Glycosylation

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Chemoenzymatic Assembly of Mammalian O-Mannose Glycans

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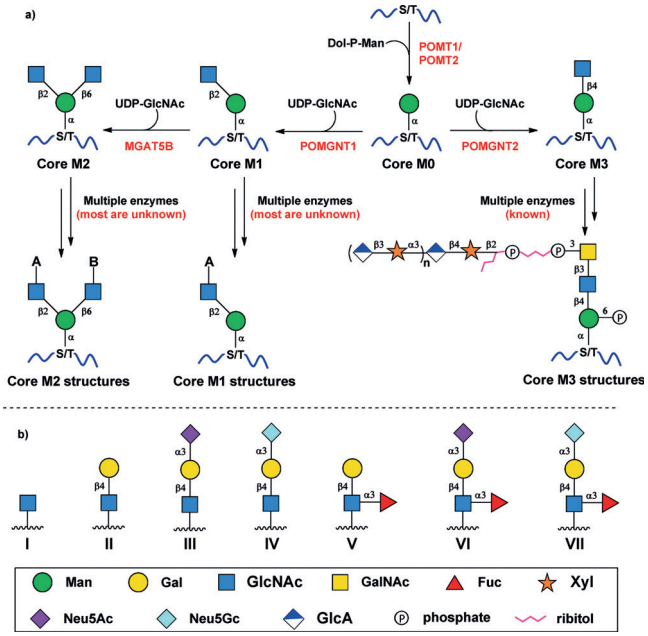
Abstract: *O-Mannose glycans account up to 30% of total O-glycans in the brain. Previous synthesis and functional studies have only focused on the core M3 O-mannose glycans of α-dystroglycan, which are a causative factor for various muscular diseases. In this study, a highly efficient chemoenzymatic 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 five judiciously designed core structures, and the diversity-oriented modification of the core structures with three enzyme modules to provide 58 complex O-mannose glycans in a linear sequence that does not exceed four steps. The binding profiles of synthetic O-mannose glycans with a panel of lectins, antibodies, and brain proteins were also explored by using a printed O-mannose glycan array.*

The protein α-dystroglycan (α-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-GalNAc glycans, O-mannose glycans have been identified as the third most common glycosylation modification on α-DG. The O-mannose glycans on α-DG are known to play essential roles in muscle structure and functions. Defects in O-mannosylation lead to hypoglycosylation of α-DG, which is a causative factor for multiple forms of congenital muscular dystrophy (CMD).[1] The O-mannose glycans of α-DG have also been demonstrated to be involved in mesenchymal stem cell differentiation, lymphocytic choriomeningitis virus (LCMV) and Lassa fever virus (LFV) infection, and cancer metastasis.[2]

The O-mannose glycan structures of α-DG are highly heterogeneous, with various branching structures linked to three O-mannose cores (cores M1–3; Scheme 1 a).[1] Core M3 can be further elaborated to a glycosaminoglycan-like polysaccharide-containing structure (Scheme 1 a).[1,3] The core M3 structures have been exclusively identified on α-DG and have been confirmed as the only O-mannose core structures capable of directly binding to extracellular matrix (ECM) components.[1] The core M1 and core M2 structures are far more abundant and diverse than the core M3 structures on α-DG, and these highly heterogeneous and complex structures have also been characterized in many other brain glycoproteins.[1,4] However, the biosynthetic pathway and the functional roles of the branching structures remain poorly understood.[1] 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; Scheme 1 b).[1,4] So far, only around 20 core M1 and core M2 structures have been well characterized. However, according to the known branching structures **I**–**VII** and their high abundance in brain glycoproteins, there are at least 56 putative non-sulfated core M1 and core M2 structures with appendant branching structures **I**–**VII** that potentially exist in various O-mannosylated proteins, including 7 core M1 structures (Scheme 4 b), 7 symmetrical core M2 structures (Scheme 4 d) and 42 asymmetrical core M2 structures (Scheme 5).



Scheme 1. a) Biosynthetic pathway of α-dystroglycan O-mannose glycans. b) Seven common non-sulfated branching structures of core M1 and core M2.

