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Reprogramming the enzymatic assembly line for site-specific fucosylation

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Fucosylated carbohydrate determinants are common components of cell surface glycoconjugates and secreted unconjugated glycans, which play pivotal roles in many physiological and pathological processes. The biosynthesis of Lewis antigens involves multiple fucosyltransferases that catalyse the fucosylation of the poly-*N*-acetyllactosamine carbohydrate backbone in a non-site-specific manner and thus generate heterogeneous and incompletely fucosylated Lewis antigen regioisomers. In this study, an α2,6-sialylation module was used to introduce α2,6-linked sialic acid to specific sites as the protecting group against fucosylation, thus precisely controlling enzymatic fucosylation of poly-*N*-acetyllactosamine glycans in a site-specific manner. The sialic acid protecting group can be easily removed by sialidase after fucosylation to provide a variety of fucosylated poly-*N*-acetyllactosamine glycans with defined fucosylation patterns. The general applicability and robustness of this reprogrammed enzymatic assembly line was exemplified in the synthesis of 22 complex Lewis antigens and chimeric histo-blood group antigens with a total of 10 enzyme modules for the construction of 10 different glycosidic linkages.

Carbohydrates are present on the surface of all types of cells as a dense layer of glycocalyx conjugated with proteins or lipids. They are also widely distributed as secreted glycoproteins and unconjugated glycans in body fluids and human milk. As ubiquitous components of all organisms, carbohydrates mediate or modulate interactions among cells and between cell and extracellular environment, and thus play essential roles in many biological and pathological processes[1,2]. The number of complete human glycan structures (human glycomes) is orders of magnitude greater than the number of their protein counterparts (human proteomes)[3]. However, the number of carbohydrate determinants that are key recognition sites during the carbohydrate-mediated interaction processes is more limited[4–6]. The carbohydrate determinant itself is a minimal element of a glycan structure that is necessary but usually not sufficient for high-affinity interactions, and it is well recognized that the topological and dynamical presentation patterns of carbohydrate determinants on the enormous number of underlying glycan structures are important for fine-tuning a myriad of biological events[1,4,7–9].

Lewis antigens are a family of fucosylated *N*-acetyllactosamine (Galβ1,4GlcNAc or LacNAc, a type-2 chain) or lacto-*N*-biose (Galβ1,3GlcNAc, LNB, a type-1 chain) structures. They are common carbohydrate determinants and ubiquitous components of various cell surface glycoconjugates and secreted unconjugated glycans[10–12]. Lewis x (Le^x, **1**), sialyl Lewis x (sLe^x, **2**) and Lewis y (Le^y, **3**) share the same type-2 chain as their biosynthetic precursor, whereas Lewis a (Le^a, **4**), sialyl Lewis a (sLe^a, **5**) and Lewis b (Le^b, **6**) are biosynthesized from the type-1 chain. The Le^y and Le^b determinants with additional α1,3-linked *N*-acetylgalactosamine (GalNAc) or galactose (Gal) at the non-reducing end are the chimeric histo-blood group antigens ALe^y (**7**), BLe^y (**8**), ALe^b (**9**) and BLe^b (**10**), respectively (Fig. 1). The structural diversity and complexity of Lewis-related antigens bestow them with a broad spectrum of significant biological functions. For example, Le^x antigen, a stage-specific embryonic antigen (SSEA-1/CD15), is abundant in the poly-LacNAc chains on the embryo cell surface during the tissue development[11]. sLe^x and sLe^a antigens are ligands for the selectins (P-, L- and E-selectin) for leukocytes rolling, cell signalling and chemotaxis in inflammation[12,13]. Le^x, sLe^x, Le^y and sLe^a are common tumour-associated carbohydrate antigens, and their high overexpression on a range of carcinomas is correlated with cancer progression and poor prognosis. These tumour-associated carbohydrate antigens have been used as biomarkers for cancer clinical diagnosis and antigenic targets for the development of carbohydrate-based cancer immunotherapies[14–16]. Furthermore, these Lewis-related antigens on the host cells have been used as receptors for various bacterial, viral and parasitic infections, and many pathogens also express these antigens as molecular mimicry to escape from the host immune surveillance[1,10]. Moreover, these Lewis-related antigens are common determinants for many well-characterized human milk oligosaccharides (HMOs), which are important for shaping the gut microbiota[17,18].

Owing to their significant biological functions and great promises for biomedical applications, the synthesis of these structurally complex Lewis-related antigens has gained significant interest for decades. Since the first chemical synthesis of the Le^a antigen by the Lemieux and Driguez in 1975[19], numerous chemical or enzymatic strategies have been explored for the assembly of these complex Lewis-related antigens, such as chemical synthesis using Schmidt's imidate[20], Nicolaou's two-stage activation[21], Danishefsky's glycal assembly[22], Kahne's sulfoxide glycosylation[23], Boons' polymer-supported solution-phase synthesis[24], Seeberger's automatic solid-phase

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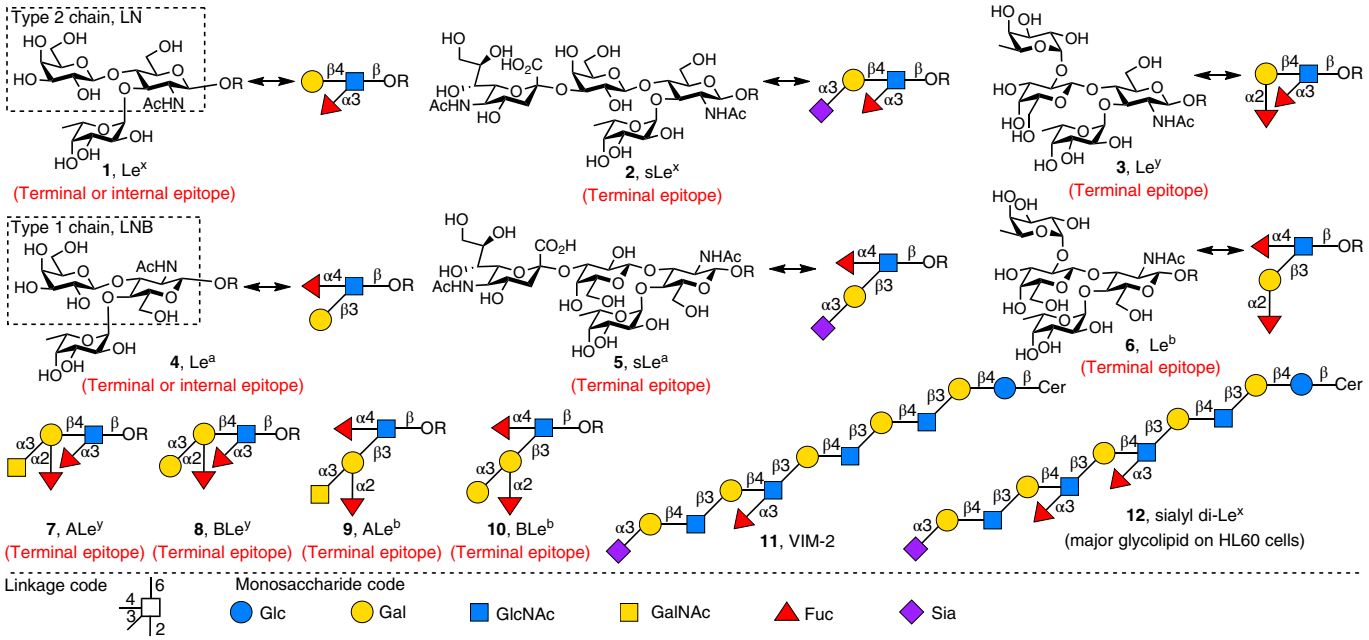

