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Facile chemoenzymatic synthesis of O-mannosyl glycans

Shuaishuai Wang,[a]§ Qing Zhang,[a]§ CongCong Chen,[b] Yuxi Guo,[a] Madhusudhan Reddy Gadi,[a] Jin Yu,[c] Ulrika Westerlind,[c,d] Yunpeng Liu,[a] Xuefeng Cao,[a] Peng G. Wang,*[a] and Lei Li*[a]

Abstract: O-Mannosylation is a vital protein modification involved in brain and muscle development, whereas the biological relevance of O-mannosyl glycans remains largely unknown, due to the lack of structurally defined glycoforms. An efficient scaffold synthesis/enzymatic extension (SSEE) strategy was thus developed to prepare such structures, by combining gram-scale convergent chemical synthesis of 3 scaffolds and strictly controlled sequential enzymatic extension catalyzed by glycosyltransferases. Totally, 45 O-mannosyl glycans were obtained, covering majority of identified mammalian structures. Subsequent glycan microarray analysis revealed fine specificities of glycan-binding proteins and specific anti-sera.

O-Mannosylation is a vital protein post-translational modification that plays essential roles in brain and muscle development and normal tissue function.[1] Alpha-dystroglycan (α-DG) is the most studied O-mannosyl protein that is widely distributed in muscle and brain tissues. Abnormal O-mannosylation on α-DG disrupts the receptor function of dystroglycan and leads to congenital muscular dystrophy.[2] In addition, O-mannosyl glycans have been reported to be involved in other human diseases including arenaviral infection, cancer and metastasis.[3]

In mammalian brain tissue, O-mannosyl glycans account for up to 30% of all O-linked glycans.[4] Regardless of the abundance, studies on mammalian O-mannosylation have been mainly focused on α-DG. Recent advances in glycoproteomics enabled discovery of an increased number of O-mannosylated proteins and various O-mannosyl glycans.[1b, 5] To date, more than 20 O-mannosyl glycans have been identified, which are classified into four core types.[5] As illustrated in **Figure 1A**, core M0, identified in cadherin family proteins,[6] represents a single structure of one mannose (Man) residue α-linked to serine (Ser) or threonine (Thr). Core M1 contains a linear β1,2-linked *N*-acetylglucosamine (GlcNAc), whereas the branched core M2 contains both β1,2- and β1,6-linked GlcNAc-residue. Core M3 is a phosphorylated trisaccharide, to which glycosaminoglycan-like polysaccharides usually are attached.[5]

The core M1 and M2 are typically extended and have been identified with structures including *N*-acetyllactosamine (Gal-β1,4-GlcNAc, LN), 3-sialyl-*N*-acetyllactosamine (Neu5Ac-α2,3-Gal-β1,4-GlcNAc, 3SLN), Lewis X [Gal-β1,4-(Fuc-α1,3-)GlcNAc, Le^X], sialyl-Le^X (sLe^X), and the human natural killer-1 (3-sulfo-GlcA-β1,3-Gal-β1,4-Glc, HNK-1) epitopes (**Fig 1B**).[1b] These terminal epitopes largely diversify core M1 and M2 based glycans and in fact, the majority of identified structures belong to these two core types.[1b] Additionally, it was found that the structures centered on core M1 and M2 account for 15% and 5% of all brain O-linked glycans, respectively.[4] Despite the abundance of core M1 and M2 structures, very little is known about their functional relevance.[5] Therefore, structurally well-defined O-mannosyl glycans not only serve as ideal standards to identify and characterize such glycoforms, but also provide unique probes to uncover their biological roles.

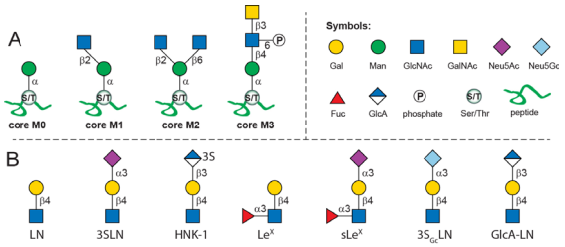

Figure 1. The 4 cores of mammalian O-mannosyl glycans (A), and identified extensions on core M1 and M2 (B). Gal, galactose; Man, mannose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Fuc, L-fucose; GlcA, glucuronic acid.