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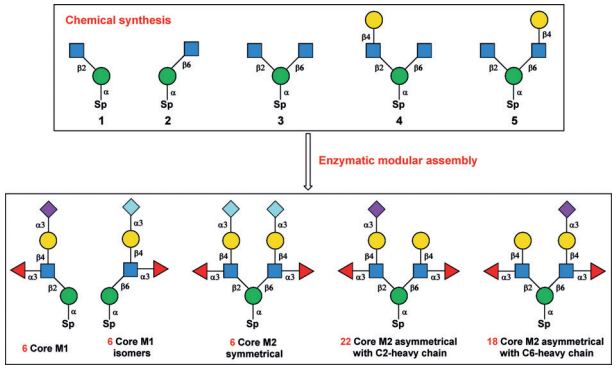
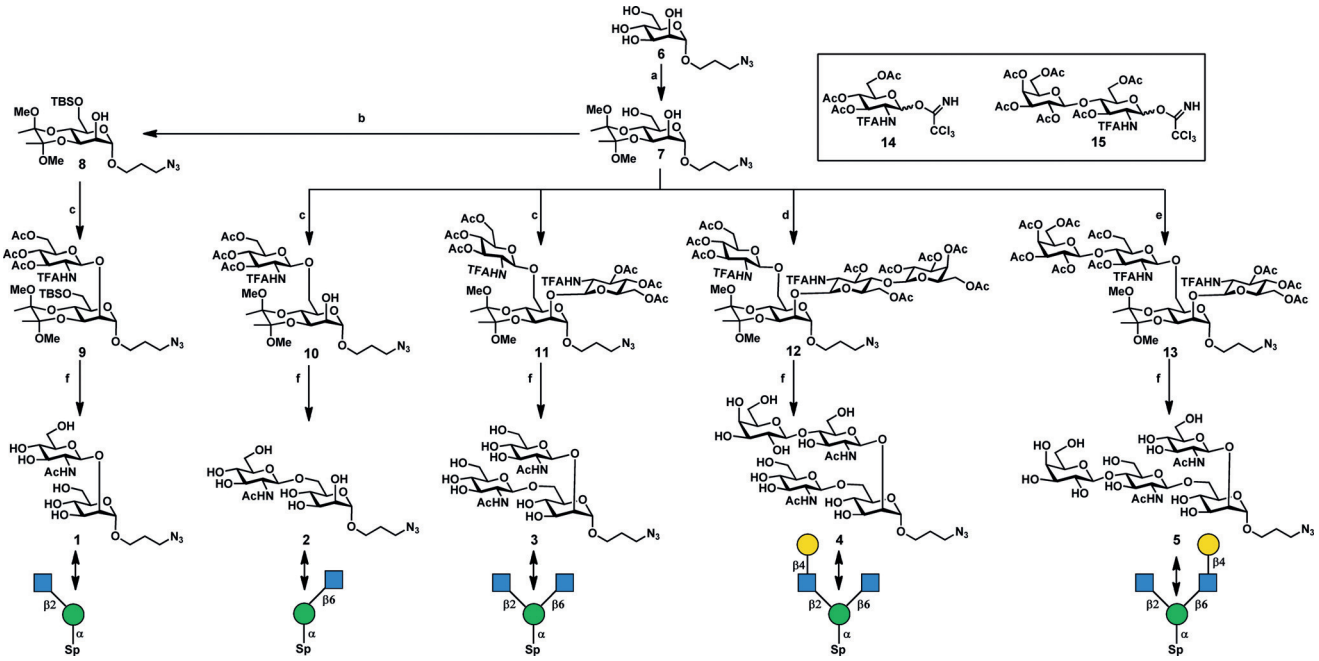
To decipher the puzzling biosynthetic pathway and elucidate the functional roles of these highly heterogeneous O-mannose glycans in many brain glycoproteins, a collection of all putative core M1 and core M2 structures in sufficient amounts is highly desirable. Unfortunately, the collective synthesis of these complex glycans, especially the asymmetrical core M2 structures is very challenging. Previous chemical or chemoenzymatic syntheses have mainly focused on the simple core M1 glycans with branching structures **I**–**III**,[5] and the phosphorylated core M3 trisaccharide.[6] Except for a symmetrical core M2 trisaccharide core structure,[7] no other core M2 symmetrical and asymmetrical structures have 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 limited availability of glycosyltransferases for the construction of core structures, 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. Trisaccharide **3** was designed to target the symmetrical core M2 structures and the asymmetrical tetrasaccharides **4** and **5** were designed for the selective enzymatic extension to generate all remaining 40 putative asymmetrical core M2 structures.