Fig. 1 | Structures of fucosylated carbohydrate determinants and incomplete fucosylated glycans. The Le^x, sLe^x and Le^y share the same type-2 chain (poly-LacNAc), whereas the Le^a, sLe^a and Le^b share the type-1 chain (LNB). The Le^y and Le^b determinants with an additional blood A or blood B antigen motif at the non-reducing end are the chimeric histo-blood group antigens ALe^y, BLe^y, ALe^b and BLe^b. Le^x and Le^a antigens are found at both internal and terminal sites of poly-LacNAc chains. However, the sLe^x, Le^y, sLe^a, Le^b, ALe^y, BLe^y, ALe^b and BLe^b antigens are exclusively present at the terminal sites of poly-LacNAc chains. ALe^y, chimeric blood group A/Lewis y antigen; BLe^y, chimeric blood group B/Lewis y antigen; ALe^b, chimeric blood group A/Lewis b antigen; BLe^b, chimeric blood group B/Lewis b antigen; VIM-2, VIM-2 antibody recognition antigen. The symbolic structures of all the glycans in this paper are annotated using the Symbol Nomenclature for Glycans system[58]. Cer, ceramide; R, carbohydrates, glycolipids or glycoproteins.

synthesis[25], Ye and Huang's preactivation glycosylation[26], Wong's reactivity-based one-pot synthesis and chemoenzymatic synthesis[27,28], and enzymatic synthesis using both mammalian and bacterial glycosyltransferases[29,30]. Most recently, chemoenzymatic syntheses of Lewis antigen branched asymmetric N-glycans[31–33], O-glycans[34,35] and human milk oligosaccharides[36,37] have also been achieved.

Despite tremendous progress on the chemical and chemoenzymatic synthesis of poly-LacNAc glycans[33,36,38–40] and complex Lewis carbohydrate determinants[19–37] in past decades, the regioselective introduction of a Lewis antigen on a poly-LacNAc glycan chain is still a formidable challenge[41]. Poly-LacNAc extensions were found to be common components of N- and O-linked glycans of glycoproteins, glycolipids and unconjugated glycans that serve as precursors for various fucosyltransferases (FucTs) for the synthesis of ABH and Lewis-related antigens. In fact, among the 13 human FucTs identified so far, ten are involved into the biosynthesis of ABH and Lewis-related antigens. Substrate specificity studies of these ten FucTs demonstrated that two α1,2-FucTs (FucT-I/II) had a very strict site specificity and can only add α1,2-linked fucose (Fuc) to the non-reducing end Gal, which results in the human blood group H antigen. Two human α1,3-FucTs (FucT-X/XI) were estimated to recognize only the innermost LacNAc sequence of poly-LacNAc in N-glycans[10,11]. Although the remaining four human α1,3-FucTs (FucT-IV/VI/VII/IX) and two human bifunctional α1,3/4-FucTs (FucT-III/V) showed a preference for the distal or internal LacNAc units of poly-LacNAc chains, they could also recognize multiple fucosylation sites of poly-LacNAc chains to produce partially α1,3- and/or α1,4-fucosylated mixtures in both in vivo and in vitro enzymatic syntheses[10,11]. For example, incompletely fucosylated glycans, such as VIM-2 antigen **11** and sialyl di-Le^x antigen **12** (Fig. 1), were identified as the major fucosylated glycolipids on human neutrophils and leukaemia HL60 cells as receptors for E-selectin-dependent binding under dynamic flow conditions[42]. Several recombinant bacterial FucTs were successfully applied for the synthesis of various fucosylated glycans. Similar to mammalian FucTs, the bacterial α1,2-FucTs can only recognize the terminal Gal to form the H antigens, whereas bacterial α1,3- or α1,3/4-FucTs have promiscuous substrate specificities that are able to add Fuc not only to the multiple fucosylation sites of poly-LacNAc glycans, but also to the lactose unit[29,43,44]. Therefore, in the current enzyme inventory neither an α1,3-FucT nor an α1,4-FucT is available from any organism for the site-specific fucosylation of poly-LacNAc glycans.

Here we describe a generally applicable modular assembly strategy by reprogramming the enzymatic glycosylation assembly line to unlock a previously inaccessible reaction pathway to precisely control the site-specific enzymatic fucosylation of poly-LacNAc glycans.

Results

Designing a strategy for site-specific fucosylation. Most mammalian glycosyltransferases are glycosylated membrane proteins that can only be overexpressed in eukaryotic cells, which renders them difficult to purify and produce on a large scale[30]. They generally have very strict substrate specificities and can be inhibited by nucleotide by-products[28]. Therefore, they are commonly used in single-enzyme-catalysed small-scale glycosylation reactions by feeding the system with an expensive nucleotide-activated sugar donor, stabilization reagent (such as bovine serum albumin) and additional enzyme(s) for the degradation of nucleotide by-products[28,36]. Unlike mammalian glycosyltransferases, recombinant bacterial glycosyltransferases can be overexpressed in a conventional *Escherichia coli* expression system. Most of them have promiscuous substrate specificities and are negligibly inhibited by nucleotide by-products, and thus they can be coupled with bacterial sugar nucleotide generation enzymes for multienzyme-catalysed cascade reactions. In recent years, several recombinant FucTs from *Helicobacter pylori*

