** (69 mg, 95%), **18** (71 mg, 96%), and trisaccharide **16** were elaborated parallelly using enzyme module 3 to yield Le<sup>x</sup>-containing structure **19** (45 mg, 89%), sLe<sup>x</sup>-containing Core M1 structures **20** (31 mg, 80%) and **21** (52 mg, 75%).

The same stepwise assembly procedure was applied to the synthesis of C6-branched Core M1 isomers **22–27** from disaccharide **2**, and symmetrical Core M2 O-mannose glycans **28–33** from trisaccharide **3**. All these compounds were excellent substrates for 3 enzyme modules affording C6-branched Core M1 isomers **22–27** (Scheme 4c) and symmetrical Core M2 O-mannose glycans **28–33** (Scheme 4d) in good yields (see Scheme S2–S3 and Experimental section in Supporting Information for details).

The asymmetric Core M2 glycans were divided into two subgroups in our synthetic plan according to the number of monosaccharide units on C2- and C6-branched structures. As shown in Scheme 5a, the first group with C2-branched “heavy chain” as the C2-branch has greater or equal number of sugar units to C6-branched structures. The C2 “heavy chain”-branched Core M2 asymmetric structures **34–55** can be derived from chemically prepared asymmetric tetrasaccharide **4** (Scheme 5a). In contrast, the C6 “heavy chain”-branched Core M2 asymmetric structures **56–73** can be assembled from tetrasaccharide **5** (Scheme 5b). The overall synthetic scheme for the assembly of C2 “heavy chain”-branched Core M2 asymmetric structures **34–49** was depicted in Scheme 5a. Starting from tetrasaccharide **4**, the sequential glycosylation using three enzyme modules (Enzyme modules 1–3 in Scheme 4a) afford 22 diverse complex Core M2 asymmetric structures **34–55** in 25–435 mg scales in a linear sequence that does not exceed 4 steps (see Experimental section in Supporting Information for details). Following the similar enzymatic assembly strategy, the C6 “heavy chain”-branched Core M2 asymmetric structures **56–73** were also obtained in good yields (Scheme 5b, see Scheme S4 and Experimental section in Supporting Information for details).

### Recognition of sialylated O-mannose glycans by human CD33-Fc (Siglec-3).

Despite the fact O-mannose glycans account for up to one-third of all O-linked glycans in the brain, and more than 50 O-mannosylated proteins have been identified,  $\alpha$ -DG is the only O-mannosylated protein that has been intensively studied. Most recently, the biosynthetic pathway of Core M3 O-mannose glycans and their functional roles in various congenital muscular dystrophies have been well established. In contrast to Core M3 O-mannose glycans which exclusively presented on  $\alpha$ -DG, the diverse and complex Core M1 and Core M2 O-mannose glycans have been identified on a number of brain proteins. However, the biosynthetic pathway of Core M1 and Core M2 O-mannose glycans have not been fully elucidated, and their biological functions or cognate receptors in the brain tissues are still unknown. The high prevalence of O-mannose glycans in brain encouraged us to screen their potential binding receptors with the printed microarray of our systematically synthesized Core M1 and Core M2 glycans. Among three tested brain proteins, only the recombinant human Fc-fusion CD33 (hSiglec-3)<sup>13</sup> showed strong binding to all the sialylated O-mannose glycans. The response is very specific for sialic acids, and no signal of binding was displayed by the non-sialylated glycans (Figure S1). Human L1 cell adhesion molecule

(LICAM/CD171) and human myelin-associated glycoprotein (MAG/Siglec-4a) were also screened with O-mannose glycan array, however, no significant binding were exhibited for these two human brain proteins.