Scheme 2. Synthetic plan.

To acquire core structures **1**–**5** at gram scales for further enzymatic diversification, a concise chemical approach was designed using 2,6-diol **7**, known monosaccharide imidate **14**,[8] and disaccharide imidate **15**[9] as the key building blocks (Scheme 3). It was envisaged that 2,6-diol **7** could serve as the only acceptor required for the synthesis of core structures **2**–**5** through regio- and stereoselective glycosylation (Scheme 3). Regioselective glycosylation at the C6-OH or double glycosylation at the 2,6-diol of **7** with imidate **14** provided C6-branched disaccharide **10** (81%) or C2,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**, 2,6-diol **7** was selectively protected with *tert*-butyldimethylsiyl (TBS) at the C6-OH. The resulting mannoside **8** was glycosylated with imidate **14**, yielding the desired disaccharide **9** in 70% yield for two steps. Next,

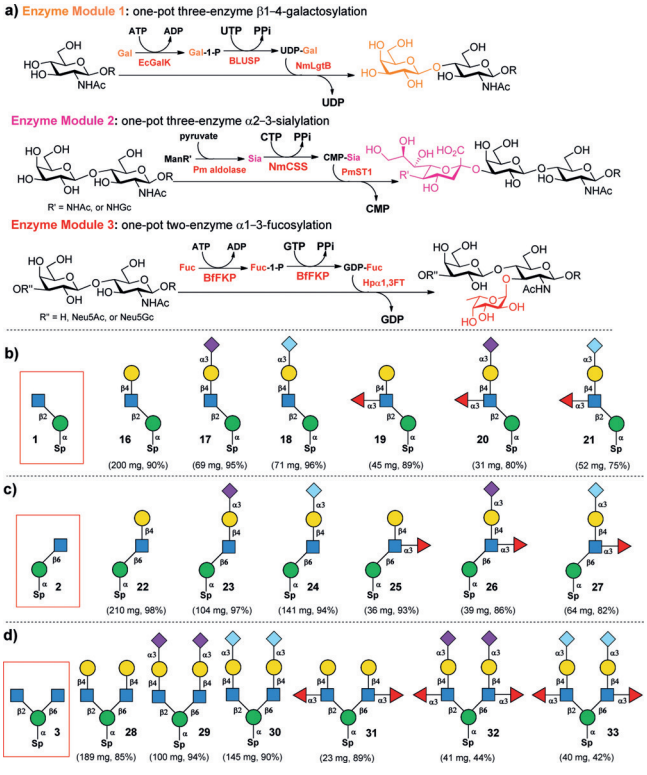


Scheme 3. Chemical assembly of core structures **1**–**5**. a) 2,3-butanedione, trimethyl orthoformate, (±)-camphorsulfonic acid, 110 °C, 12 h, 58%. b) TBSCl, imidazole, DMF, RT, 1 h, 84%. c) **14**, TMSOTf, 4 Å MS, −50 °C to −20 °C, 83% for **9**, 86% for **10**, 85% for **11**. d) **14** (1.0 equiv), 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_2O (9:1, v/v); 2) NaOMe/MeOH, then 1.0 M LiOH; 3) Ac_2O/MeOH. Yield over 3 steps: **1** (1.8 g) 87%; **2** (1.88 g) 90%; **3** (1.13 g) 84%; **4** (1.58 g) 91%; **5** (1.41 g) 90%.

a unified three-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 five designed core structures **1**–**5** was achieved, which makes it possible to explore the enzymatic diversification in the next stage.