To date, only a few O-mannosyl glycans were prepared.[7] A core M1 tetrasaccharide (**Fig 2**, **M102**) was firstly synthesized in 1999,[7c, 7d] and later two lower homologs (**M100**, **M101**). Most recently, Cao and co-workers prepared six core M1 structures (**M100**, **M101**, **M102**, **M104**, **M105**, and **M102G**) employing a chemoenzymatic approach.[7a] Glycopeptides harboring basic O-mannosyl structures (e.g., **M101**,[8] **M102**,[9] core M0,[7e] **M000**,[10] or core M3[11]) were also generated. Nevertheless, the majority of O-mannosyl glycans (especially core M2 branched structures) are still not accessible, which hampered in-depth understanding of O-mannosylation. Here an efficient strategy was developed employing scaffold synthesis/enzymatic extension (SSEE) to prepare 45 structurally well-defined core M1 and M2 based O-mannosyl glycans (**Fig 2**), covering all identified glycoforms with the exception of the HNK-1 epitope.[1b]

Considering the structural signatures of O-mannosyl glycans, it is concluded that all core M1 and M2 structures can be enzymatically elaborated from the following three scaffolds, **M100**, **M201** and **M301** (**Fig 2**). We envisioned that the protected Man derivative **1** (**Fig 3A**) would serve as a versatile precursor for the synthesis of these scaffolds. It has a free hydroxyl group at the C-2 position and the 4,6-hydroxyl groups are protected as the benzylidene acetal. This arrangement allows performing chemical glycosylation at the C-2 position followed by the C-6 position with the deprotection of the acetal protecting group.

[a] S, Wang; Q, Zhang; Y, Guo; Dr. M, Gadi; Dr. Y, Liu; Dr. X, Cao; Prof. Dr. P.G. Wang, and Dr. L. Li
Department of Chemistry & Center for Diagnostics & Therapeutics, Georgia State University, Atlanta, GA 30303 (USA)
E-mail: lli22@gsu.edu (L. Li) or pwang11@gsu.edu (P.G. Wang)
[b] C. Chen
National Glycoengineering Research Center, School of Pharmaceutical Science, Shandong University, Jinan 250012, China
[c] Dr. J Yu and Prof. Dr. U. Westerlind
Leibniz-Institut für Analytische Wissenschaften - ISAS - e.V. 44227, Dortmund, Germany
[d] Prof. Dr. U. Westerlind
Department of Chemistry, Umeå University, 901 87 Umeå, Sweden
§ S. Wang and Q. Zhang contributed equally to this work, list in alphabetical order by last name.

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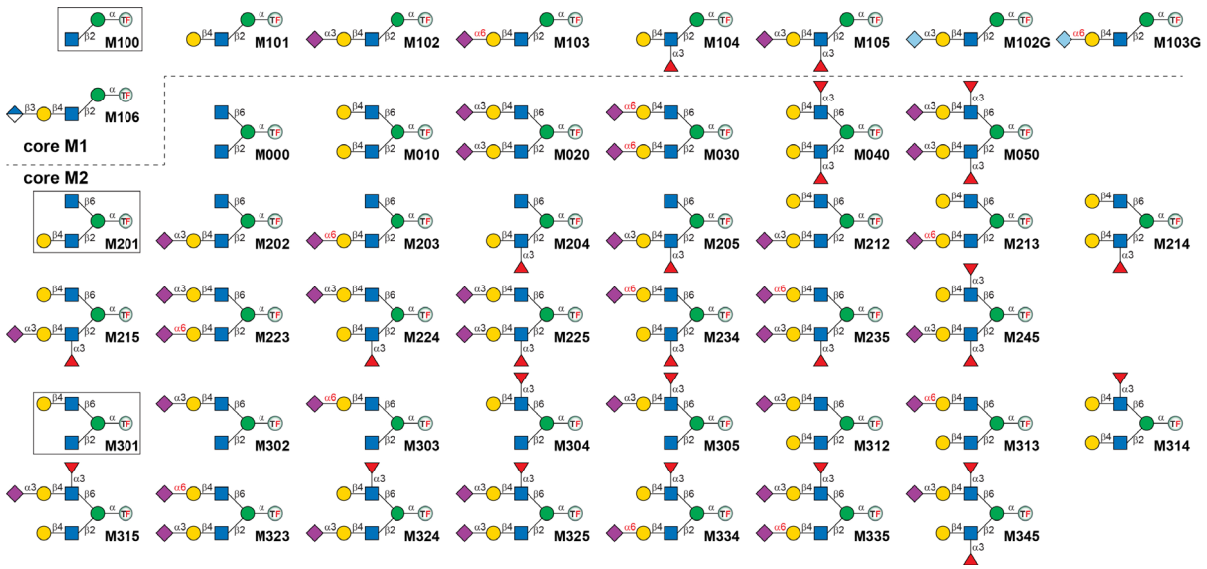