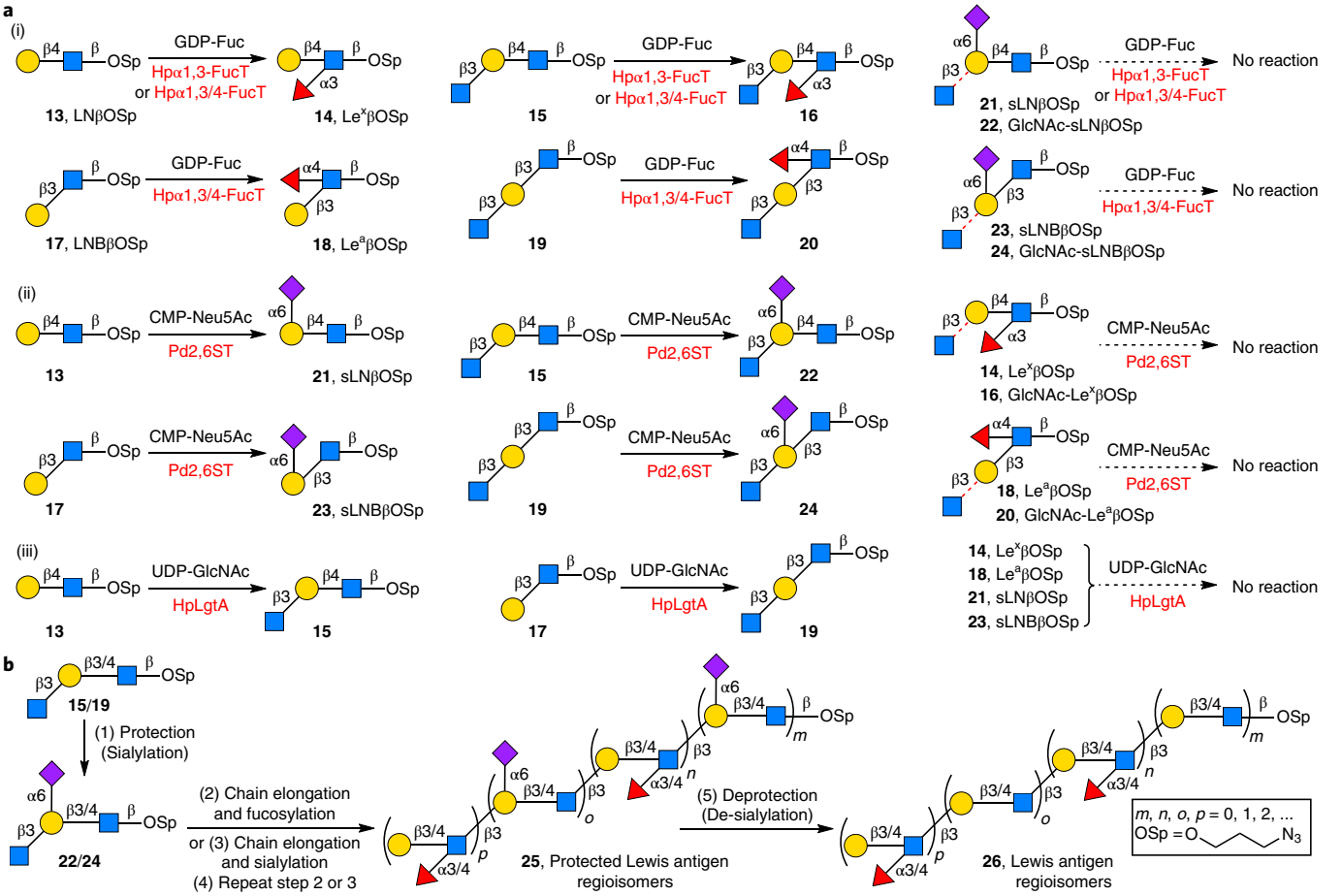

Fig. 2 | Enzyme substrate specificities and strategy design. a, Substrate specificities of glycosyltransferases of Hpα1,3-FucT (**i**), Hpα1,3/4-FucT (i), Pd2,6ST (ii) and HpLgtA (iii). **b**, Outline of reprogrammed enzymatic assembly line for the site-specific fucosylation of poly-LacNAc glycan. The α2,6-linked sialic acid was introduced as a protecting group during the enzymatic glycan assembly process to direct the site-specific enzymatic fucosylation at non-sialylated LacNAc unit(s). The protecting groups can be readily removed using a sialidase to provide site-specifically fucosylated Lewis antigens.

that exhibit higher expression levels and superior catalytic efficiencies than other FucTs were successfully applied to the synthesis of α1,2-fucosylated human blood group ABH antigens[45], α1,3-fucosylated Le^x- and sLe^x-containing O-mannose glycans[34,35] and α1,4-fucosylated Le^a antigens[29] using a one-pot multienzyme-based modular assembly strategy[46].

To design a general enzymatic strategy for site-specific α1,3- and/or α1,4-fucosylation of poly-LacNAc glycan chains, we first examined the substrate specificities of four bacterial glycosyltransferases. As summarized in Fig. 2a, a recombinant α1,3-FucT from *H. pylori* (Hpα1,3-FucT)[43,44] could use type-2 glycan **13** and GlcNAc-extended type-2 glycan **15** as substrates to form the Le^x determinant **14** and internal fucosylated Le^x determinant **16**, respectively. A bifunctional *H. pylori* α1,3/4-FucT (Hpα1,3/4-FucT)[29] was able to use both type-1 and type-2 glycans as substrates to provide Le^a antigens **18** and **20** and Le^x antigens **14** and **16**, respectively. Although α2,6-sialylation was not part of the Lewis antigen biosynthetic pathway, previous substrate-specificity studies indicated that the C6-hydroxyl group of Gal in both type-1 and type-2 glycan acceptors was essential for a successful fucosylation by α1,3- and α1,3/4-FucTs from either mammalian or bacterial sources[10,32,36]. We also confirmed that the α2,6-sialylated type-1 and type-2 glycans **21**–**24** were not substrates for Hpα1,3-FucT and Hpα1,3/4-FucT. The recombinant bacterial α2,6-sialyltransferase from *Photobacterium damselae* (Pd2,6ST)[47] was shown to have a promiscuous substrate specificity, which could recognize both terminal and internal Gal and GalNAc units to give multisialylated products[48,49], but α1,3-fucosylated type-2 glycans, such as Le^x **14** and **16**, and α1,4-fucosylated type-1 glycans, such as Le^a **18** and **20**, were not substrates for Pd2,6ST. Moreover, a recombinant *H. pylori* β1,3-*N*-acetylglucosaminyltransferase (HpLgtA)[50] was known to be able to use both type-1 and type-2 glycan receptors for poly-LacNAc chain elongation, but none of the α1,3-fucosylated type-2, α1,4-fucosylated type-1 or α2,6-sialylated LacNAc glycans **14**, **18**, **21** and **23** were substrates for HpLgtA for chain extension (Fig. 2a; Supplementary Figs. 2–11 give details).