Next, we turned our attention to enzymatic diversification of core structures **1**–**5** for the collective synthesis of core M1 and core M2 O-mannose glycans **16**–**71**. Previously, 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.[8] By taking advantage of the promiscuous substrate specificity of bacterial enzymes, we anticipated that these enzyme modules could also be applied for the synthesis of sterically hindered core M2 structures. As shown in Scheme 4a, enzyme module 1 is a one-pot three-enzyme system that was designed for β1-4-galactosylation to form the Galβ1-4GlcNAc moiety.[10] Enzyme module 2 is another one-pot three-enzyme system, which was designed for α2-3-sialylation to form both Neu5Acα2-3Gal and Neu5Gcα2-3Gal units.[11] Enzyme module 3 is a one-pot two-enzyme system that is applied for α1-3-fucosylation to form the Fucα1-3GlcNAc linkage.[12]

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 the Supporting Information). Accordingly, disaccharide **1** was firstly converted into LacNAc-terminated

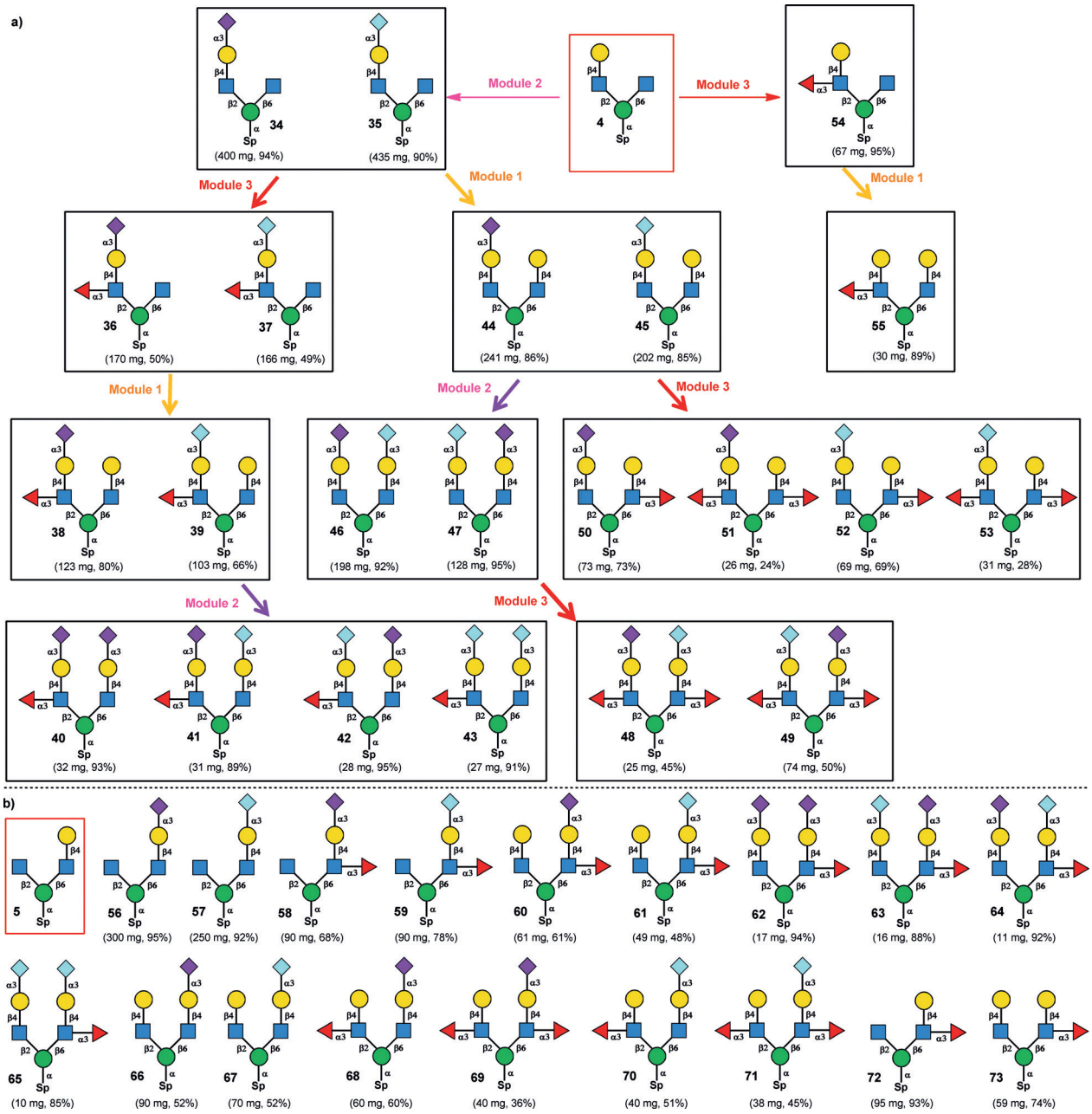


Scheme 4. a) The three enzyme modules for enzymatic diversification. b) Enzymatic assembly of core M1 O-mannose glycans **16**–**21** from **1**. c) Enzymatic assembly of C6-branched core M1 O-mannose glycan isomers **22**–**27** from **2**. d) Enzymatic assembly of symmetrical core M2 O-mannose glycans **28**–**33** from **3**.