Figure 2. The 45 core M1 and M2 O-mannosyl glycans prepared in this study. The three scaffold structures were squared. TF, Fmoc protected threonine.

As shown in **Figure 3**, the three scaffolds required for the enzymatic extension were synthesized in a convergent approach and were assembled by three simple building blocks, **1**, **2** and **3** (please refer **Supporting Information I** for synthetic details) and the Fmoc-Thr(OH)-O*t*Bu **4**. To prepare the glyco-amino acid **M100**, donor **2** was chemically glycosylated to the glycosyl acceptor **1** at the C-2 position to obtain the disaccharide **5** in 85% yield. An *N*-phenyltrifluoroacetimidate donor was used instead of the typical trichloroacetimidate, as the latter can generate over 50% of a glucosamine thioether by-product through thioether migration. Typical deprotection and protection strategies were employed to convert the benzylidene acetal, 4-methoxybenzyl ether (PMB), and trichloroethoxycarbonyl (Troc) protecting groups on **5** to acetyl (Ac) groups to afford **6** over three steps, with a total yield of 80%. The obtained thiophenyl glycosyl donor **6** was coupled to the protected threonine amino acid **4** at 0 °C in the presence of *N*-iodosuccinimide (NIS) and AgOTf to obtain the protected glyco-amino acid derivative **7** in excellent yield. The *t*-butyl (*t*Bu) and Ac groups were then removed successively, followed by reintroduction of the partially cleaved Fmoc to provide the final product **8** (**M100**) in gram-amounts with a total yield of 90% over three steps.

To synthesize **M301**, the PMB group on the disaccharide **5** was initially converted to Ac, followed by deprotection of the benzylidene acetal to achieve the diol **9**. Glycosylation of **9** with the glycosyl donor **3** yielded the protected target tetrasaccharide **10** in 79% yield. Successively, the Troc protecting groups were converted to Ac groups and provided compound **11,** which was used as glycosyl donor for coupling with the amino acid **4** to obtain the tetrasaccharide **12** in 89% yield. Complete deprotections of *t*Bu and Ac groups were performed under standard reaction conditions and reprotection of the partially Fmoc deprotected amine afforded gram-amounts of compound **13** (**M301**), in a total yield of 85% over three steps. Similarly, **19** (**M201**) was prepared, by coupling of disaccharide donor **3** to the C-2 position of acceptor **1** followed by coupling of **2** to the C-6 position., Then coupling of the obtained oligosaccharide thiosglycoside **18** with amino acid **4** gave the desired glycosylated-amino acid **19** in a total yield of 29% over 11 steps.