Taken together, the inherent substrate specificities of these four bacterial glycosyltransferases inspired us to design a general strategy for the site-specific α1,3- and/or α1,4-fucosylation of poly-LacNAc glycan chains by integrating the α2,6-sialylation module into the Lewis antigen enzymatic assembly line. As depicted in Fig. 2b, the α2,6-linked sialic acid can be enzymatically introduced as the protecting group to block a specific LacNAc unit from fucosylation. The glycan can be further enzymatically extended and the nascent GlcNAcβ1,3,Galβ1,3/4GlcNAc sequence can be either protected by α2,6-sialylation at the Gal unit or α1,3- or α1,4-fucosylation at the internal GlcNAc unit. The distal GlcNAc unit is essential for poly-LacNAc backbone elongation, and it is inert to both α1,3- and α1,4-fucosylation. By repeating the chain elongation through β1,4-galactosylation and β1,3-*N*-acetylglucosaminylation with sialylation (protection) or fucosylation, and finally de-sialylation using a sialidase, poly-LacNAc glycans with all the defined fucosylation patterns can be enzymatically assembled (Fig. 2b).

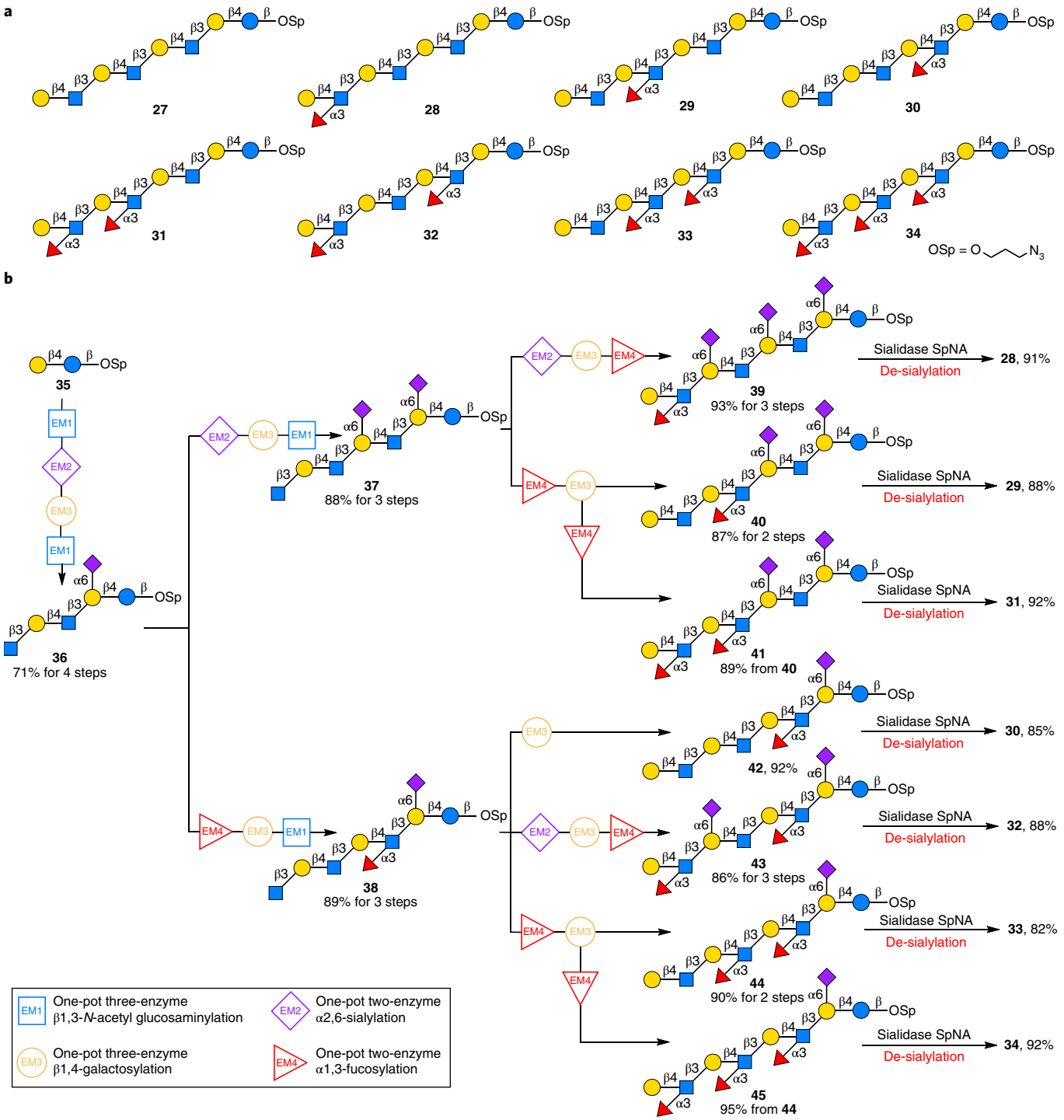

Fig. 3 | Site-specific α1,3-fucosylation of type-2 glycan chains. a, Structures of all the putative α1,3-fucosylated products of type 2 poly-LacNAc glycan **27**. **b**, Site-specific α1,3-fucosylation of type-2 glycan chains using a reprogrammed enzymatic assembly line. The enzymatic modular assembly of α2,6-sialic-acid-protected key intermediates **39**–**45** and de-protection with sialidase SpNA from *S. pneumoniae* (Supplementary Figs. 12–20 give details).