In summary, an efficient chemoenzymatic approach has been developed for the diversity-oriented assembly of complex Core M1 and Core M2 O-mannose glycans. Five core structures **1–5** designed to target different O-mannose glycan subgroups were obtained at gram-scale through concise chemical synthesis. The enzymatic diversification of 5 readily accessible core structures **1–5** were performed with 4 simple monosaccharides (Gal, ManNAc, ManNGc and Fuc) as donor precursors and 3 enzyme modules through diversity-oriented parallel enzymatic glycosylation. These provided 58 Core M1 and Core M2 structures in a linear sequence that does not exceeded 4 steps. All 8 recombinant enzymes in 3 enzyme modules are cloned from bacteria, have broad substrate specificities, and can be expressed at large scales in a conventional *E. coli* expression system, thus making the described synthesis of complex O-mannose glycans readily accessible. The 63 diverse chemoenzymatically synthesized O-mannose glycans, 55 of them are synthesized for the first time, constitute the most comprehensive O-mannose glycan library to date. These systematically synthesized O-mannose glycans enable to explore their unknown functions in brain. Using the printed O-mannose glycan array, the human brain protein CD33, a key factor in the modulation of Alzheimer's disease, was identified as strongly binding to sialylated Core M1 and Core M2. This unique binding profile observed here indicates the O-mannose glycans may play roles in the neurodegenerative disease, and also provides important information on design O-mannose glycan-based probes to target the sterically rigid binding pocket of CD33.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