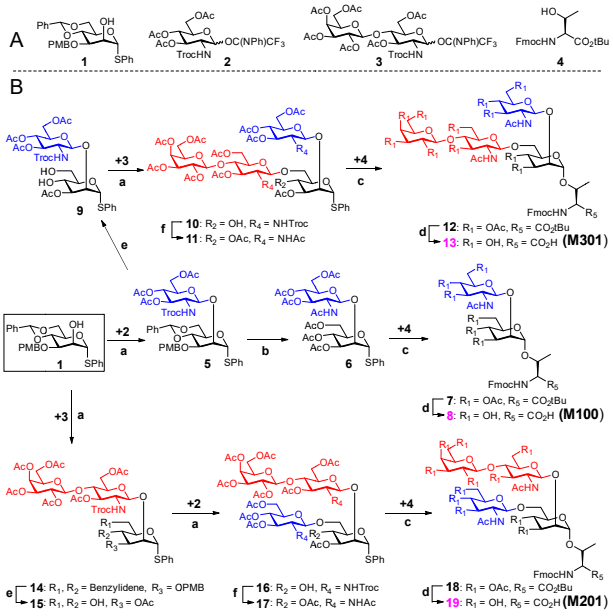

Figure 3. The building blocks (A) for the assembly of the 3 O-mannosyl scaffold structures, and the synthetic scheme (B). Reagents and conditions: (a) TMSOTf, DCM, -60 °C, **5**: 85%; **10**: 79%; **14**: 75%; **16**: 82%; (b) (i) TFA, DCM; (ii) Zn, AcOH; (iii) Ac_2O, Py, 80% over three steps; (c) NIS, AgOTf, DCM/Et_2O=1:1, 0 °C, **7**: 93%; **12**: 89%; **18**: 83%; (d) (i) TFA, DCM; (ii) NaOMe, MeOH; (iii) FmocOSu, $NaHCO_3$, Acetone/H_2O=3:1, **8**: 90%; **13**: 85%; **19**: 87%; (e) (i) DDQ, DCM/PBS Buffer=9:1; (ii) Ac_2O, Py; (iii) EtSH, TsOH, DCM, **9**: 77%; **15**: 81%; (f) (i) Zn, AcOH; (ii) Ac2O, Py, **11**: 87%; **17**: 82%.