Site-specific α1,3-fucosylation of type-2 poly-LacNAc chains. Type-2 poly-LacNAc structures, a type of glycan chain with repeating *N*-acetyllactosamine units, are ubiquitous components of various O-glycans, N-glycans and glycolipids in human cells, parasites and bacteria. The incomplete α1,3-fucosylated type-2 poly-LacNAc chains were identified in various mammalian and parasite (such as *Schistosomal mansoni*) glycoconjugates, and the O-antigens of the lipopolysaccharide of *H. pylori*[10,42,51]. To evaluate the fidelity of proposed reprogramming enzymatic assembly strategy, the site-specific α1,3-fucosylation modification of type-2 poly-LacNAc glycan **27** was examined first. As shown in Fig. 3a, glycan **27** has seven putative α1,3-fucosylated products—three monofucosylated Le^x antigens **28**–**30**, three fucosylated di-Le^x antigens **31**–**33** and one fucosylated tri-Le^x antigen **34**. As illustrated in Fig. 3b, four one-pot multienzyme-based enzyme modules reported previously[34,52] were reprogrammed for the modular assembly of all seven Le^x antigens

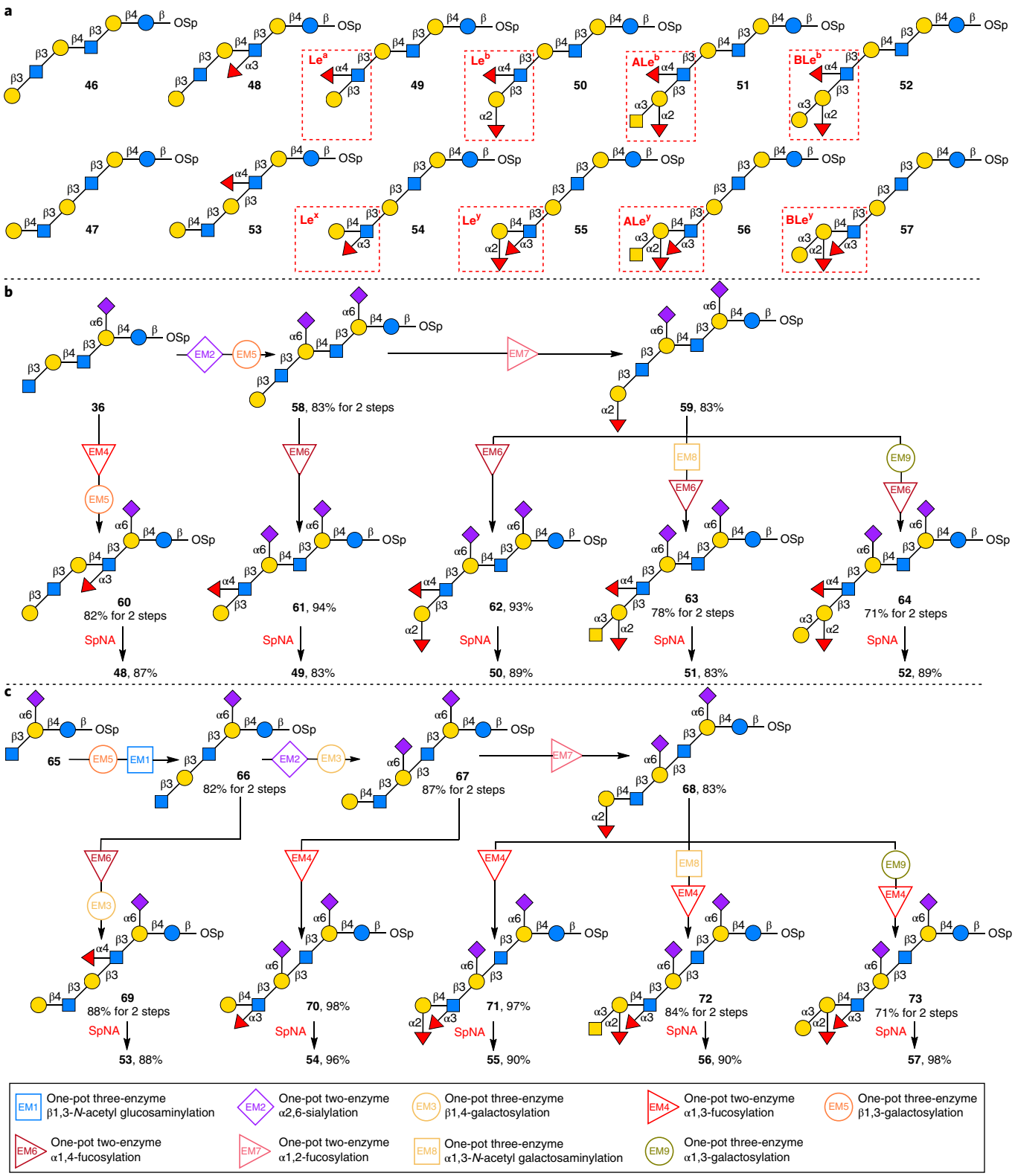

Fig. 4 | Site-specific α1,3- or α1,4-fucosylation of type-1 and type-2 hybrid poly-LacNAc chains. a, Glycans **48**–**52**, which comprise Le^x, Le^a, Le^b, ALe^b and BLe^b determinants, respectively, share the same hybrid poly-LacNAc backbone as **46**. Glycans **53**–**57**, which contain Le^a, Le^x, Le^y, ALe^y and BLe^y determinants, respectively, share the same hybrid poly-LacNAc backbone as **47**. **b**, Enzyme modular assembly of antigens **48**–**52**. **c**, Enzyme modular assembly of antigens **53**–**57**. Supplementary Figs. 21–27 give details.

28–**34**. Lactoside **35** was first extended with a β1,3-linked GlcNAc using enzyme module 1 (EM1), then the Gal unit of trisaccharide product was protected with α2,6-linked Neu5Ac using enzyme module 2 (EM2). The resulting Neu5Ac-protected tetrasaccharide was sequentially extended using enzyme module 3 (EM3) and EM1 to form Neu5Ac-protected hexasaccharide **36** in a 71% yield with