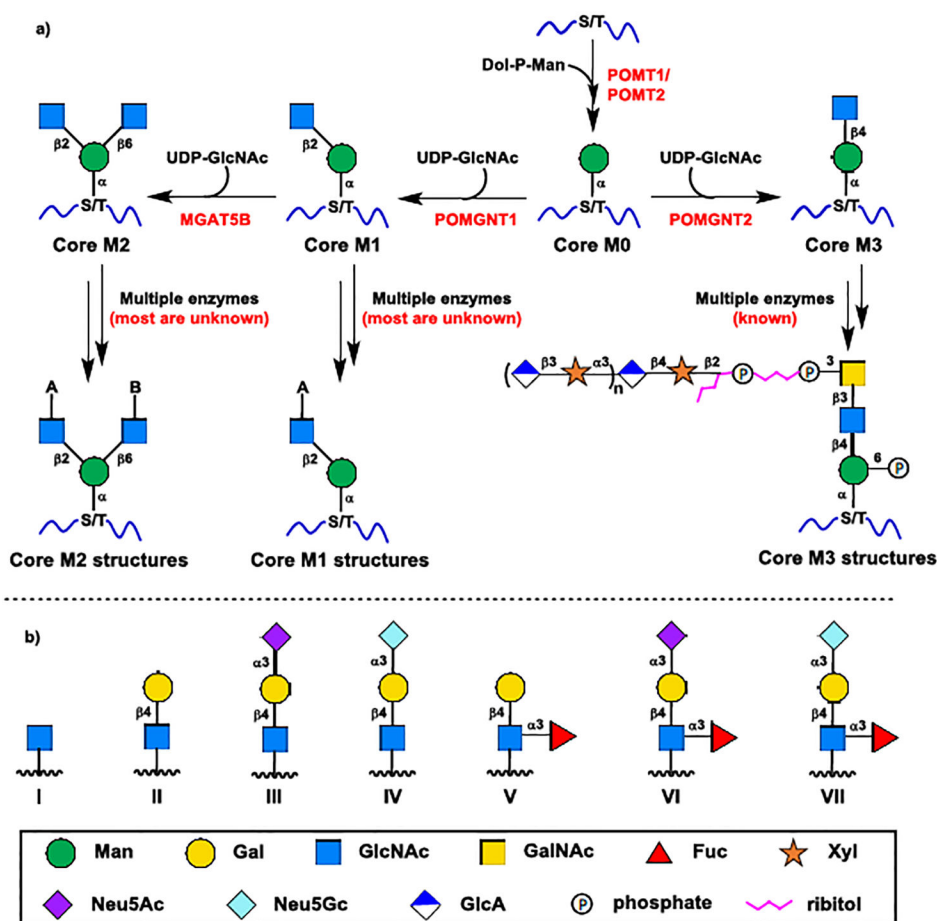
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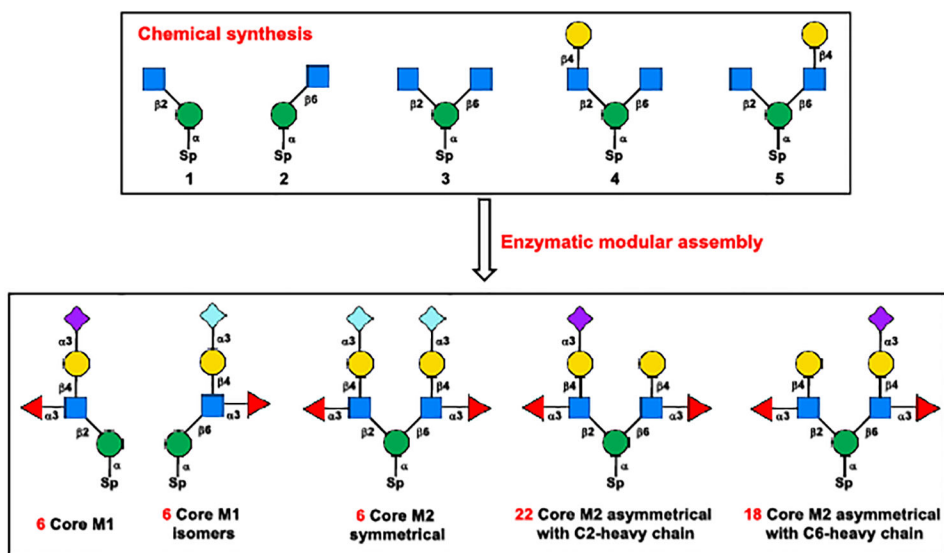
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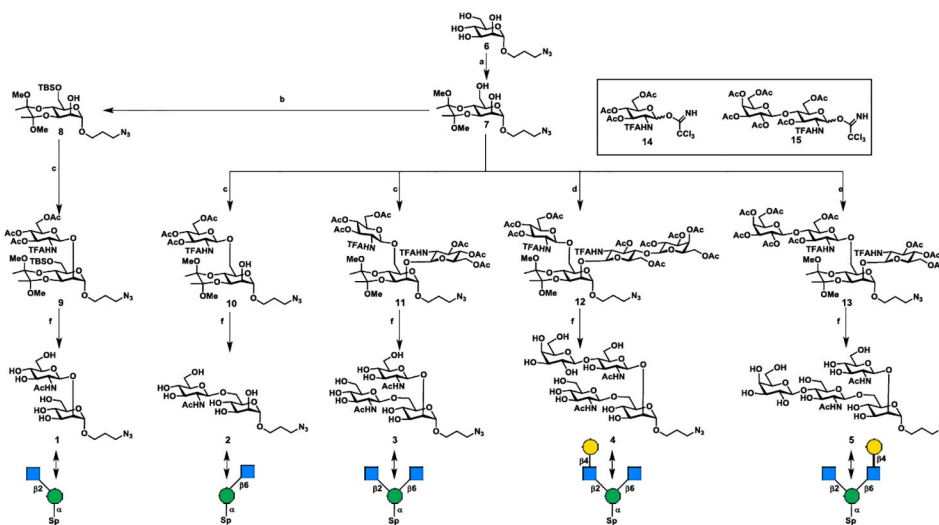
Scheme 1.

a) Biosynthetic pathway of  $\alpha$ -dystroglycan O-mannose glycans; b) Seven common non-sulfated branching structures of Core M1 and Core M2