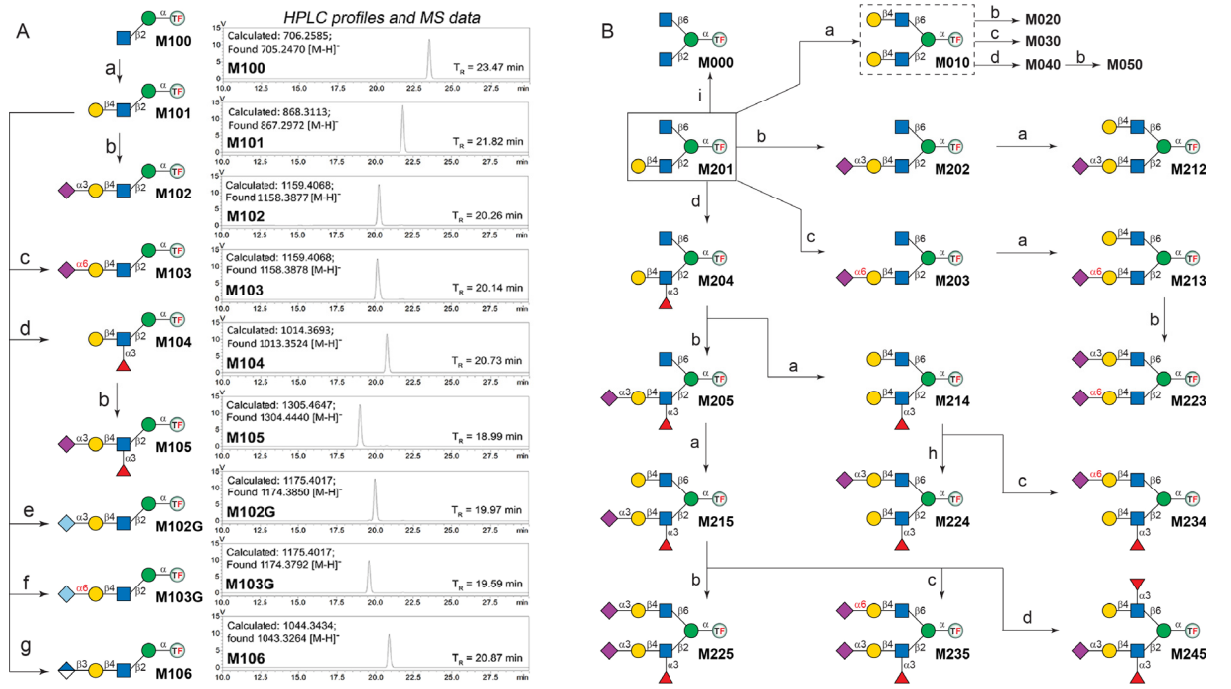

Figure 4. Enzymatic extension of core M1 (A) and core M2 (B) structures. (a) NmLgtB, UDP-Gal, Mg^{2+}; (b) PmST1-M144D, NmCSS, Neu5Ac, CTP, Mg^{2+}; (c) Pd26ST, NmCSS, Neu5Ac, CTP, Mg^{2+}; (d) Hp3FT, GDP-Fuc, Mg^{2+}; (e) PmST1-M144D, NmCSS, Neu5Gc, CTP, Mg^{2+}; (f) Pd26ST, NmCSS, Neu5Gc, CTP, Mg^{2+}; (g) GlcAT-P, UDP-GlcA, Mg^{2+}; (h) PmST1m, NmCSS, Neu5Ac, CTP, Mg2+; (i) βGalD.

To note the Fmoc was reintroduced to facilitate product detection and purification, as Fmoc has both UV (260 nm) and fluorescence (Ex. 260 nm, Em. 310 nm) absorbance.[12] The bulky hydrophobic group also enhanced retention and separation of hydrophilic O-mannosyl glycans on reversed phase chromatography. Herein, HPLC was used to detect, purify and quantify enzymatically extended O-mannosyl glycans (**Supporting Information II**).

Four robust bacterial GTs and a human β1,3-glucuronyltransferase (GlcAT-P) were used to extend the scaffold **M100** to eight core M1 O-mannosyl glycans (**Fig 4A**). The bacterial GTs include, β1,4-galactosyltransferase from *Neisseria meningitidis* (NmLgtB),[13] mutant M144D of α2,3-sialyltransferase 1 from *Pasteurella multocida* (PmST1-M144D) with decreased donor hydrolysis plus reduced sialidase activity,[14] α2,6-sialyltransferase from *Photobacterium damselae* (Pd26ST),[15] and C-terminal 66 amino acids truncated α1,3-fucosyltransferase from *Helicobacter pylori* (Hp3FT) with increased solubility.[16] These GTs are highly active and recognize minimal motifs (e.g., GlcNAc for NmLgtB, LN for Pd26ST and Hp3FT, LN or Le^X for PmST1-M144D) on oligosaccharide acceptors, and have been previously applied for preparing complex glycans.[17]

As illustrated in **Figure 4**, **M101** was prepared by the NmLgtB-catalyzed reaction, containing **M100** (10 mM), uridine 5'-diphosphogalactose (UDP-Gal) (15 mM), $MgCl_2$ (10 mM), and an appropriate amount of NmLgtB. After overnight incubation, an *m*/*z* peak of 867.2972 was observed on the ESI mass spectrum, corresponding to **M101** $[M-H]^-$. Meanwhile, on the HPLC profile, a new peak (T_R = 21.71 min) was observed, of which the area underneath increased while the peak corresponding to **M100** (T_R = 23.32 min) became smaller over time. After over 90% conversion (monitored by HPLC with a 4.6 × 250 mm Inertsil ODS-4 column), **M101** was isolated by HPLC using a semi-preparative column (Inertsil ODS-4, 20 × 250 mm). The purified **M101** was then lyophilized, and further extended to afford **M102** and **M104**, catalyzed by PmST1-M144D and Hp3FT, respectively (**Fig 4**). To make glycosylation reactions more efficient and less costly, the one-pot two-enzyme (OPTE)[18] approach was adopted when adding sialic acid residues. For example, in the PmST1-M144D-catalyzed reaction to generate **M102**, Neu5Ac, cytidine 5'-triphosphate (CTP), $MgCl_2$, and *N. meningitidis* CMP-sialic acid synthetase (NmCSS)[19] were added to achieve *in situ* generation of the sugar donor CMP-Neu5Ac. Finally, **M105** was prepared from **M104** via the same OPTE approach. Employing a bacterial α2,6-sialyltransferase (Pd26ST), we were also able to generate a not yet identified structure, **M103**, harboring the 6-sialyl-*N*-acetyllactosamine (Neu5Ac-α2,6-Gal-β1,4-GlcNAc, 6SLN) epitope (**Fig 4**), which may serve as an ideal standard for mining such structures.