trisaccharide **16** (200 mg, 90%) using enzyme module 1, then purified trisaccharide **16** was used as an acceptor by enzyme module 2 for the parallel synthesis of Neu5Acα2-3-linked sialoside **17** and Neu5Gcα2-3-linked sialoside **18** by using ManNAc or ManNGc as the sialic acid precursor, respectively. Isolated sialosides **17** (69 mg, 95%), **18** (71 mg, 96%), and trisaccharide **16** were elaborated in a parallel fashion using enzyme module 3 to yield Le^x-containing structure **19** (45 mg, 89%) and sLe^x-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 the three 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 Schemes S2,S3, and the 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** is depicted in Scheme 5a. Starting from tetrasaccharide **4**, sequential glycosylation using the three enzyme modules afforded 22 diverse complex core M2 asymmetric structures (**34**–**55**) in 25–435 mg quantities. It is known that LacNAc is a better acceptor than the sialyl LacNAc for Hpα1-3-FucT,[12e] therefore, fucosylation of **44** or **45** with enzyme module 3 affords both the mono-fucosylated **50** or **52** as the major products along with the double fucosylated **51** or **53** in one pot, respectively (Scheme 5a). Following a 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 the Supporting Information for details).

Despite the fact that 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, α-DG is the only O-mannosylated protein that has been intensively studied.[1] Most recently, the biosynthetic pathway of core M3 O-mannose glycans and their functional roles in various congenital muscular dystrophies have been well established.[1] In contrast to core M3 O-mannose glycans, which exclusively presented on α-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 pathways 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 the brain encour-



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).

aged us to screen their potential binding receptors with a printed microarray of our systematically synthesized core M1 and core M2 glycans. The binding affinities of plant lectins MAL-I, MAL-II, and SNA with O-mannose glycans were examined, and only MAL-I showed moderate binding with all the α2-3-sialic acid-capped LacNAc structures (Figure S1). The recombinant human Siglec-9 (hSiglec-9-Fc), chicken polyclonal anti-Neu5Gc antibody IgY (pChGc), human serum rich in anti-Neu5Gc antibody, and His-tagged typhoid toxin (PltB-His) were also screened on the array. The hSiglec-9-Fc exhibited moderate to strong binding to all sialylated O-mannose glycans (Figure S2). Both pChGc and anti-Neu5Gc-rich human serum showed strong binding for all the Neu5Gc-sialylated O-mannose glycans but not their Neu5Ac-counterparts (Figure S2). Selective binding with PltB-His was observed for all the Neu5Ac-containing O-mannose glycans (Figure S2). The binding profiles of three brain proteins, namely the recombinant human Fc-fusion CD33 (hSiglec-3), human L1 cell adhesion molecule (L1CAM/CD171), and human myelin-associated glycoprotein (MAG/Siglec-4a), were also screened with O-mannose glycan array, and only CD33 showed strong binding to all the sialylated O-mannose glycans (Figure S3).

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 on a gram scale through concise chemical synthesis. The enzymatic diversification of core structures **1**–**5** with three enzyme modules through diversity-oriented parallel enzymatic glycosylation provided 58 core M1 and core M2 structures in a linear sequence that does not exceeded four steps. The 63 diverse chemoenzymatically synthesized O-mannose glycans, 55 of which were synthesized for the first time, constitute the most comprehensive O-mannose glycan library to date. These systematically synthesized O-mannose glycans enabled exploration of their binding profiles with different lectins, antibodies, and brain proteins by using a printed O-mannose glycan array. A cross-comparison of O-mannose glycans with other conventional O-GalNAc glycans and N-glycans, as well as further functional studies of O-mannose glycans on brain proteins, are currently under investigation.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: chemoenzymatic synthesis · dystroglycan · glycosylation · glycosyltransferases · O-mannose glycans

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