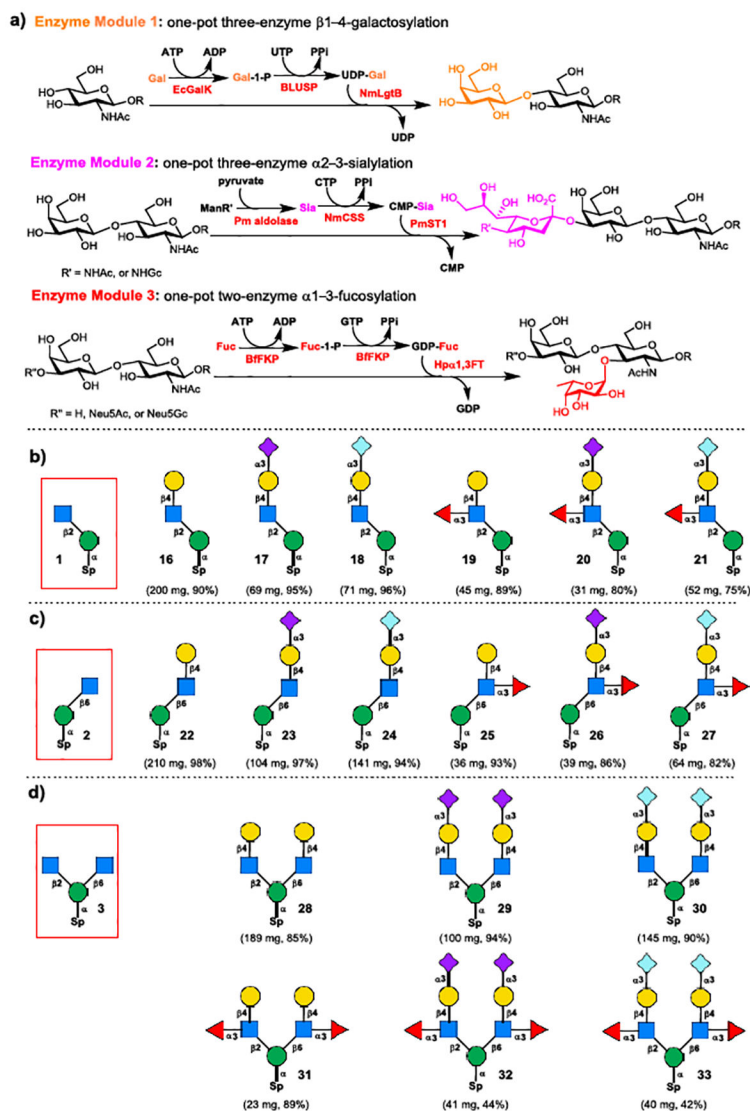
Scheme 2.  
Synthetic plan.