Core M1 glycans terminated with *N*-glycolylneuraminic acid (Neu5Gc) (e.g. $3S_{Gc}LN$, **Fig 1**) were also observed on animal α-DG.[4, 20] Structures with a Neu5Gc residue (**M102G** and **M103G**) were thus prepared, using PmST1-M144D- and Pd26ST-catalyzed OPTE system similar to the synthesis of **M102** and **M103**. The HNK-1 epitope is a unique sulfated trisaccharide that highly expressed in the nervous system and plays critical roles in neuronal plasticity and diseases.[21] We optimized the codon

and cloned the β-glucuronosyltransferase gene (GlcAT-P) for heterogeneous expression in *E. coli*. Even though pure soluble proteins were not obtained, we were able to synthesize a precursor structure of the HNK-1 epitope on core M1 (**Fig 4**, **M106**) using GlcAT-P-containing cell lysate (**Supporting Information II**). Research in obtaining a 3-sulfotransferase for generating HNK-1 epitope is undergoing.

Core M2 O-mannosyl glycans can be classified into symmetric (with same motifs on both β1,2- and β1,6-branches, e.g., **M0X0**) and asymmetric (with different motifs on the branches, e.g., **M2XX** and **M3XX**) structures. To generate symmetric structures, chemically prepared **M201** was firstly galactosylated by NmLgtB to afford **M010**. Similar as described for the synthesis of core M1 glycans, **M020**, **M030** and **M040** were then prepared starting from **M010** via reactions catalyzed by PmST1-M144D, Pd26ST, and Hp3FT, respectively. On the other hand, **M050** was prepared starting from **M040** via the PmST1-M144D-catalyzed reaction (**Fig 4B**). Specifically, **M000** was generated by treating **M201** with a β-galactosidase from *Streptococcus pneumoniae* (βGalD).[20]

The synthesis of asymmetric core M2 O-mannosyl glycans was performed starting with **M201** or **M301**, by enzymatic extension of the Gal-containing branch first and then the other, in a strictly controlled sequential manner. The synthesis of **M2XX** is illustrated in **Figure 4B**. Such synthetic routes were designed according to substrate specificities of corresponding GTs to avoid undesirable glycosylation. With **M234** as example, the scaffold **M201** was first fucosylated to form **M204**, followed by galactosylation on the β1,6-branch to provide **M214**. Such a synthetic sequence eliminates the addition of Fuc onto the β1,6-branch, as Hp3FT requires an LN disaccharide motif for its activity.[16] Lastly, **M234** was formed by the Pd26ST-catalyzed reaction, which attaches a Neu5Ac residue selectively onto the terminal Gal on the β1,6-branch, as α1,3-fucosylation prevents Pd26ST-catalyzed α2,6-sialylation[22] on the β1,2-branch. The synthetic scheme of **M3XX** is shown in **Figure S1**. It should be noted that **M224** and **M324** were synthesized from **M214** and **M314** (**Fig 4B**, **Fig S1**) by using mutant E271F/R313Y of PmST1 (PmST1m) instead of PmST1-M144D, which greatly prefers the LN disaccharide over the Le^X trisaccharide,[23] to avoid undesired sialylation. Collectively, all 36 possible combinations of core M2 glycans that harboring LN, 3SLN, 6SLN, Le^X or sLe^X motifs were prepared. These glycans were purified by HPLC, and characterized by ESI/MALDI-MS & NMR to confirm the structures (**Supporting Information IV** & **V**).