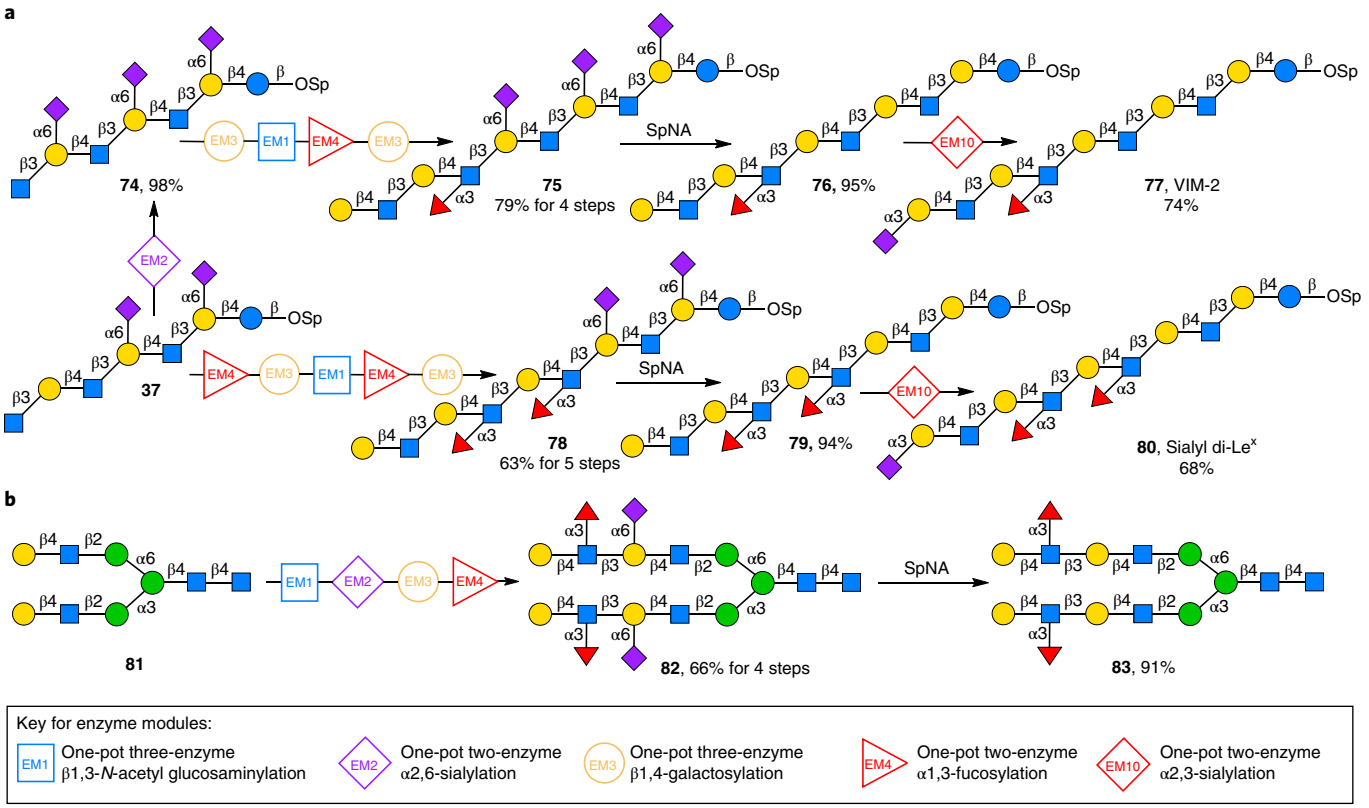

Fig. 5 | Enzymatic synthesis of VIM-2 antigen and fucosylated N-glycan. a, Enzymatic synthesis of VIM-2 antigen **77** and sialyl di-Le^x antigen **80**. **b**, Enzymatic synthesis of site-specifically fucosylated N-glycan **83** (Supplementary Figs. 28–30 give details).

four steps. The sequential glycosylation of hexasaccharide **36** using EM2, EM3 and EM1 led to the Neu5Ac-protected nonasaccharide **37** in an 88% yield with three steps. The advanced intermediate **37** was protected with a third α2,6-linked Neu5Ac by EM2, extended by a β1,4-linked Gal with EM3 and a further α1,3-fucosylation using enzyme module 4 (EM4) to provide Neu5Ac-protected glycan **39** with a terminal Le^x epitope. The nonasaccharide **37** was also elaborated to give the Neu5Ac-protected glycan **40** with an internal Le^x epitope by sequential glycosylation with EM4 and EM3. **40** was α1,3-fucosylated by an additional EM4 step to form the Neu5Ac-protected glycan **41** with a terminal di-Le^x epitope. Also, the hexasaccharide **36** could be transformed to give Neu5Ac-protected poly-LacNAc glycan **42** with an innermost Le^x epitope, Neu5Ac-protected di-Le^x glycans **43** and **44**, and Neu5Ac-protected tri-Le^x glycan **45** following different glycosylation sequences with the same four enzyme modules as depicted in Fig. 3b (Supplementary Methods gives details). The α2,6-linked Neu5Ac protecting groups of glycans **39**–**45** were readily removed by a recombinant sialidase (SpNA) from *Streptococcus pneumoniae*[53] to yield α1,3-fucosylated type-2 poly-LacNAc glycans **28**–**34**, which comprise precisely controlled α1,3-fucosylation patterns.

Site-specific α1,3- or α1,4-fucosylation of type-1 and type-2 hybrid glycan chains. Having established the reprogrammed enzymatic modular assembly strategy for the site-specific α1,3-fucosylation of type-2 poly-LacNAc glycans, we turned our attention to evaluate this strategy for the site-specific fucosylation of type-1 glycans by reprogramming nine enzyme modules (EM1–EM9) for the synthesis of complex Le^x antigens **48** and **54**, Le^a antigens **49** and **53**, Le^b antigen **50**, Le^y antigen **55** and the chimeric histo-blood group antigens **51**, **52**, **56** and **57** (Fig. 4). Unlike the type-2 chain, for which tandem repeats of the poly-LacNAc glycan chain are common, type-1 LNB-containing glycans are usually found as hybrid poly-LacNAc glycans that cap with LNB. As shown in Fig. 4b, enzyme module 5 (EM5) comprises a recombinant β1,3-galactosyltransferase from *E. coli* O55 (WbgO)[54] and two enzymes for the in situ uridine 5'-diphosphate galactose (UDP-Gal) generation used to convert terminal GlcNAc into the type-1 LNB unit. Enzyme module 6 (EM6), which contains the α1,3/4-FucT[29], and the guanosine 5'-diphosphate fucose (GDP-Fuc) generating enzyme were used for α1,4-fucosylation of the type-1 LNB sequence. It is known that Hpα1,3/4-FucT could recognize type-1 and type-2 glycans as well as lactosides as substrates to form α1,4-fucosylated Le^a, α1,3-fucosylated Le^x or α1,3-fucosylated lactoside products, respectively[29]. As illustrated in Fig. 4b, the protecting/de-protecting-based reprogramming enzymatic assembly strategy was explored for the site-specific fucosylation modification of poly-LacNAc glycans **46** and **47**, which contain either a terminal or internal type-1 LNB unit. For the site-specific fucosylation modification of **46**, the Neu5Ac-protected hexasaccharide **36** was α1,3-fucosylated using EM4, and then extended with EM5 to give poly-LacNAc glycan **60** with an internal Le^x. In parallel, hexasaccharide **36** could be sequentially protected and extended by EM2 and EM5 to form octasaccharide **58**, and further α1,4-fucosylation by EM6 to afford Neu5Ac-protected poly-LacNAc glycan **61** with a terminal Le^a epitope. After de-protection (de-sialylation) using sialidase SpNA, the site-specific fucosylated poly-LacNAc glycans **48** and **49** were obtained in 87% and 83% yields, respectively. To synthesize complex Le^b antigen **50**, chimeric histo-blood group ALe^b antigen **51** and BLe^b antigen **52**, three enzyme modules (EM7–EM9) designed previously[45] for the assembly of human blood group ABH antigens were adopted. As shown in Fig. 4b, the Neu5Ac-protected octasaccharide **58** was first