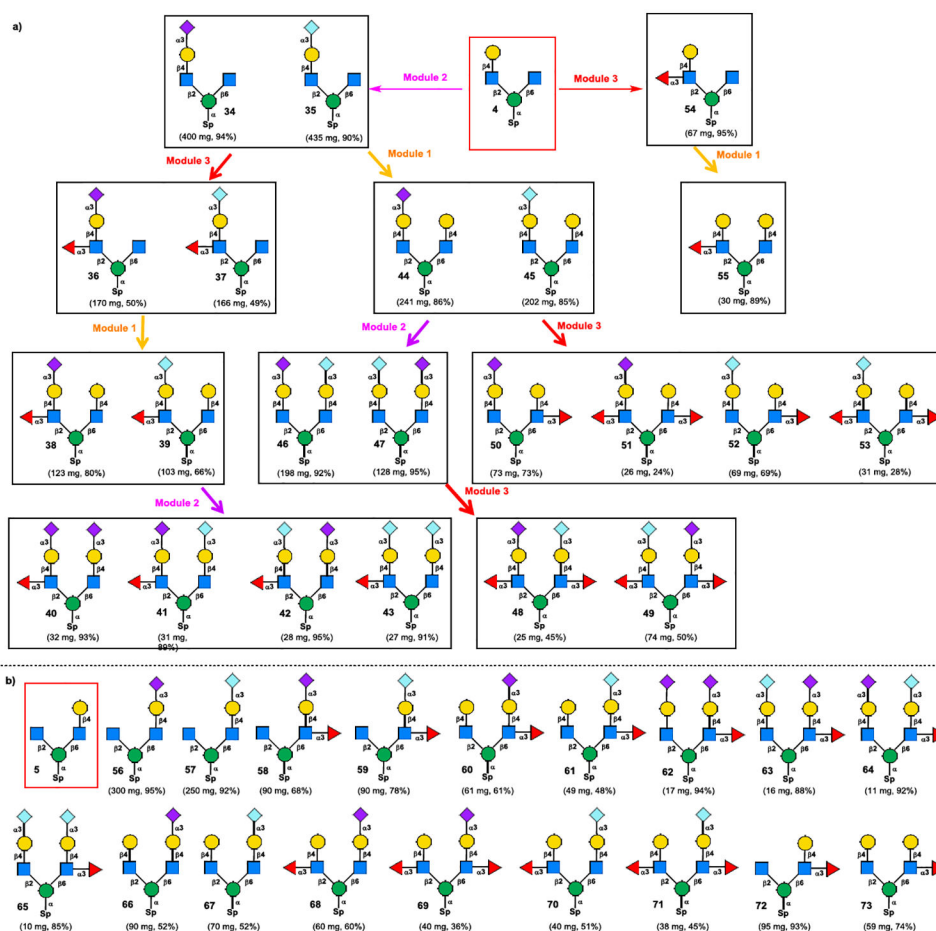
### Scheme 3.

Chemical assembly of core structures **1–5**. (a) 2,3-butanedione, trimethyl orthoformate, ( $\pm$ )-camphorsulfonic acid, 110 °C, 12 h, 58%; (b) TBSCl, imidazole, DMF, rt, 1 h, 84%; (c) **14**, TMSOTf, 4 Å MS,  $-50$  °C to  $-20$  °C, 83% for **9**, 86% for **10**, 85% for **11**; (d) **14** (1.0 equiv), TMSOTf, 4 Å MS,  $-50$  °C, 1 h, then **15** (1.5 equiv),  $-20$  °C, 1 h, 65% for 2 steps; (e) **15** (1.0 equiv), TMSOTf, 4 Å MS,  $-50$  °C, 1 h, then **14** (1.5 equiv),  $-20$  °C, 1 h, 74% for 2 steps; (f) 1) TFA/H<sub>2</sub>O (9:1, v/v); 2) NaOMe/MeOH, then 1.0 M LiOH; 3) Ac<sub>2</sub>O/MeOH, **1** (1.8 g) 87% for 3 steps; **2** (1.88 g) 90% for 3 steps; **3** (1.13 g) 84% for 3 steps; **4** (1.58 g) 91% for 3 steps; **5** (1.41 g) 90% for 3 steps.



#### Scheme 4.

a) Three enzyme modules for enzymatic diversification; b) Enzymatic assembly of Core M1 O-mannose glycans **16–21** from **1**; c) Enzymatic assembly of C6-branched Core M1 O-mannose glycan isomers **22–27** from **2**; d) Enzymatic assembly of symmetrical Core M2 O-mannose glycans **28–33** from **3**.

**Scheme 5.**

a) Enzymatic modular assembly of C2 “heavy chain”-branched asymmetrical Core M2 glycans 34–55 from tetrasaccharide 4 (50 and 51 were derived from 44 in one-pot, 52 and 53 were derived from 45 in one-pot); b) Enzymatic modular assembly of C6 “heavy chain”-branched asymmetrical Core M2 glycans 56–73 from tetrasaccharide 5 (68 and 69 were derived from 66 in one-pot, 70 and 71 were derived from 67 in one-pot).