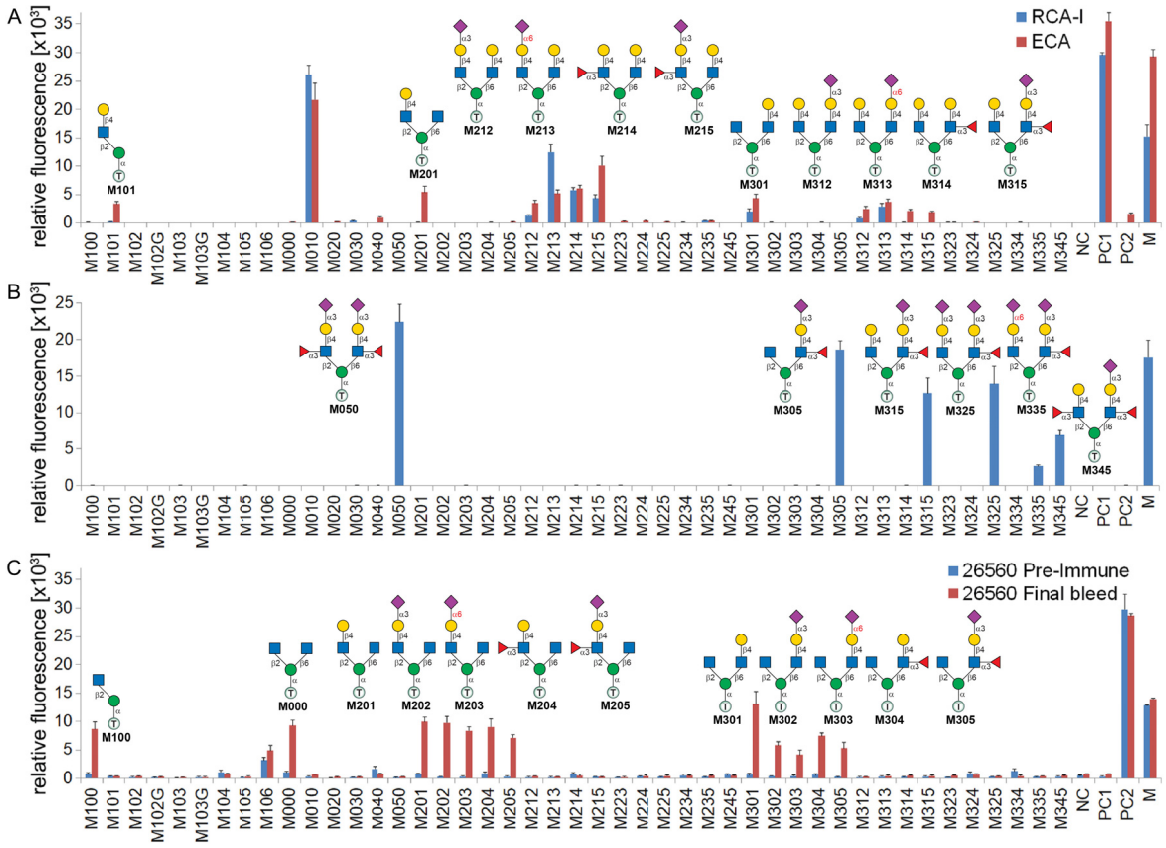

Figure 5. Microarray analysis and evaluation of binding to spotted O-mannosyl glycans against *Ricinus communis* lectin I and *Erythrina crystagalli* lectin (A), anti-CD15s antibody (B), and core M2 polyclonal rabbit sera 26560 (C). The x-axis shows the glycans and the y-axis shows the relative fluorescence, readout by (A) Cy3-streptavidin, (B) goat anti-mouse IgG-Alexa Fluor 647 conjugate, and (D) goat anti-rabbit IgG-Alexa Fluor 647 conjugate. NC, printing buffer; PC1, Biotin-PEG2-Amine; PC2, Rabbit IgG; M, human IgG-Cy3 conjugate and human IgG Alexa647 conjugate.