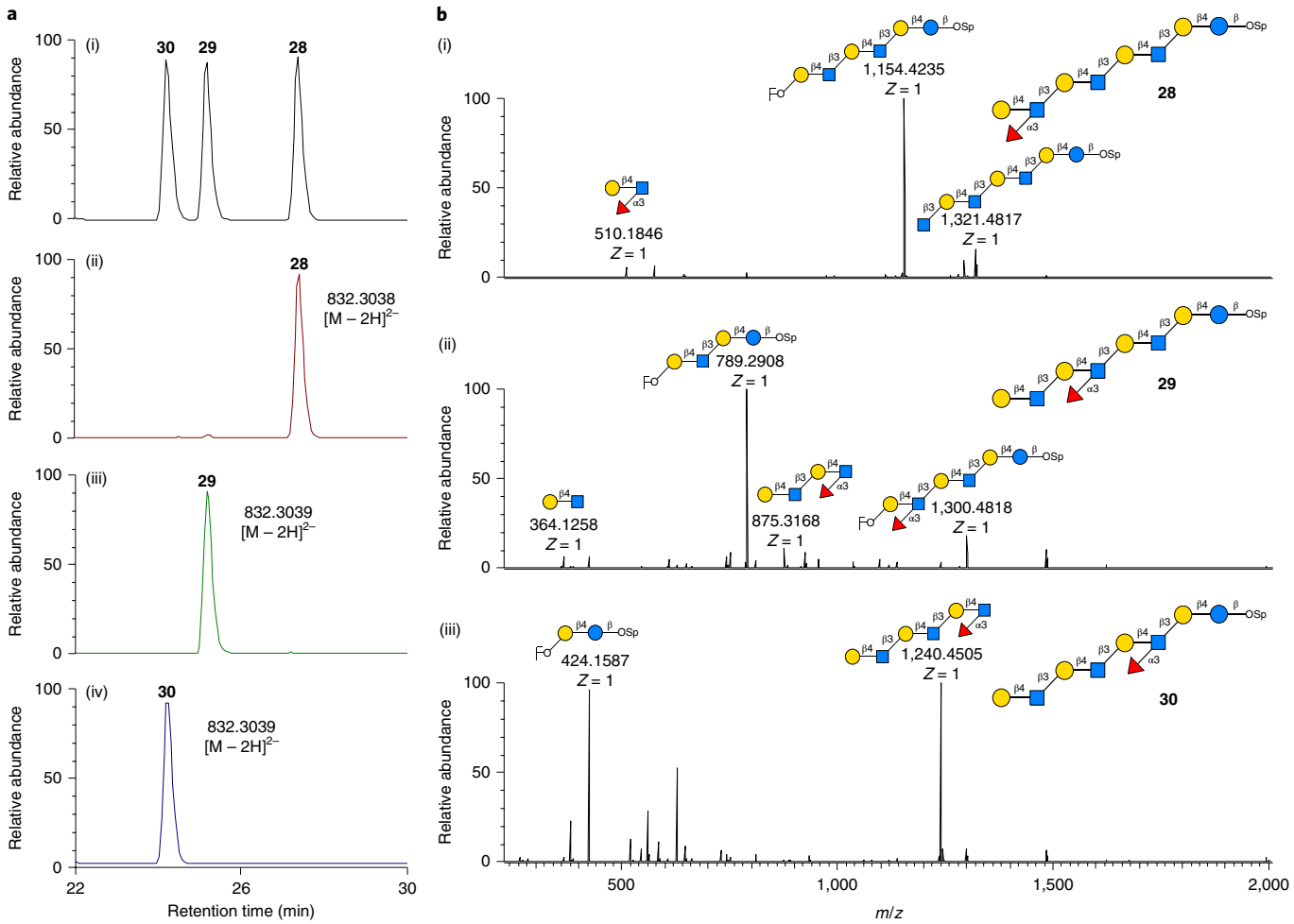

Fig. 6 | LC–MS and MS² of Lewis antigen regioisomers 28–30. a, LC–MS (total ion current, negative mode) of a mixture of Lewis antigen regioisomers **28**–**30** (i) and Lewis antigen regioisomers **28** (ii), **29** (iii) and **30** (iv). **b**, Tandem mass spectrometry (negative mode) of **28** (i), **29** (ii) and **30** (iii).

converted into H antigen **59** by EM7, and this H antigen was modified by either α1,3-linked GalNAc using EM8 or α1,3-linked Gal using EM9 to provide blood group A antigen or B antigen intermediates **S12** and **S13**, respectively (Supplementary Methods gives details). To our delight, the Neu5Ac-protected H antigen **59**, A antigen **S12** and B antigen **S13** were all well tolerated by Hpα1,3/4-FucT in EM6 for further α1,4-fucosylation to yield Neu5Ac-protected Le^b (**62**), ALe^b (**63**) and BLe^b (**64**) antigens in 93%, 78% and 71% yields, respectively. After de-sialylation using sialidase SpNA, the Le^b (**50**), ALe^b (**51**) and BLe^b (**52**) antigens that share the same hybrid poly-LacNAc backbone as **46** were achieved in good yields (Fig. 4b). Following a similar strategy, the site-specific fucosylation and derivatization of hybrid poly-LacNAc **47** was achieved starting from tetrasaccharide **65**. The internal Le^a antigen **53**, terminal Le^x antigen **54**, Le^y antigen **55**, ALe^y antigen **56** and BLe^y antigen **57** were obtained in 12–80 mg following different assembly lines with nine enzyme modules and final de-sialylation, respectively (Fig. 4c). Note that EM6, which contains a bifunctional Hpα1,3/4-FucT, can be used to replace EM4 for the α1,3-fucosylation of type-2 LacNAc sequences. EM6 was only used for the α1,4-fucosylation of type-1 lacto-*N*-biose sequences in this study as the expression level of Hpα1,3/4-FucT is five times lower than that of Hpα1,3-FucT[29,44].