The synthesized O-mannosyl glycans are well-defined and closely related glycoforms (**Fig 2**), providing unique probes for mining fine specificities of glycan-binding proteins (GBPs). As shown in **Figure 5A**, microarray analysis (**Supporting Information III**) showed that both *Ricinus communis* lectin I (RCA-I) and *Erythrina cristagalli* lectin (ECA) strongly bound to **M010**, consistent with its primary specificity towards terminal LN epitope.[24] Moreover, ECA exhibited a broader specificity towards all O-mannosyl glycans harboring a free terminal LN (**M101**, **M201**, **M21X**, **M301**, and **M31X**), whereas RCA-I seems to prefer terminal LN on the β1,6-branch (**M301** and **M21X**) over the β1,2-branch (**M101**, **M201**, **M314** and **M215**) (**Fig 5A**). Such a branch preference was also found for the anti-CD15s antibody (specific to sLe^X epitope), which bound to glycans that contain sLe^X on the β1,6-branch (**M050**, **M3X5**) but not to that with sLe^X only on the β1,2-branch (**M105**, **M2X5**) (**Fig 5B**). On the other hand, *Aleuria aurantia* lectin (AAL, specific to α-Fuc) exhibited a preference towards the β1,2-branch, as well as other fine specificities (**Supporting Information III**, **Fig S2**).

ConA, an α-Man specific lectin commonly used for enriching tryptic O-mannosyl peptides, strongly bound to **M100** but not to other natural core M1 or any core M2 structures (**Fig S3**). It thus can be speculated that detection or enrichment of O-mannosyl glycans using ConA may miss a substantial amount of complex structures. Interestingly, weak bindings of ConA to unnatural α2,6-sialylated core M1 glycans (**M103**, **M103G**) was observed (**Fig S3**), suggesting that such modification may result in conformational changes that facilitated ConA binding.

Anti-glycan antibodies, on the other hand, may present as better detection tools. For example, anti-serum from two rabbits (26559, 26560) that immunized with a core M2 glycan (**M000**) conjugate bound to a broad range of core M2-containing glycopeptides regardless of varied peptide sequences.[10] We further evaluated the anti-sera toward synthesized O-mannosyl glycans. Our results showed that both sera exhibit binding specificities towards core M2 O-mannosyl glycans that containing at least one free terminal GlcNAc residue (**M000**, **M20X**, **M30X**) as well as the core M1 disaccharide **M100** (**Fig 6C**, **Fig S4**). In addition, anti-sera from rabbit 26560 exhibited comparable bindings to all those glycans, whereas anti-sera from rabbit 26559 showed stronger binding to glycans contain a free GlcNAc on the β1,6-branch (**M20X**) compared with binding to the β1,2-branch (**M100**, **M30X**) (**Fig S4**). The results imply that antibodies generated from different hosts may posses certain individual heterogeneity (or individual difference). Nevertheless, our results revealed fine specificities of the anti-sera towards O-mannosyl glycans. Comparing with ConA, these anti-sera are advantageous in the detection of branched O-mannosyl glycans.

In summary, by combining convergent chemical synthesis with strictly programmed enzymatic synthesis in a stepwise manner, an efficient scaffold synthesis/enzymatic extension (SSEE) strategy was developed to access 45 mammalian O-mannosyl glycans. Such unique glycoforms provide not only standards for identifying O-mannosyl glycans and revealing their biological roles, but also ideal probes for mining fine details of protein-glycan interactions.

Experimental Section

Detailed synthetic procedures, enzymatic reactions, microarray analysis, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry data, and NMR spectra for products are available in the supporting information.

Acknowledgements

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Keywords: carbohydrates • chemoenzymatic synthesis • glycosylation • microarrays • O-mannosyl glycans

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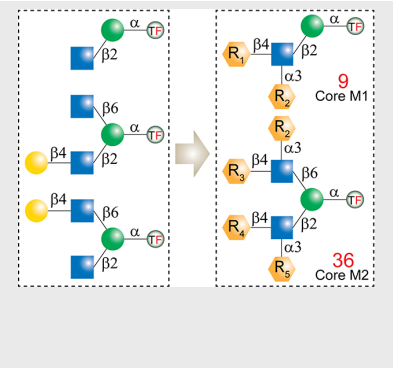
Layout 1:

COMMUNICATION

An efficient scaffold synthesis/enzymatic extension (SSEE) strategy was developed to prepare 45 structurally well-defined O-mannosyl glycans, combining convergent chemistry and strictly programmed enzymatic extension catalyzed by glycosyltransferases. Glycan microarray analysis was also performed to mine fine specificities of glycan-binding proteins using these glycoforms.



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Facile chemoenzymatic synthesis of O-mannosyl glycans