Total synthesis of VIM-2 antigen and fucosylated N-glycan. To further highlight the power of this reprogrammed enzymatic assembly strategy, the total synthesis of complex VIM-2 antigen dodecasaccharide **77**, sialyl di-Le^x antigen tridecasaccharide **80** and regioselectively difucosylated biantennary N-glycan **83** were explored. As shown in Fig. 5a, the Neu5Ac-protected poly-LacNAc glycan **37** was again protected by a third Neu5Ac to form decasaccharide **74**, which was sequentially glycosylated by EM3, EM1, EM4 and EM3 to yield Neu5Ac-caged glycan **75** in a 79% overall yield for the four steps. After de-protection by sialidase SpNA, the monofucosylated undecasaccharide **76** was obtained in 95% yield. The enzyme module 10 (EM10)[46,52], which contains a mutated recombinant α2,3-sialyltransferase from *Pasteurella multocida* (PmST1 M144D)[55] and an enzyme for in situ cytidine 5'-monophosphate *N*-acetylneuraminic acid (CMP-Neu5Ac) generation, was used for the α2,3-sialylation of glycan **76**, which resulted in VIM-2 dodecasaccharide **77** in 85% yield. Previously, a β1,6-linked GlcNAc was enzymatically introduced as a protecting group for the site-specific α1,3-fucosylation of the type-2 tetrasaccharide chain[56]. However, this method is limited by the strict substrate specificity of mammalian β1,6-*N*-acetylglucosaminyltransferase. For the synthesis of sialyl di-Le^x antigen tridecasaccharide **80**, a major fucosylated glycolipid on human neutrophils and leukaemia HL60 cells, the advanced intermediate **37** was sequentially glycosylated by EM4, EM3, EM1, EM4 and EM3 to give Neu5Ac-protected di-Le^x tetradecasaccharide **78**. **78** was converted into di-Le^x antigen **79** by the removal of two Neu5Ac protecting groups following the standard

de-sialylation conditions and finally transformed into sialyl di-Le^x antigen **80** by EM10 (Fig. 5a). The α2,3-sialyltransferase PmST1 is a multifunctional enzyme that also exhibits a very weak α2,6-sialyltransferase activity[55]. Therefore, trace amounts of α2,6-sialylated by-products were also observed in the α2,3-sialylation of **76** and **79** using EM10. The synthesis of site-specifically difucosylated biantennary N-glycan **83** started from N-glycan **81**, which is readily available from a glycopeptide of egg yolk following a reported procedure[57]. The N-glycan **81** was sequentially treated with EM1, EM2, EM3 and EM4. The resulting Neu5Ac-protected N-glycan **82** was de-sialylated using SpNA to yield the desired difucosylated biantennary N-glycan **83** (Fig. 5b). The past few years have witnessed a tremendous advance in the enzymatic and chemoenzymatic synthesis of complex N-glycans. However, a method for the selective fucosylation modification of the internal LacNAc residues of N-glycan is still unknown[41]. The reprogrammed enzymatic modular assembly strategy described here provides a general approach to access these complex fucosylated N-glycans, which can be used for the synthesis of N-glycan-modified glycoproteins according to well-established methods[57].

Liquid chromatography–mass spectrometry and tandem mass spectrometry of Lewis antigen regioisomers. With the availability of the diverse Lewis antigens produced from synthesis, one potential application is to use these compounds as valuable standards. The isolation and characterization of complex glycans, especially their regioisomers from a microheterogeneous natural source, are very challenging. These systematically synthesized structurally well-defined glycans could be used to establish the fingerprints for various glycomic purposes. To develop a convenient method to profile these Lewis antigen isomers, both liquid chromatography–mass spectrometry (LC–MS) and tandem mass spectrometry (MS^2) were explored. After screening several analytical chromatography columns, a porous graphitic carbon Hypercarb column (150 × 2.1 mm, 3.0 μm, Thermo Scientific) equipped with a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific) exhibited superior separation results. As shown in Fig. 6a, a mixture of three monofucosylated poly-LacNAc glycans **28**–**30** can be readily separated and detected by a coupled Thermo LTQ-Orbitrap XL MS (Thermo Fisher Scientific). All the other fucosylated poly-LacNAc isomers could also be differentiated by their retention time under the same LC–MS conditions (Supplementary Figs. 41–62 give the LC-MS profiles for all the final products). Under optimized conditions, the negative-ion electrospray MS^2 of all the isomeric structures displayed unique fragmentation patterns. As shown in Fig. 6b, the most abundant ion peaks for isomers **28**–**30** at *m*/*z* 1,154, 789 and 1,240, respectively, all resulted from the glycosidic bond cleavage at the reducing end of the Le^x sequences, and the corresponding intact Le^x fragments are found at *m*/*z* 510 (for **28**), 875 (for **29**) and 1,240 (for **30**). These informative fragment peaks along with other distinct ion peaks (Supplementary Figs. 41–62 give the MS^2 profiles for all the final products) provided a simple approach to structure identification. This LC–MS/MS^2 method can also be applied to the sequencing of other complex glycans without the need for derivation.

Conclusion

With the tremendous diversity of Lewis determinant structures, a thorough understanding of their biological functions demands a library of structurally well-defined oligosaccharides. Chemoenzymatic strategies can greatly expedite the synthesis of these complex structures. However, no α1,3- or α1,4-FucTs are available to install the Fuc unit at specific locations within a poly-LacNAc chain, which presents tremendous synthetic challenges in the high-yielding synthesis of precise target structures. To overcome this obstacle, a reprogrammed enzymatic modular assembly strategy was developed that enabled the site-specific α1,3- and/or α1,4-fucosylation of poly-LacNAc glycans. The successful integration of a non-related α2,6-sialylation module into the Lewis antigen enzymatic assembly line is the key for this reprogrammed system. Taking advantage of enzyme-substrate selectivities, ten enzyme modules were orchestrated in a successive manner for the assembly of Lewis-related complex antigens with defined fucosylation patterns. The ten enzyme modules comprise ten glycosyltransferases and six enzymes for the in situ generation of five nucleotide-activated sugar donors (UDP-GlcNAc, UDP-GalNAc, UDP-Gal, GDP-Fuc and CMP-Neu5Ac). This reprogrammed glycan assembly line serves as a platform for the synthesis of complex Lewis antigens with diverse glycan structures. The availability of these well-defined structures could provide valuable standard compounds for the characterization and quantification of complex oligosaccharides isolated from nature, but could also expedite a thorough understanding of their important biological functions.

Data availability

The NMR spectroscopy, LC-MS, MS^2 and other data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

H.C. conceived and designed the experiments. J.Y., H.X., N.S., C.-C.L. and X.-W.L. carried out the enzymatic synthesis. A.S. and L.C. performed the LC-MS and MS^2 experiments. J.Y., A.S., L.C., C.-C.L. and H.C. analysed the data. G.G., S.W., J.Z., P.W., M.X. and F.W. contributed materials and/or analysis tools. C.-C.L. and H.C. wrote the paper with input from all the authors.

Competing interests

The authors declare no competing interests.

Additional information

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